Expression of *Bombyx mori* Nucleopolyhedrovirus ORF4 under the Control of Baculovirus Iel Promoter by a Novel Bac-to-Bac/BmNPV Baculovirus Expression System

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Open reading frame 4 of *Bombyx mori* nucleopolyhedrovirus (BmNPV), designated as *Bm4*, is a gene whose function is completely unknown. With the recently developed BmNPV bacmid and a modified pFastBac1 whose polyhedrin promoter was replaced with iel promoter, a recombinant bacmid expressing Bm4-EGFP fusion protein under the control of iel promoter in BmN cells was successfully constructed. The result not only showed that the polyhedrin promoter can be replaced efficiently with other promoters to direct the expression of foreign gene in BmN cells by using Bac-to-Bac/BmNPV baculovirus expression system but also laid the foundation for rescue experiment of *Bm4* deletion mutant due to the ability of iel promoter to direct gene expression throughout the infection cycle.

Key words: expression, iel promoter, Bac-to-Bac/BmNPV baculovirus expression system, *Bombyx mori* nucleopolyhedrovirus, open reading frame 4, rescue experiment

Introduction

The baculovirus expression system has been employed widely as a powerful expression vector for the production of recombinant proteins under the control of the powerful very late promoters, p10 or polyhedrin promoter. Traditionally, recombinant baculoviruses were generated by homologous recombination in insect cells which takes at least 40 days because of multiple rounds of purification and amplification of viruses. Recently, a BmNPV bacmid system had been developed (Motohashi et al., 2005). In this system, recombinant baculoviruses were generated by site specific transposition in *E. coli* which needs no more than 10 days due to the elimination of multiple rounds of purification of viruses, and the BmNPV bacmid is infectious to Bm cell lines and silkworm. Some recombinant proteins have been produced in Bm cell lines or silkworms under the control of polyhedrin promoter by using this novel system, such as spider flagelliform silk protein and Superoxide dismutase (Miao et al., 2006; Yue et al., 2006). However, there is no recombinant protein reported to be produced under the control of promoters other than polyhedrin promoters by using this system. To investigate the feasibility of utilizing other promoters to drive the expression of recombinant proteins in this system, and thus to lay the foundation for the rescue experiment of *Bm4* deletion mutant, we generated a recombinant bacmid expressing Bm4-EGFP fusion protein under the control of iel promoter (ielp) rather than polyhedrin promoter.

Materials and methods

Materials

pFastBac1 and the *E. coli* DH10Bac/BmNPV were supplied by Prof. E.Y. Park and Prof. K. Maenaka. FuGENE 6 transfection reagent was the product of Roche Applied Science, USA. The Grace's insect cell culture medium (GIBCO) was purchased from Invitrogen.

Cell line

*B. mori* cell line, BmN originated from ovary, was preserved in our laboratory and cultured at 27°C with GIBCO medium.

Construction of recombinant donor plasmid pFastBac-
ie1p-Bm4-EGFP

With BmNPV genomic DNA as template, ie1 promoter and Bm4 were PCR amplified by using the following primers: primers for ie1p were (Forward: 5'-TCTTACG-TAGATTTCAGTTCGGGAGATAAA-3'; reverse: 5'-GTTTCAGGATCCCTGCGGCAATGTCAAA-3'; SnaBI and BamHI sites were underlined), and primers for Bm4 were (Forward: 5'-ATAGGATCCATGTTCTCGTGCAAAAGTTTAT-3'; reverse: 5'-GGGGTACCTTGAAAATGTTTATTTAAAA-3'; BamHI and KpnI sites were underlined). PCR product of ie1p promoter was digested with SnaBI and BamHI and then cloned into the SnaBI-BamHI sites of pFastBac to generate pFastBac-ie1p. PCR product of Bm4 was digested with BamHI and KpnI and ligated with pBacPAK-EGFP which was also digested with the same restriction enzymes to generate pBacPAK-Bm4-EGFP. Bm4-EGFP was excised from pBacPAK-Bm4-EGFP by digestion with BamHI and EcoRI, and then cloned into the BamHI- EcoRI sites of pFastBac-ie1p, to generate pFastBac-ie1p-Bm4-EGFP. Fig. 1 shows the construction of pFastBac-ie1p-Bm4-EGFP.

Construction and isolation of recombinant bacmid
pFastBac-ie1p-Bm4-EGFP was transformed into E. coli DH10Bac/BmNPV where transposition occurred. After a 6 h incubation at 37°C in SOC, transformed cells were
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Plated onto media containing 50 µg/ml of kanamycin, 7 µg/ml of gentamicin, 10 µg/ml of tetracycline, 100 µg/ml of Bluo-gal, and 40 µg/ml of isopropyl-β-D-thiogalactopyranoside (IPTG). Plates were incubated at 37°C for a minimum of 24 h. White colonies resistant to kanamycin, gentamicin, and tetracycline were selected, streaked onto fresh plates to verify the phenotype, and then confirmed by PCR with the M13 forward and M13 reverse primers. The PCR conditions were 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 5 min; and 1 cycle at 72°C for 10 min.

Recombinant bacmid confirmed by PCR was transfected into BmN cells using transfection reagent according to manual.

Detection of the expression of Bm4-EGFP fusion protein

BmN cells transfected with Recombinant bacmid were examined under fluorescent microscope for expression of Bm4-EGFP 72 h post transfection.

