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Bombyx mori Nucleopolyhedrovirus Bacmid Enabling Rapid Generation of Recombinant Virus by *In Vitro* Transposition

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A novel recombinant bacmid, bEasyBm, that enables the easy and fast generation of pure recombinant baculovirus without any purification step was constructed. In bEasyBm, *att*R recombination sites were introduced to facilitate the generation of a recombinant viral genome by *in vitro* transposition. Moreover, the extracellular RNase gene from *Bacillus amyloliquefaciens*, barnase, was expressed under the control of the *Cotesia plutellae* bracovirus early promoter to negatively select against the nonrecombinant background. The bEasyBm bacmid could only replicate in host insect cells when the barnase gene was replaced with the gene of interest by *in vitro* transposition. When bEasyBm was transposed with pDualBac-EGFP, the resulting recombinant virus, EasyBm-EGFP, showed high levels of EGFP expression efficiency compared with that of non-purified recombinant virus BmGOZA-EGFP, which was constructed using the bBmGOZA system. In addition, nonrecombinant backgrounds were not detected in unpurified EasyBm-EGFP stocks. Based on these results, a high-throughput system for the generation of multiple recombinant viruses at a time was established.

Keywords: Baculovirus expression system, EasyBm, *in vitro* transposition, barnase, high throughput

Introduction

The baculovirus expression vectors have been extensively used for the expression of foreign genes because they do not only enable high-level expression but also support most eukaryotic post-translational modifications authentically [13]. Although a number of methods for the convenient and rapid generation of recombinant baculoviruses expressing a gene of interest have been reported [1, 14, 17, 18], a major drawback of these methods is the tedious efforts required to purify recombinant viruses from nonrecombinant backgrounds.

Recently, we constructed a novel recombinant bacmid, bEasyBac, which enabled the rapid generation of recombinant virus without any processes to purify target recombinant viruses from a nonrecombinant background [2]. In this recombinant bacmid, the barnase gene from *Bacillus amyloliquefaciens*, which is lethal to insect cells, was introduced into the *Autographa californica* nucleopolyhedrovirus (AcMNPV) genome under the control of early promoters from *Cotesia plutellae* bracovirus (CpBV). In addition, bacteriophage lambda site-specific attachment (*att*) sites were introduced into the genome for the fast generation of a recombinant viral genome by *in vitro* transposition. The bEasyBac can only replicate in transfected insect cells when the CpBV promoter-barnase cassette is replaced with the gene of interest by *in vitro* transposition. The bEasyBac is suitable for the high-throughput expression of heterologous genes, because the replication of the nonrecombinant bacmids in insect cells is blocked by host cell death at the early stage of viral replication, and therefore time-consuming selection steps are not required.

Most baculovirus expression vectors have been based on AcMNPV or *Bombyx mori* nucleopolyhedrovirus (BmNPV). For the large-scale production of foreign proteins, BmNPV-based vectors have a unique advantage of having an advanced system, the silkworm *B. mori*, that can be used for *in vivo* expression [9]. The silkworm can be easily mass-cultured at low cost by an automatic feeding machine and advanced artificial diets [11].

Although methods for the generation of recombinant BmNPV directly in silkworm using the BmNPV bacmid have been recently reported [10, 12], they are not suitable for the high-throughput expression of heterologous genes because these systems require time-consuming steps for purification and amplification of recombinant viruses. In this study, we introduced *att* sites and the barnase gene under the control of the CpBV early promoter into the BmNPV genome for the fast generation of a recombinant viral genome by *in vitro* transposition, and the effectiveness of this system for the high-throughput expression of foreign genes was investigated.

