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Subcellular Localization of Bm4, the Protein Product of ORF4 of Bombyx Mori Nucleopolyhedrovirus

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Abstract

Bm4, the protein product of open reading frame 4 (*Bm4*) of *Bombyx mori nucleopolyhedrovirus* (BmNPV), is a protein whose function is completely unknown. To unravel its biological function, its subcellular localization is a first crucial step. In order to study the subcellular localization of Bm4 in the context of BmNPV infection, we used a recently developed BmNPV bacmid, which is infectious to Bm cell lines, to express Bm4-EGFP fusion protein. With *EGFP* fused to the 3' terminus of *Bm4* as the reporter gene and by using a confocal scanning microscope, we found that the green fluorescent signal was localized primarily in the nucleus of Bm cells, however, a weaker signal was also observed in the membrane. Furthermore, bioinformatic analysis showed that there was no nuclear localization signal (NLS) or transmembrane helices (TMHs) in the amino acid sequence of Bm4, indicating that Bm4-EGFP fusion protein may be colocalized with host- or virus-encoded factors in the nucleus, and that Bm4 may have the ability to bind to certain protein located in the membrane. The western blot analysis using the antibody against EGFP detected only a specific band of about 67 kDa, which is consistent with the theoretical size of Bm4-EGFP fusion protein, confirming that *EGFP* was correctly fused to *Bm4*. The localization of Bm4-EGFP fusion protein in the nucleus and membrane implies that Bm4 may have two different functions.

Key words: Bac-to-Bac/BmNPV Baculovirus Expression System; EGFP; fusion protein; nuclear localization; membrane localization

INTRODUCTION

The *Baculoviridae* is a large family of viruses that infect invertebrates, particularly insects of the order *Lepidoptera*, and is characterized by large, double stranded DNA genomes and rod-shaped enveloped nucleocapsids [1,2]. According to the occlusion body morphology, the family can be divided into two genera: granuloviruses (GVs) and nucleopolyhedroviruses (NPVs). The latter can be further divided into single-nucleopolyhedroviruses (SNPV) and multiple-nucleopolyhedroviruses (MNPV) based on the number of nucleocapsids per virion [3]. *Bombyx mori nucleopolyhedrovirus* (BmNPV) is a representative of SNPV, which has a genome of 128 kb that encodes approximately 136 genes [4]. Due to the high identity in nucleotide sequences, functions of many of these genes are deduced from their counterparts in *Autographa californica nucleopolyhedr ovirus* (AcNPV), which is the prototype member of the Baculoviridae family. However, many genes of AcNPV have not been characterized so far, including the open reading frame 11(*Ac11*), the counterpart of *Bm4* [4]. *Bm4 and Ac11* are both 1020 bp in size [4]. Using BLAST in NCBI, we found that *Bm4 and Ac11* share 96% identity in nucleotide sequence and have their counterparts in three other baculoviruses including *Plutella xylostella multiple nucleopolyhedr ovirus, Rachiplusia ou multiple nucleopolyhedr ovirus and Maruca vitrata* MNPV. Furthermore, in the promoter region of *Bm4*, there exists an early promoter motif (TATA box followed by CAGT motif 20-25 bp downstream), which is consistent with the fact that its expression level is high in the early stage of infection and decreases in the late stage [4, 5]. Accordingly, *Bm4* is an early gene, which may play an important role in the early stage of virus infection.

Like AcNPV bacmid system, the recently developed BmNPV bacmid system uses site specific transposition in *E. coli* which takes no more than 10 days to generate recombinant baculovirues, however, BmNPV bacmid is infectious to Bm cell lines and silkworm [6]. Some recombinant proteins have been produced in Bm cell lines or silkworms by using this novel system, such as spider flagelliform silk protein and Superoxide dismutase [7, 8], showing the system is a good expression system to express recombinant proteins in Bm cell lines and silkworm.

In order to gain more knowledge of *Bm4*, we localized its protein product Bm4 by using BmNPV bacmid as expression vector and *EGFP* as reporter gene.

MATERIALS AND METHODS

Materials

pFastBac1 and the *E .coli* DH10Bac/BmNPV were supplied by Prof. E.Y. Park and Prof. K. Maenaka. pBacPAK-EGFP and recombinant bacmid expressing EGFP was constructed by our lab. FuGENe 6 transfection reagent was the product of Roche Applied Science, USA. The Grace's insect cell culture medium (GIBCO) was purchased from Invitrogen.

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-Bm4-EGFP

With BmNPV genomic DNA as template, *Bm4* was PCR amplified by using the following primers: Forward: 5'-ATAGGATCCATGTCTCTCGCTGCAAAGT-3'; reverse: 5'-ACCTTGTAAATGTTTATTATTTAAAA-3' (*Bam*HI and *Kpn*I sites were underlined). The PCR product of *Bm4* was digested with *Bam*HI and *Kpn* I 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-Bm-EGFP by digestion with *Bam*HI and *Eco*RI, and then cloned into the *Bam*HI- *Eco*RI sites of pFastBac1, to generate pFastBac-Bm4-EGFP.