Results

Generation of pFastBac-ie1p-Bm4-EGFP

621-bp ie1 promoter and 1032-bp Bm4 were PCR amplified from BmNPV genomic DNA (Fig. 2, lane 4, 5, respectively). According to Lin and Blissard (2001), to replace polyhedrin promoter with ie1 promoter, pFastBac1 was digested with SnaBI and BamHI, removing a 153 bp fragment containing polyhedrin promoter, and then ligated with SnaBI-BamHI-digested ie1 promoter, leaving the multiple cloning site intact. pFastBac-ie1p-Bm4-EGFP digested with SnaBI and BamHI generated ~ 6.4 kbp pFastBac-Bm4-EGFP and 621 bp ie1 promoter instead of 153 bp fragment (Fig. 2, lane 2). When digested with BamHI and KpnI, pFastBac-ie1p-Bm4-EGFP generated three fragments: pFastBac-ie1p (~ 5.2 Kbp), Bm4 (1032 bp) and a fragment containing EGFP and the sequences between EcoRI site and KpnI site of pFastBac-ie1p-Bm4-EGFP (800 bp) (Fig. 2, lane 3). This is because there is another KpnI site besides the one between Bm4 and EGFP due to the cloning of Bm4-EGFP into BamHI-EcoRI sites of pFastBac-ie1p (Fig. 1). These results showed the successful construction of pFastBac-ie1p-Bm4-EGFP.

Identification of recombinant bacmid

Recombinant bacmid DNA is greater than 128 kb in size, so restriction analysis is difficult to perform with DNA of this size. PCR analysis was used to verify the presence of your gene of interest in the recombinant bacmid. The bacmid contains M13 Forward (-40) and M13 Reverse priming sites flanking the mini-artTn7 site within the lacZa-complementation region to facilitate PCR analysis (Fig. 3). Accordingly, PCR product of non-recombinant Bacmid was about 300 bp (Fig. 4, lane 3), and PCR product of recombinant bacmid was about 2800 bp (2300 + (621-
expression of Bm4-gfp fusion protein, these cells were examined by fluorescent microscope. Fluorescent signal was detected in most of the cells (Fig. 5), showing the successful expression of Bm4-EGFP fusion protein under the control of ie1 promoter in BmN cells.

**Discussion**

Given that His-tags in the N-termini of expressed proteins were susceptible to proteolytic removal in insect cells (Hu et al., 2006), and to quickly and easily detect the expression of Bm4-EGFP fusion protein, we used EGFP fused to the 3' terminus of Bm4 as reporter. The expression of Bm4-EGFP fusion protein could be detected by using fluorescent microscope without complicated assays (Fig. 5), and showed that ie1 promoter we used to replace polyhedrin promoter successfully drove gene expression.

Polyhedrin and p10 promoters, the most powerful very late promoters, are not always the best candidate promoters for driving gene expression. Jarvis et al. (1990) reported that, during the late phase of virus infection, the host cell secretory pathway was compressed by showing that t-PA expressed under ie1 promoter was processed faster and more efficiently in transformed Sf9 cells. Higgins et al. (2003) proved that the use of weaker promoters increase the expression of correctly assembled Shaker potassium channel in insect cells. Therefore, for those proteins that need extensive post-translational modifications, early promoters like ie1 promoter or weaker promoters may have advantages over polyhedrin promoter. Moreover, Hofmann et al. (1995) showed, for the first time, that recombinant baculoviruses containing a cytomegalovirus (CMV) promoter were able to transduce human hepatocytes and drive expression of a reporter gene under the control of this promoter. The ease of replacing polyhedrin promoter with other promoters by this novel system will for sure contribute to the discovery of other shuttle promoters between insect cells and mammalian cells. In addition, the ability of ie1 promoter to drive gene expression throughout the infection cycle makes it a perfect candidate promoter to be used in rescue experiment. Bm4 is a gene whose function is completely unknown. To study its function, a Bm4 deletion mutant should be generated first, and then a copy of Bm4 under the control of its own promoter or ie1 promoter should be inserted into the polyhedrin locus of the Bm4 deletion mutant in rescue experiment. However, because the promoter of Bm4 has not been characterized yet, a well-characterized promoter like ie1 promoter should be used as a positive control. According to Iwanaga et al. (2004), the expression level of Bm4 is high in early stage of infection.

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**Fig. 4.** Recombinant bacmid was analyzed by PCR with the M13 forward and M13 reverse primers. PCR products were electrophoresized on a 0.8% agarose gel. Lane 1, molecular marker (λ/HindIII); lane 2, PCR product of recombinant Bacmid; lane 3, PCR product of non-recombinant Bacmid; lane4, molecular marker (DL-2000).

**Fig. 5.** Expression of Bm4-EGFP fusion protein detected by fluorescent microscope

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Expression of Bm4-EGFP fusion protein

BmN cells transfected with recombinant bacmid showed signs of infection 72 h post transfection. To detect the...
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and peaks at 6 h post infection, making ie1 promoter a perfect promoter to drive the expression of Bm4 in rescue experiment due to the fact that ie1 promoter is active at the onset of infection without the help of viral factors. Accordingly, the successful expression of Bm4 under the control of ie1 promoter by the Bac-to-Bac/BmNPV baculovirus expression system lays the foundation for the rescue experiment of Bm4 deletion mutant.

To conclude, the ease of replacing polyhedrin promoter with the promoter of interest by this novel Bac-to-Bac/BmNPV baculovirus expression system will greatly facilitate promoter-related studies performed in Bm cell lines and silkworm.

References