Materials and Methods

Bacterial Strains and Transformation

Escherichia coli strain JM109 (Takara, Japan) was used for the transformation of high-copy plasmids. Transformation of high-copy plasmids was carried out by a heat-shock protocol using chemically treated competent cells according to the manufacturer's instruction. For transformation of low-copy bacmids, *E. coli* strain DH10b (Invitrogen, USA) was used. Low-copy bacmids were transformed into electrocompetent cells by electroporation as follows. Competent cells (200 µl) were mixed with 1 µg of bacmid DNA and single shocked in a 0.1 cm electroporation cuvette (Bio-Rad, USA) using a Bio-Rad Gene Pulser under the following conditions: volts, 1.8 kV; resistance, 200 Ω; and capacitance, 25 µF. All of the restriction endonucleases and modifying enzymes were from Roche Applied Science (Germany).

Insects, Insect Cells, and Baculoviruses

B. mori larvae were reared on artificial diets under 25°C, 65 ± 5% relative humidity, and 16 h:8 h light:dark cycles. *B. mori* cell line Bm5 was maintained in TC-100 medium (JBI, Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (JBI, Korea), incubated at 27°C, and subcultured every 4−5 days. Wild-type BmNPV and all of the recombinant BmNPVs used in this study were propagated in Bm5 cells maintained in Tc-100 medium.

Construction of Plasmid Vectors

To construct pBmKSK3-attBLacZ, 467 bp of the *lacZ* gene fragment was amplified from pGEM-5Zf(-) (Stratagene, USA) using oligonucleotides attB1LacZ-EcoRF (5′-GGCGAATTCACAAGT TTGTACAAAAAAGCAGGCTTTCCATTCGCCATTCAGGC-3′) and attB2LacZ-KpnR (5′-GGCGGTACCACCACTTTGTACAAGAAA GCTGGGTAGCGCAACGCAATTAATGTG-3′) and digested with *Eco*RI. This PCR-amplified and *Eco*RI-digested attB1-*lacZ*-attB2 cassette was introduced into the *Eco*RI and *Sma*I sites of pBmKSK3 [4]. The pBm101-MF plasmid was constructed by inserting the *Sac*I-digested pMiniF-Kan [7] into the *Sac*I site of pBm101 [6].

In Vitro Transposition

The transposition reaction was carried out using Gateway LR Clonase II Enzyme Mix (Invitrogen, USA). In the transposition reaction, 150 ng of donor vector was mixed with 150 ng of bEasyBm bacmid DNA. In total, 2 µl of LR Clonase II Enzyme Mix (Invitrogen, USA) was added to the transposition reaction, and the mixture was mixed well by vortexing briefly twice. Then, it was centrifuged briefly and incubated at 25°C overnight (approximately 12−16 h). A total of 1 µl of proteinase K solution was added to the reaction (to terminate transposition) and incubated at 37°C for 10 min.

Transfection

Approximately 5 × 10^4 Bm5 cells per well were seeded in a 24-well tissue culture plate and incubated at 27°C for 30 min to allow the cells to attach. In total, 11 µl of LR recombination reaction was added to 100 µl of incomplete TC-100 medium in a polystyrene tube. In another polystyrene tube, 10 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 1 ml of incomplete TC-100 medium and refreshed with 0.5 ml of the same medium. The 1/5 or 1/10 fractions of Cellfectin-DNA complexes were added dropwise per well to the medium covering the cells while the plate was gently swirled. After incubation at 27°C for 5 h, each well was refreshed with 2 ml of TC-100 medium supplemented with 10% FBS, and the transfected cells were incubated at 27°C. At 5 days post-transfection (p.t.), the transfection supernatant was harvested by centrifugation at 500 ×*g* for 5 min and stored at 4°C.

RNA and RT-PCR

Total RNA was isolated from Bm5 cells transfected with recombinant bacmids at 4 days p.t. using TRIZOL Reagent (Invitrogen, USA) according to the manufacturer's instructions. RT-PCR was carried out using *AccuPower* RT/PCR Premix (Bioneer, Korea) in a 20 µl volume according to the manufacturer's instructions. To amplify the *gp64* gene, the oligonucleotides gp64-F (5′-CCAAACATGAACGAAGTC-3′) and gp64-R (5′-GACACT GTGCTTCATCG-3′) were used. The *vp39* gene was amplified using oligonucleotides Bm-vp39-F (5′- AGGCGGCTACACCTCCA-3′) and Bm-vp39-R (5′- GTATGATGCAAGCCGAA-3′).

