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Expression of *Bombyx mori* Nucleopolyhedrovirus ORF76 in Permissive and Non-permissive Cell Lines by a Novel Bac-to-Bac/BmNPV Baculovirus Expression System

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Abstract

Open reading frame 76 of *Bombyx mori* nucleopolyhedrovirus (BmNPV), designated as *Bm76,* is a gene whose function is completely unknown. With *EGFP* fused to the 3' terminal of *Bm76* as the reporter gene and BmNPV bacmid as the expression vector, a recombinant bacmid was successfully constructed expressing Bm76-EGFP fusion protein under the control of polyhedrin promoter in *Bombyx mori* cells (Bm cells), BmNPV's permissive cell line, laying the foundation for rescue experiment of *Bm76* deletion mutant. Moreover, the supernatant from Bm cells transfected with the recombinant bacmid was used to infect *Trichoplusia Ni* cells (Tn cells), BmNPV's non-permissive cell line. Unexpectedly, the expression of Bm76-EGFP fusion protein in some Tn cells was detected, implying that viral DNA was replicated in these cells. The causes are being studied for the inability of BmNPV to produce enough viable budded viruses in Tn cells despite of viral DNA replication.

Key words: Bac-to-Bac/BmNPV baculovirus expression system; EGFP; host range; ORF76; 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 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 of viruses. Luckow *et al.* (1993) developed an AcNPV bacmid system. In this system, recombinant baculoviruses were generated by site specific transposition in *Escherichia coli* which needs no more than 10 days due to the elimination of multiple rounds of purification of viruses. However, AcNPV bacmid is not infectious to Bm cells and silkworm, and thus AcNPV bacmid system can not be applied in Bm cells and silkworm. Recently, a BmNPV bacmid system had been developed, and the BmNPV bacmid is infectious to *Bombyx mori* cells (Bm cells) and silkworm (Motohashi *et al.*, 2005). Using this novel system, some recombinant proteins have been produced in Bm cell lines or silkworms, such as spider flagelliform silk protein and Superoxide dismutase (Miao *et al.*, 2006; Yue *et al.*, 2006).

Open reading frame 76 of BmNPV (*Bm76*, nt 71263-71748 of GenBank accession number NC_001962) is 486-bp in size and its function is completely unknown (Gomi *et al.*, 1999). Using BLAST in NCBI, we found that *Bm76* has homologues in almost all the sequenced baculoviurses. However, none of these homologues has been characterized. Moreover, there is no conserved motif in the predicted amino acid sequence of *Bm76* product. Therefore, in order to study its role, a *Bm76* deletion mutant should be generated first. There are many steps in the construction of a *Bm76* deletion mutant, and mutations may arise in other genes. Accordingly, we should confirm that the observed phenotype resulted from the deletion, not from second site mutations. To achieve this, we should construct a repair virus in which a copy of *Bm76* under the control of its native promoter or a positive promoter is inserted into the polyhedrin locus of the *Bm76* deletion mutant (Lin and Blissard, 2002). However, *Bm76* promoter has not been characterized yet, and a positive promoter should be used. According to Iwanaga

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et al. (2004), *Bm76* has a similar expression pattern with polyhedrin gene. Therefore, polyhedrin promoter can be used as a positive promoter in repair virus.

In this study, in order to lay the foundation for the rescue experiment of *Bm76* deletion mutant, we investigated the feasibility of expressing *Bm76* under the control of polyhdrin promoter in Bm cells by using this novel system. Moreover, with Bm76-EGFP as reporter gene, we explored the infection of *Trichoplusia Ni* cells (Tn cells) with the recombinant bacmid expressing Bm76-EGFP.

Experimental

Material and Methods

Plasmids and cell line. Plasmid pFastBac1 and the *E. coli* DH10Bac/BmNPV were supplied by Prof. E.Y. Park and Prof. K. Maenaka (Motohashi *et al.*, 2005). pBacPAK-EGFP was previously constructed in our laboratory (unpublished). Bm cell line, originated from ovary, was preserved in our laboratory and cultured at 27°C with GIBCO medium supplemented with 10% fetal bovine serum.

Reagent and medium. FuGENE 6 transfection reagent was the product of Roche Applied Science, USA. The Grace's insect cell culture medium (GIBCO) was purchased from Invitrogen.

