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Increased productivity of the baculovirus expression vector system by combining enhancing factors

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ABSTRACT

The baculovirus expression vector system uses the polyhedrin promoter for the expression of foreign proteins. To increase the polyhedrin promoter activity, vectors were constructed by combining two transcription enhancers and the vp39 promoter. The transcription enhancers included homologous region 5 (hr5) and burst sequences (BSs). Seven vectors were constructed using combinations of these transcription enhancers, and their expression levels were compared in *Autographa californica* multicapsid nuclepolyhedrovirus (AcMNPV) and *Bombyx mori* NPV (BmNPV) using enhanced green fluorescent protein (EGFP). Our results indicate that most of the constructs increased the expression level of EGFP using the polyhedrin promoter. In particular, the vp39 promoter and BSs were the most effective in BmNPV and AcMNPV, respectively. Synergistic effects of the enhancing factors were observed only in AcMNPV by the vp39 promoter and BSs. The hr5 negatively affected the expression level of EGFP using the polyhedrin promoter and other enhancers in both viruses. The enhancing effect obtained by combining the three factors was higher in BmNPV than in AcMNPV. This was the first study to perform a comparative evaluation of enhancing factors that affect the expression efficiency of foreign protein using the polyhedrin promoter in both AcMNPV and BmNPV.

Introduction

The baculovirus expression vector system (BEVS) is widely used for the production of recombinant proteins using insect cells or larvae (Bae et al., 2017; Kidd and Emery, 1993; Kim et al., 2012; Morokuma et al., 2015). One of the great advantages of BEVS is that it produces recombinant proteins through post-translational modifications in the insect cell environment (Kidd and Emery, 1993; King and Possee, 1992). Unlike other expression systems, BEVS is based on the strong promoter of polyhedrin, which is abundantly produced by baculovirus (Ayres et al., 1994; Smith et al., 1985). However, the production efficiency of recombinant proteins using the polyhedrin promoter is not as high as that of polyhedrin (Bae et al., 2017). Numerous studies have aimed to solve this limitation and several methods have been proposed, such as the alteration of promoter sequences, the use of transcription enhancers, fusion expression with various tagging signals that show increased effects, and co-expression of regulatory proteins (Bae et al., 2017; Bleckmann et al., 2016; Gómez-Sebastián et al., 2014; Ishiyama and Ikeda, 2010; Kato et al., 2012; Liu et al., 2008; Manohar et al., 2010; Marumoto et al., 1987; Roh et al., 2010; Theilmann and Stewart, 1992; Tiwari et al., 2010). Although these methods increased the expression efficiency of foreign proteins using the polyhedrin promoter, they were not very effective. Among these studies, we noted the effects of transcription enhancers and the vp39 promoter due to their potentially synergistic effects (Bleckmann et al., 2016; Ishiyama and Ikeda, 2010; Kato et al., 2012; Manohar et al., 2010). The transcription enhancers involved in improving the polyhedrin promoter activity have been reported, included the homologous region (hr) (Bleckmann et al., 2016; Gómez-Sebastián et al., 2014; Theilmann and Stewart, 1992), burst sequence (BSs) (Kato et al., 2012; Manohar et al., 2010), and very late transcriptional factor 1 (VLF-1) (McLachlin and Miller, 1994). The hrs are dispersed throughout the baculovirus genome and are believed to act as origins of DNA replication. Among them, hr1, hr5, and hr6 have been reported to be related to an increase of the expression efficiency of foreign proteins using the polyhedrin promoter (Bleckmann et al., 2016; Gómez-Sebastián et al., 2014; Theilmann and Stewart, 1992). A BS is an A/T rich sequence located downstream of very late promoters consisting of approximately 50 bp and is required for a burst

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of expression. Using mutation analysis, it was shown that the BS is very important for the expression efficiency of foreign proteins using the polyhedrin promoter (Ooi et al., 1989). In addition, it has been reported that the expression efficiency of foreign proteins using the polyhedrin promoter could be increased by repeated BSs (Kato et al., 2012; Manohar et al., 2010). VP39 is a major baculovirus capsid protein of approximately 42 kDa and has a late gene promoter motif (Thiem and Miller, 1990). It has been reported that the vp39 promoter can be expressed at an earlier stage than the polyhedrin promoter, and thus the expression efficiency of foreign proteins using the vp39 promoter is higher than that using the polyhedrin promoter due to decreased damage to the host cell (Thiem and Miller, 1990; Ishiyama and Ikeda, 2010).

Therefore, in this study, we aimed to construct vectors with highly efficient expression through the combination of these transcription enhancers and the vp39 promoter. In addition, the increased expression efficiency was compared within *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* NPV (BmNPV). The results showed that the various combinations of these transcription enhancers and the vp39 promoter showed increased expression efficiency of foreign protein compared with the standard vector, and the effect was even greater in BmNPV. The BS and vp39 promoter were the most effective in AcMNPV and BmNPV, respectively, and hr5 showed a negative effect in both viruses. Our results suggest the construction of effective vectors for AcMNPV and BmNPV using several factors involved in enhancing the expression efficiency of foreign protein using the polyhedrin promoter.

Materials and methods

Cells and viruses

The *Spodoptera frugiperda* cell line IPLBSF-21(Sf21 cells) was maintained at 25 °C in SF900 II serum-free medium (Gibco). The *B. mori* cell line (Bm5) was maintained at 25 °C in TC-100 insect medium (WelGENE) supplemented with 10% fetal bovine serum. AcMNPV C6 and BmNPV-K1 were used in this study. Routine cell culture maintenance and virus production procedures were performed according to published procedures (King and Possee, 1992).