PCR and Primers

For the amplification of the barnase-barstar cassette, oligonucleotides

BaBa-XhoF (5′-CCCGCTCGAGATGGCACAGGTTATCAACACG-3′) and BaBa-EcoRR (5′-CCCGGAATTCTTAAGAAAGTATGATGGTGA-3′) were used in the PCR. Additionally, the kanamycin resistance gene-MiniF replicon cassette was amplified using oligonucleotides Ka-F (5′-ATTGTCGCACCTGATTGCC-3′) and Mini-R (5′-GTCATCTGCATCAAGAACTAG-3′). For PCR, the recombinant bacmid DNAs were subjected to 33 PCR cycles (1 min at 94°C, 30 sec 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).

Infection of Cells with Baculoviruses

Insect cells were seeded in a 6-well plate at a density of 5×10^5 cells/well and incubated at 27°C for 30 min to allow cells to attach. Attached cells were washed twice with 3 ml of incomplete TC-100 medium and inoculated with each recombinant virus at a multiplicity of infection of 10 plaque-forming units (PFU)/cell. After incubation at 27°C for an hour with gentle rocking, the medium was replaced with 3 ml of fresh medium and incubated at 27°C. At 3 days post-infection, infected cells were harvested by centrifugation at 500 ×*g* for 5 min, washed twice with phosphate buffered saline (PBS), and stored at 4°C.

Quantification of EGFP

Insect cells infected with recombinant viruses were harvested and washed, as mentioned above. Cells were sonicated in PBS containing 1/100 volume of protease inhibitor cocktail (Sigma, USA) and centrifuged at 22,000 ×*g* for 15 min. The fluorescence of the resulting supernatant was measured using a SPECTRAmax GEMINI XS Microplate spectrofluorometer (Molecular Device Inc., USA) with an excitation filter of 450 nm and an emission filter of 510 nm. EGFP levels were calculated from measured relative fluorescence units (RFU) and compared with a standard curve (r^2 = 0.999), which was plotted as a log function of 2-fold serial dilutions of EGFP (BD Biosciences, USA). SigmaStat ver. 2.0 software (SPSS Inc., USA) was used for detecting significant differences by Duncan's multiple range test.

Luciferase Activity Assay

Insect cells infected with recombinant virus were harvested and washed, as mentioned above. The intracellular luciferase assay was performed using the Luciferase Assay System (Promega, USA) according to the manufacturer's protocols.

Results

Construction of the Recombinant Bacmid bEasyBm

We aimed to construct a recombinant viral genome, bEasyBm, that could replicate in *E. coli* cells but not insect cells and express barnase under the control of the CpBV ORF3005 promoter between the site-specific recombination sites attR1 and attR2 (Fig. 1). For this purpose, a recombinant virus, vBpLacZ, was constructed by co-transfection of bBpGOZA [8] with pBmKSK3-attBLacZ into Bm5 cells. vBpLacZ was purified from infected Bm5 cells by plaque purification. Genomic DNA of vBpLacZ was co-transfected with pBm101-MF into Bm5 cells, and viral DNAs were