Construction of recombinant donor plasmid pFastBac-Bm76-EGFP. With BmNPV genomic DNA as template, *Bm76* was PCR amplified by using the following primers: forward: 5'-ATAGGATCCATGGC GACTAGCAAAAC-3'; reverse: 5'-GACGGTACCA TTTACAATTTCAATTCCAAT-3' (BamHI and KpnI sites were underlined). The PCR product of *Bm76* was digested with BamHI and KpnI and then cloned into BamHI-KpnI sites of PUC18 to generate PUC-Bm76. PUC-Bm76 was sequenced, and then *Bm76* was excised from PUC-Bm76 by digestion with BamHI and KpnI. The excised *Bm76* was cloned into BamHI-KpnI sites of pBacPAK-EGFP to generate pBacPAK-Bm76-EGFP. *Bm76-EGFP* was excised from pBacPAK-Bm-EGFP by digestion with BamHI and EcoRI, and then cloned into the BamHI-EcoRI sites of pFastBac1, to generate pFastBac-Bm76-EGFP.

Construction and isolation of recombinant bacmid. pFastBac-Bm76-EGFP was transformed into *E. coli* DH10Bac/BmNPV where transposition occurred. After 6-h incubation at 37°C in SOC medium, transformed cells were plated onto LB media containing 50 μg/ml of kanamycin, 7 μg/ml of gentamicin, 10 μg/ml of tetracycline, 100 μg/ml of X-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. Bacmid DNA was isolated by using the FlexiPrep kit (Amersham Pharmacia Biotech) and then analyzed by PCR with the M13 forward and M13 reverse primers. The PCR conditions were 1 cycle at 94°C for 5 min; 35 cycles

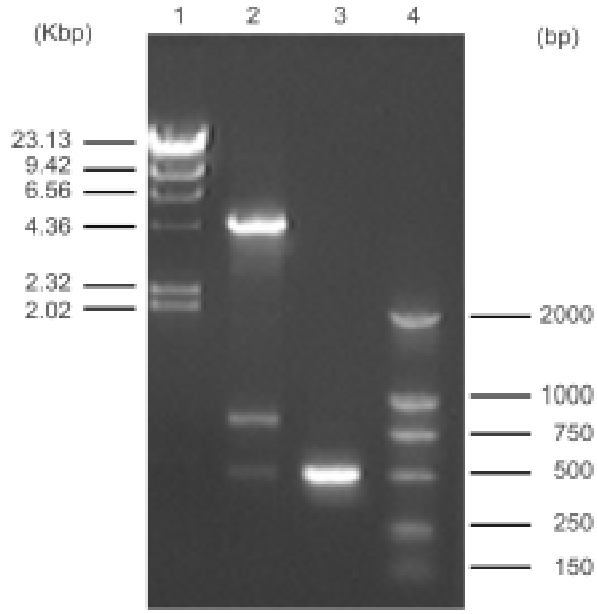

Fig. 1. Electrophoresis identification of recombinant pFastBac-Bm76-EGFP and PCR product. Lane 1, molecular marker (λ/HindIII); Lane 2, pFastBac-Bm76-EGFP digested by BamHI and KpnI; Lane 3, PCR product of *Bm76*; Lane 4, molecular marker (DL-2000)

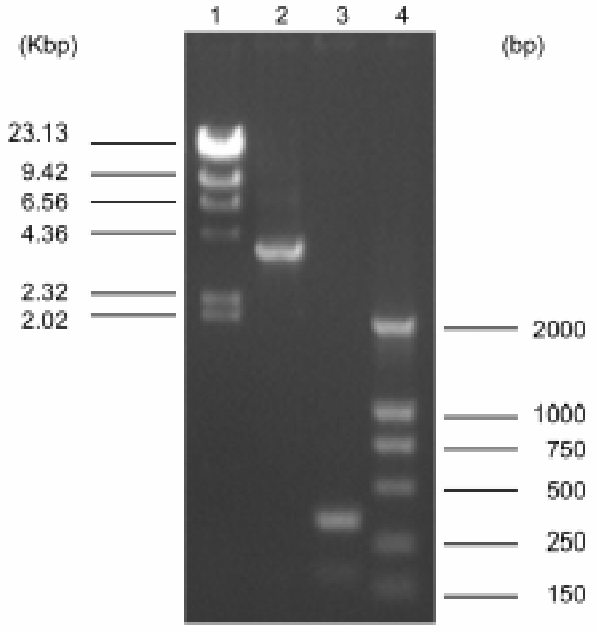

Fig. 2. Recombinant bacmid was analyzed by PCR with the M13 forward and M13 reverse primers. PCR products were electrophoresized on a 0.8% agarose gel. Lane 1, molecular marker (λ/HindIII); lane 2, PCR product of recombinant bacmid; lane 3, PCR product of non-recombinant bacmid; lane 4, molecular marker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