Construction of transfer vectors

The vp39 promoter, the polyhedrin promoter and 4 repeated BS sequences were synthesized (Macrogen), including several restriction enzyme sites required for cloning (Fig. 1, Fig. S1). The synthesized DNA fragment was digested with *Bam*HI and *Sma*I and was subsequently cloned into the corresponding restriction enzyme sites of the pHIP vector, which contains a hr5 sequence, resulting in pPol-1 (Fig. 1). The EGFP gene, which was amplified from pEGFP (Clontech) with the primer set EGFP-F (5'CCCGGGATGGTGAGCAAGGGC3')/EGFP-R (5' CTGCAGTTACTTGTACAGCTCGTC3'), was cloned into pMD20-T (Takara), resulting in pT-EGFP. pT-EGFP was digested with *Sma*I and *Pst*I and was subsequently cloned into the corresponding restriction enzyme sites of pPol-1 and pBacPAK9 (Clontech) to construct pPol-1-EGFP and pPol-EGFP, respectively. pPol-2-EGFP was constructed by the self-ligation of pPol-1-EGFP after digestion with *Nhe*I. pPol-1-EGFP was digested with *Bgl*II and *Bam*HI and then was self-ligated, resulting in pPol-3-EGFP. pPol-4-EGFP was constructed by the self-ligation of pPol-1-EGFP using a DNA blunting kit (Takara) after digestion with *Bam*HI and *Spe*I. pPol-5-EGFP was constructed by self-ligation of pPol-1-EGFP using the DNA blunting kit (Takara) after digestion with *Bgl*II and *Spe*I. pPol-2-EGFP was digested with *Bgl*II and *Bam*HI and then self-ligated, resulting in pPol-6-EGFP. pPol-7-EGFP was constructed by the self-ligation of pPol-2-EGFP using the DNA blunting kit (Takara) after digestion with *Bam*HI and *Spe*I.

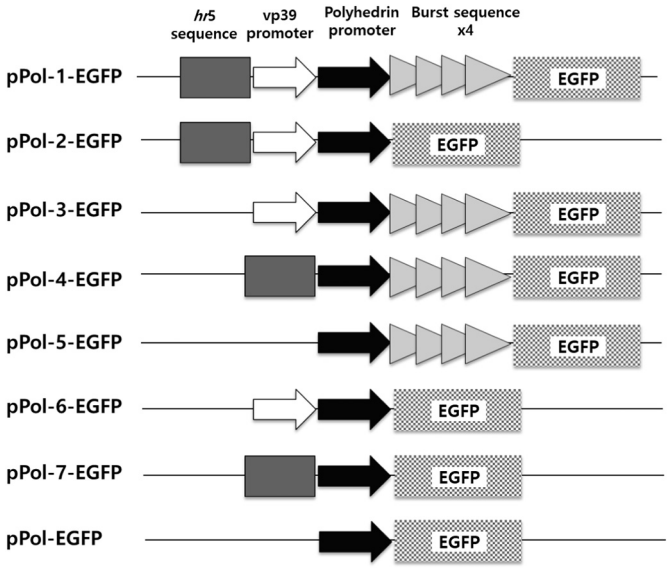

Fig. 1. Schematic representation of the transfer vector structure.

Generation of recombinant viruses

Recombinant AcMNPVs and BmNPVs were generated by co-transfection with each transfer plasmid and a defective viral genome, bApGOZA and bBpGOZA DNA, respectively (Je et al., 2001). Transfection was performed using Cellfectin II™ (Invitrogen) reagent according to the manufacturer's instructions, and the recombinant viruses were purified and propagated as described previously (O'Reilly et al., 1992).

SDS-PAGE and Western blot analysis

The infection for the analysis of recombinant EGFP was carried out at 1×10^6 cells per well in 6-well plates and infected at an MOI (multiplicity of infection) of 1. The expression of EGFP by the infected cells was observed using a fluorescence microscope (Sundew MCX1600, Micros) for 5 days with a one-day interval. For the protein analysis, the infected cells were collected at 3 days p.i. (post-infection) and washed with ice cold PBS. The cell lysate was prepared by incubating the cells with PBST (0.1% Triton-X 100 with PBS) containing a protease inhibitor cocktail (Sigma-Aldrich) for 30 min on ice followed by sonication. Next, the lysate was mixed with protein sample buffer and then boiled. The protein samples were subjected to 12% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 and probed with GFP monoclonal antibody (abm). The membrane was then incubated with horseradish peroxidase-coupled anti-mouse IgG antibody (Cell Signaling), and the bound antibodies were detected using the enhanced chemiluminescence system (Merck Millipore) according to the manufacturer's instructions.

Fluorescence spectrometer

Virus-infected cells were harvested by centrifugation at 1000 × *g* for 10 min, and the cell pellet was resuspended in 1 ml of PBS after washing with the same buffer. The lysate was prepared by incubating the cells with 900 µl of lysis buffer (20 mM Tris-HCl, 500 mM Nacl, 1 mM EDTA, 0.1% Tween 20, pH 7.0) for 30 min on ice followed by sonication. Immediately, 100 µl of 1 M sodium carbonate was added. The resulting mixture was incubated at 37 °C for up to 1 h, and then 2 ml PBS was added. Measurements were performed at room temperature in quartz cuvettes with a minimum test volume of 3 ml. The fluorescence