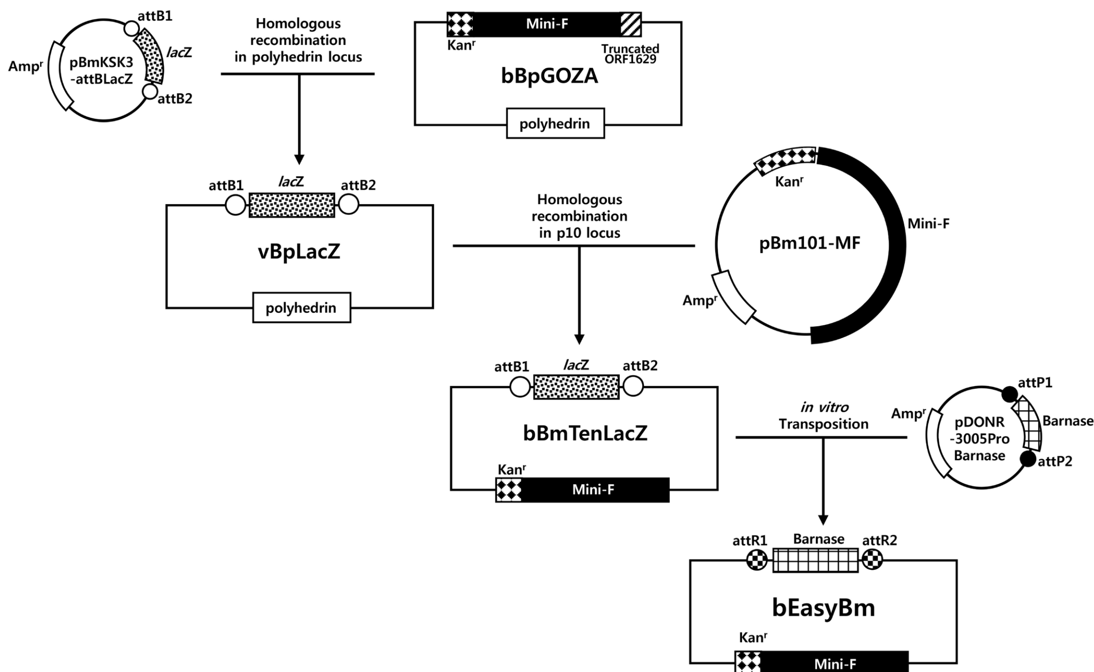

Fig. 1. Schematic construction map of the recombinant bacmid bEasyBm.

extracted from the transfected cells. These viral DNAs were transformed into *E. coli* DH10b cells, and the recombinant bacmid, bBmTenLacZ, was purified by the selection of blue colonies in blue/white screening of the transformant. The CpBV ORF3005 promoter-barnase cassette from pDONR-3005ProBarnase [2] was introduced into the polyhedrin gene loci of bBmTenLacZ by *in vitro* transposition using BP Clonase (Invitrogen, USA). The transposed DNAs were transformed into *E. coli* DH10b cells, and the recombinant bacmid, bEasyBm, was purified by the selection of white colonies in blue/white screening of the transformant.

Replication of bEasyBm in Insect Cells

To examine the cytopathic effect of barnase expressed by bEasyBm under the control of the CpBV ORF3005 promoter, bEasyBm DNA was transfected into Bm5 cells, and the growth of transfected cells was investigated (Fig. 2). While a reduced cell growth rate was observed at 1–2 days p.t., no obvious difference in cell growth rate was observed after 3 days p.t. in Bm5 cells transfected with bEasyBm compared with mock-transfected Bm5 cells. In contrast, Bm5 cells transfected with bBmTenLacZ showed dramatically reduced growth rates compared with mock-transfected cells at all of the time points investigated.

To investigate the replication of bEasyBm in insect cells, bEasyBm or bBmTenLacZ DNA was transfected into Bm5 cells to produce recombinant virus particles, and the resulting transfection medium was passaged four times in Bm5 cells. At each passage in Bm5 cells, the total RNA was extracted from infected Bm5 cells, and RT-PCR analysis using primer sets specific for the *gp64* and *vp39* genes, which are essential for BmNPV replication, was performed. Although both of the genes were amplified from the total RNAs extracted from Bm5 cells infected with bBmTenLacZ, neither of the *gp64* or *vp39* gene-specific primer sets amplified any specific fragments from the total RNA extracted from Bm5 cells infected with bEasyBm (Fig. 3).