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 comfirmed by PCR was transfected into Bm cells using FuGENE 6 transfection reagent according to manual.

Results

Generation of pFastBac-Bm4-EGFP. 483-bp *Bm76* was PCR amplified from BmNPV genomic DNA (Fig. 1, lane 3). pFastBac-Bm76-EGFP digested with BamHI and KpnI generated three fragments: pFastBac (4.7 kp), *Bm76*(483 bp) and a KpnI-KpnI fragment containing EGFP (800 bp) (Fig. 1, lane 2). This is because EGFP is flanked by two KpnI sites due to the cloing of Bm76-EGFP into BamHI-EcoRI sites of pFastBac1, leaving the KpnI site of pFastBac1 intact. These results showed the successful construction of pFastBac-Bm76-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 identify recombinant bacmid. The bacmid contains M13 forward (–40) and M13 reverse priming sites flanking the mini-*att*Tn7 site, facilitating PCR analysis. If bacmid is not transposed with donor plasmid, PCR product of the bacmid (non-recombinant bamcid) was about 300 bp (Fig. 2, lane 3). If the bacmid is transposed with donor plamid, PCR product of the bacmid (recombinant bacmid) was about 2000 + 300 bp plus the size of the insert. Therefore, PCR product of recombinant bacmid, the bacmid transposed with pFastBac-Bm76-EGFP, was about 3.5 kb (Fig. 2, lane 2).

Expression of Bm76-EGFP fusion protein in Bm cells. At 72 h post transfection, Bm cells transfected with recombinant bacmid showed signs of infection such as detachment of cells from the disk and rounding of cells (Fig. 3A). To detect the expression

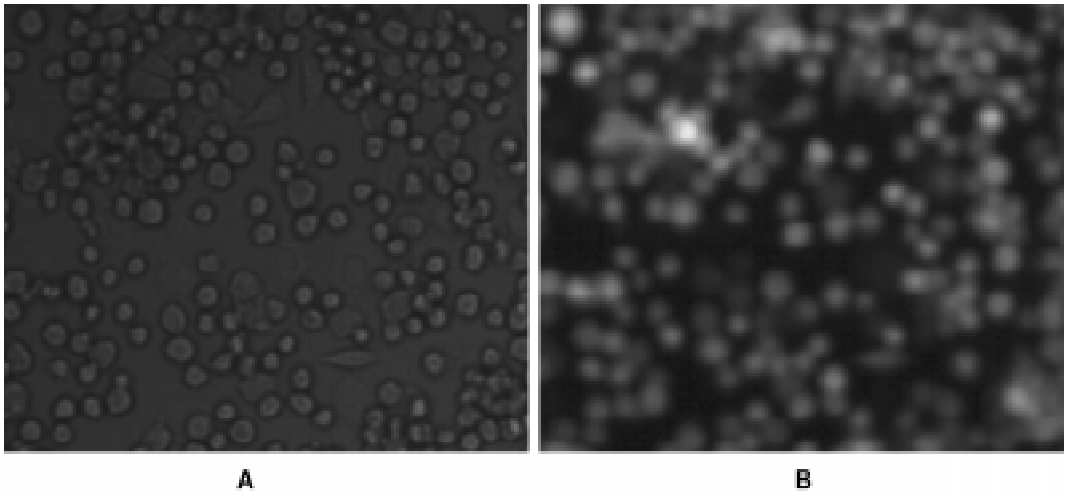

Fig. 3. Bm cells transfected with the recombinant bacmid were observed under bright field illumination (A) and under blue light illumination (B)