Generation of Recombinant Baculovirus Using bEasyBm

To assess the generation efficiency of recombinant virus using bEasyBm as a parental viral genome by *in vitro* transposition between attR sites contained in bEasyBm and attL sites contained in the donor vector pDualBac [2], an LR recombination reaction was performed with 150 ng of bEasyBm DNA purified from *E. coli* and 150 ng of donor vector, pDualBac-EGFP [2] or pDualBac-Luc [2], in which the *egfp* or *luciferase* reporter gene, respectively, was contained under the control of the *polyhedrin* promoter. When the 1/5 fractions (equivalent to 30 ng of bEasyBm and donor vector) were transfected into Bm5 cells, recombinant viruses EasyBm-EGFP and EasyBm-Luc expressing EGFP and luciferase, respectively, were generated with an efficiency of approximately 83.3–91.7% (Fig. 4).

To verify the absence of nonrecombinant backgrounds in non-purified viral stocks, the transfection media of the recombinant viruses EasyBm-EGFP and BmGOZA-EGFP, which were generated by co-transfection of bBmGOZA [8] and pDualBac-EGFP into Bm5 cells, were passaged five

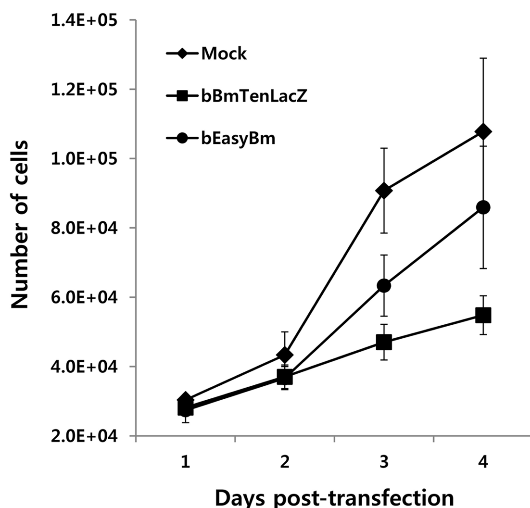

Fig. 2. Replication of the recombinant bacmid bEasyBm in insect cells.

The growth of Bm5 cells transfected with bEasyBm expressing barnase under the control of the CpBV early promoter.

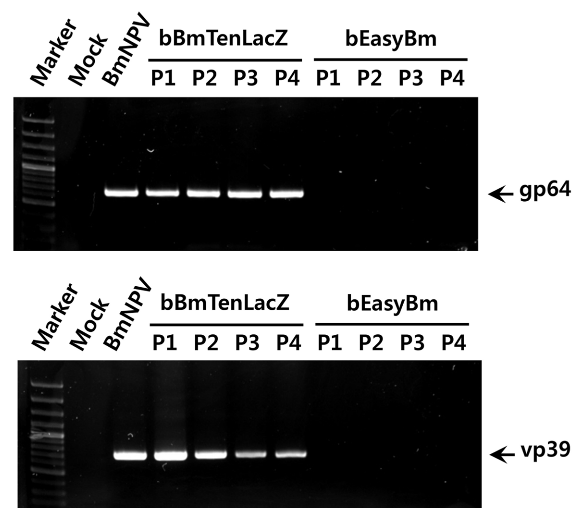

Fig. 3. Detection of viral mRNAs in Bm5 cells infected with bEasyBm during serial passages.

Passage numbers are indicated as “Pn”: “P” for passage and “n” for number.

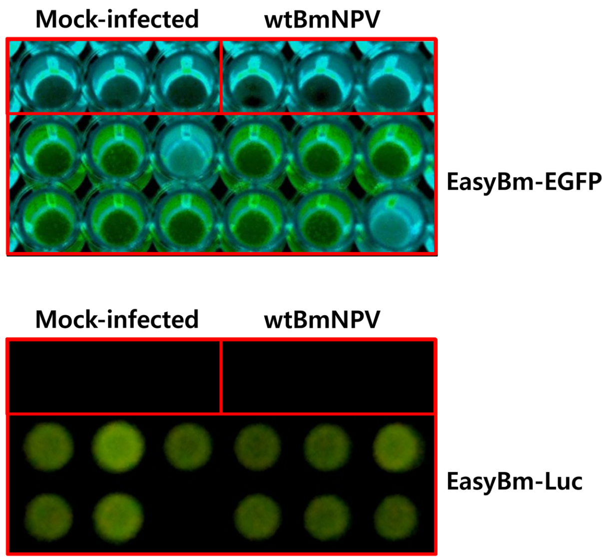

Fig. 4. Expression of foreign protein using the recombinant bacmid bEasyBm.