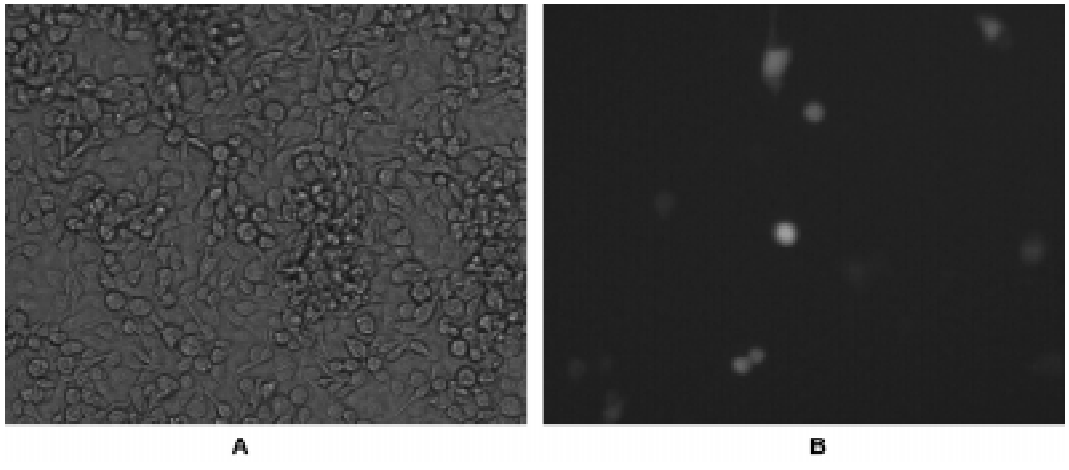

Fig. 4. Tn cells infected with the supernatant from Bm cells transfected with the recombinant bacmid were observed under bright field illumination (A) and under blue light illumination (B)

of Bm76-EGFP fusion protein, cells were examined by fluorescent microscope. Fluorescent signal was detected in most of the cells (Fig. 3B), showing the successful expression of Bm76-EGFP fusion protein under the control of polyhedrin promoter in Bm cells.

Expression of Bm76-EGFP fusion protein in Tn cells. The supernatant from Bm cells transfected with the recombinant bacmid was used to infect Tn cells. At 72 h post infection, Tn cells showed no notable sighs of infection (Fig. 4A). However, fluorescent signal was observed in some Tn cells when Tn cells were examined by fluorescent microscope (Fig. 4B).

Discussion

To quickly and easily detect the expression of *Bm76*, we used *EGFP* fused to the 3' terminal of *Bm76* as the reporter gene. The expression of Bm76-EGFP fusion protein could be detected by using fluorescent microscope without complicated assays (Fig. 3B and 4B).

The strong fluorescent signal in Bm cells (Fig. 3B) showed that the expression of Bm76-EGFP fusion protein could be driven efficiently by polyhedrin promoter at the polyhedrin locus, laying the foundation for the rescue experiment of *Bm76* deletion mutant. According to Iwanaga *et al.* (2004), apart from *Bm76*, many BmNPV genes such as ORF47, ORF121 and ORF122 have a similar expression pattern with polyhedrin promoter. Therefore, polyhedrin promoter can be also used as a positive promoter to drive the expression of these genes in repair viruses.

Late and very late promoters are activated after viral DNA replication (Durantel *et al.*, 1998), and polyhedrin promoter is very late promoter. Therefore, the expression of Bm76-EGFP fusion protein under the control of polyhedrin promoter in some Tn cells (Fig.4B) showed that viral DNA replication was carried out in these cells. However, fluorescent signal was observed in only a small number of Tn cells, indicating that few viable budded viruses, if any, were produced despite of viral DNA replication. The result is consistent with the lack of cytopathic effects observed (Fig.4A). However, our result is a little different from that of Woo *et al.* (2007) who observed pronounced cytopathic effects in Tn cells infected with recombinant BmNPV strain (BmNPV-K1) harboring the *E. coli* lacZ gene rather than the polyhedrin gene. The difference may be caused by different BmNPV strains we used. The possibility is supported by the fact that the BmNPV-K1 Woo *et al.* used also caused more pronounced cytopathic effects in Sf9 cells than the strain used by Martin and Croizier (1997). To gain more knowledge on host range, the causes are being studied for the inability of BmNPV to produce enough viable budded viruses in Tn cells.

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