In situ assay of EGFP and luciferase in Bm5 cells infected with EasyBm-EGFP or EasyBm-Luc, respectively.

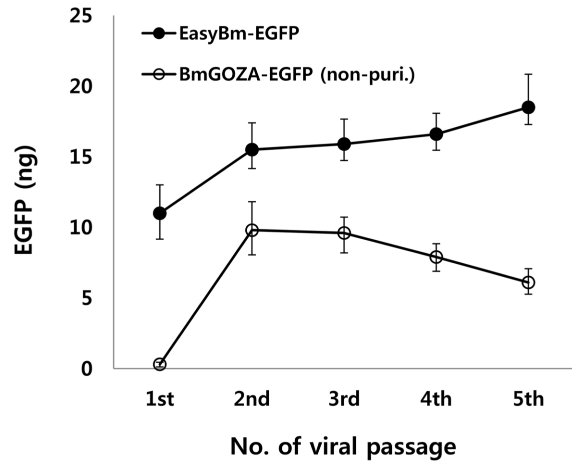

Fig. 6. Expression of EGFP in Bm5 cells infected with recombinant BmNPVs throughout serial passages.

EasyBm-EGFP and BmGOZA-EGFP were constructed using bEasyBm and bBmGOZA as parent viral genomes, respectively.

times in Bm5 cells. After the fifth passage in Bm5 cells, the viral DNA of EasyBm-EGFP was extracted from infected Bm5 cells, and PCR analysis was carried out for the barnase gene (EasyBm-EGFP) or the kanamycin resistance gene-Mini-F replicon (Mini-F) cassette (BmGOZA-EGFP), which are replaced by the *egfp* gene through *in vitro* transposition or homologous recombination. Although the kanamycin resistance gene-Mini-F replicon cassette was amplified from viral DNA of BmGOZA-EGFP, barnase gene-specific primers did not amplify any specific fragments from the viral DNA of EasyBm-EGFP after the second passage (Fig. 5).

To investigate the expression efficiency of foreign genes using bEasyBm, Bm5 cells were infected with recombinant viruses (EasyBm-EGFP and non-purified BmGOZA-EGFP), and the expression of EGFP in infected Bm5 cells during serial passages was compared (Fig. 6). Whereas Bm5 cells infected with non-purified BmGOZA-EGFP showed dramatically reduced levels of EGFP after the 3rd passage, Bm5 cells infected with EasyBm-EGFP showed high levels of EGFP from the 1st passage.

Discussion

A major bottleneck in expressing foreign genes using baculovirus expression systems is that these systems require somewhat complex and laborious processes for

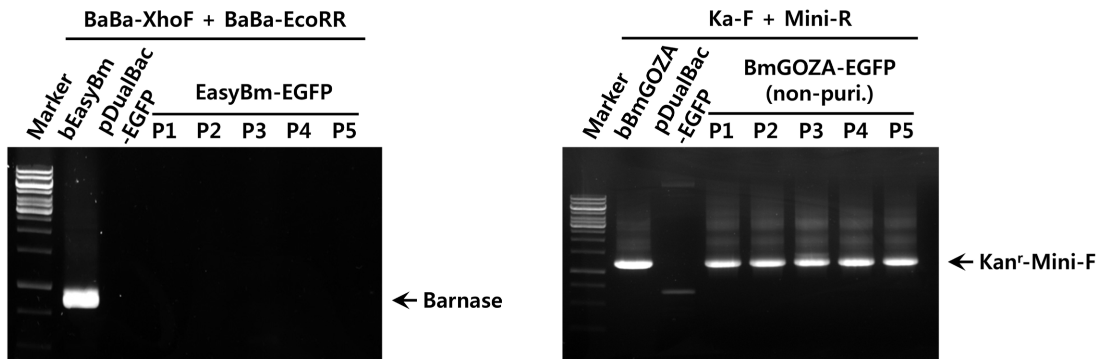

Fig. 5. Verification of the genomic structure of the recombinant viruses EasyBm-EGFP and BmGOZA-EGFP throughout serial passages.

Passage numbers are indicated as “Pn”: “P” for passage and “n” for number.

purification of target recombinant viruses from mixed populations produced by either transposition in *E. coli* cells or homologous recombination in insect cells. Previously, to achieve the fast and easy generation of recombinant baculovirus by removing extra steps for purification, we have constructed a recombinant bacmid, bEasyBac, which is the recombinant AcMNPV genome that could replicate in *E. coli* cells but not in insect cells and contained bacteriophage lambda site-specific attachment (*att*) sites for *in vitro* transposition [2].

Although cultured insect cells have been used most frequently as hosts for the production of foreign proteins in baculovirus expression systems, infected insect larvae could also be used. In this regard, one of the significant advantages of the BmNPV-based expression system is that its host insect, *Bombyx mori*, is characterized extremely well and methods for mass rearing of *B. mori* larvae are very well established. In addition, there are a number of possible advantages to produce foreign proteins in infected insect larvae. First, proteins expressed in insect larvae could be subject to a more diverse range of post-translational modifications than those expressed in cultured cells because there are a variety of different tissues having typically different capacities for post-translational modifications in insect larvae. This could have benefits for the production of functional or authentic proteins [8]. Second, the yields obtained in insect larvae could be up to 500-fold higher than those obtained in cultured cells [4, 11, 16]. Finally, expression using insect larvae represents a relatively low cost alternative to the production of a foreign protein using cultured cells [4, 8].

In the bEasyBm constructed in this study, the barnase gene was introduced into the BmNPV genome under the control of the CpBV early promoter. Barnase, a major extra-cellular ribonuclease isolated from *B. amyloliquefaciens*, is lethal to cells in which it is expressed [5, 15]. The CpBV promoter used for the expression of barnase showed activity earlier than the polyhedrin promoter, and the activity of some of these promoters was superior to the AcMNPV ie-1 promoter [3]. The bEasyBm can only replicate in transfected insect cells when the CpBV promoter-barnase cassette is replaced with the gene of interest by *in vitro* transposition. Therefore, no plaque purification or drug selection steps are required, and only recombinant virus is obtained after transfection of the *in vitro* recombination reaction between bEasyBm DNA and donor vector harboring the gene of interest, because the replication of nonrecombinant bEasyBm in insect cells is blocked by host cell death at an early stage of viral replication.

Insect cells showed cytopathic effects with reduced cell growth at 1–2 days p.t. when transfected with bEasyBm (Fig. 2). However, thereafter, the cells recovered growth rates comparable to mock-transfected cells because of the absence of viral replication and transmission through the death of insect cells in which barnase was expressed by bEasyBm. The replication of bEasyBm in transfected cells was not observed when RT-PCR on the total RNA extracted from transfected cells was carried out against baculovirus genes essential for viral replication (Fig. 3). This lack of replication was further proved by the result that the nonrecombinant bEasyBm background was not detected in non-purified viral stocks of EasyBm-EGFP (Fig. 5). Additionally, although the expression efficiency of non-purified stocks of BmGOZA-EGFP was reduced, the expression efficiency of non-purified stocks of EasyBm-EGFP was consistently maintained at a high level along serial passages. In addition, target recombinant viruses were generated with approximately 83.3–91.7% efficiency when the insect cells in a 12-well plate format were concomitantly transfected with 30 ng of the recombination reaction (Fig. 4). These results suggest that bEasyBm has an effective benefit, allowing for a high-throughput baculovirus expression vector without purifying recombinant viruses. Additionally, bEasyBm could be applied to automated systems for the concomitant expression of proteins from various origins.

Acknowledgments

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