Construction and isolation of recombinant bacmid

pFastBac-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 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 comfirmed by PCR was transfected into BmN cells using transfection reagent according to manual.

Western blot of Bm4-EGFP fusion protein

BmN Cells transfected with recombinant bacmid expressing EGFP and Bm4-EGFP fusion protein were harvested at 72 h post transfection, washed twice in 1× phosphate-buffered saline (PBS) and lysed in SDS-PAGE loading buffer by boiling for 5 min to completely denature the proteins. The cellular debris was removed by centrifugation at 12,000 rpm for 10 min. The samples were separated by SDS-PAGE using 10% polyacrylamide and analyzed by Western blot with antibody against EGFP.

RESULTS

Generation of pFastBac- Bm4-EGFP

1020-bp *Bm4* was PCR amplified from BmNPV genomic DNA (Figure.1, lane 3). pFastBac-Bm4-EGFP digested with *Bam*HI and *Kpn*I generated three fragments: pFastBac (4.7 kp), *Bm4* (1020 bp) and a *Kpn* I-*Kpn*I fragment containing EGFP (800 bp) (Figure.1, lane 2). This is because EGFP is flanked by two *Kpn*I sites due to the cloing of Bm4-EGFP into *Bam*HI-*Eco*RI sites of pFastBac1, leaving the *Kpn*I site of pFastBac1 intact. These results showed the successful construction of pFastBac-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 the gene of interest in the recombinant bacmid. The bacmid contains M13 Forward (-40) and M13 Reverse priming sites flanking the mini-*att*Tn7 site which is a target site for bacterial transposition, facilitating PCR analysis. Accordingly, PCR product of non-recombinant Bacmid was about 300 bp (Figure.2, lane 3), and PCR product of recombinant bacmid was about 2300bp plus the size of the insert. Therefore, PCR product of recombinant bacmid (the bacmid transposed with pFastBac-Bm4-EGFP) was about 4.1 kb (Figure.2, lane 2), showing the successful construction of recombinant bacmid.

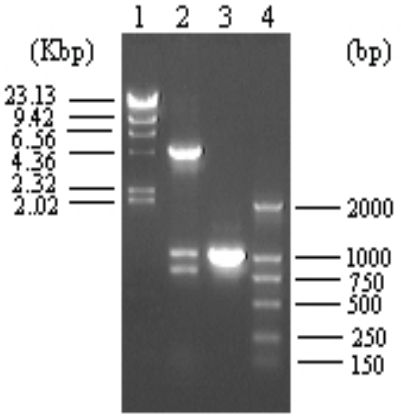

Figure 1. Electrophoresis identification of recombinant pFastBac-Bm4-EGFP and PCR products. Lane 1, molecular marker (λ/Hind III); Lane 2, pFastBac-Bm4-EGFP digested by *Bam*HI and *Kpn*I; Lane 3, PCR product of *Bm4*; Lane 4, molecular marker(DL-2000).

Subcellular localization of Bm4-EGFP fusion protein

To study subcellular localization of Bm4-EGFP fusion protein, BmN cells transfected with recombinant bacmid expressing Bm4-EGFP fusion protein were examined by a confocal laser scanning microscope at 72 h post transfection. Figure.3 showed that green fluorescent signal was primarily localized in the nucleus of BmN cells, however, a weaker signal was also observed in the membrane.

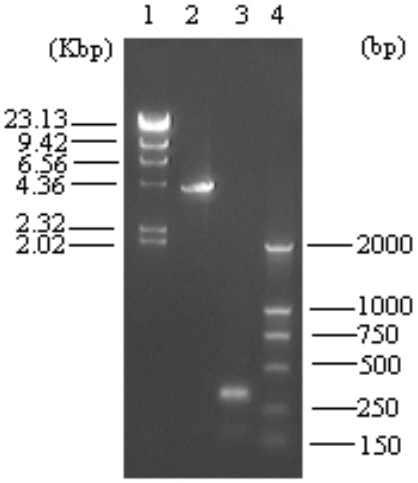

Figure 2. Recombinant bacmid was analyzed by PCR

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

Analysis of Bm4-EGFP fusion protein by western blot

To confirm the correct fusion of Bm4 with EGFP, a western blot analysis was performed using antibody against EGFP. While a band of about 27 Kda was detected in the total proteins of BmN cells tranfected with recombinant bacmid expressing EGFP (Figure.4, lane 1), a band of about 67 Kda was detected in the total proteins of BmN cells transfected with recombinant bacmid expressing Bm4-EGFP fusion protein(Figure.4, lane 2). According to the amino acids sequence of Bm4, Bm4 has a predicted molecular weight of 39.6 Kda. Therefore, the result confirmed the correct fusion of Bm4 with EGFP.

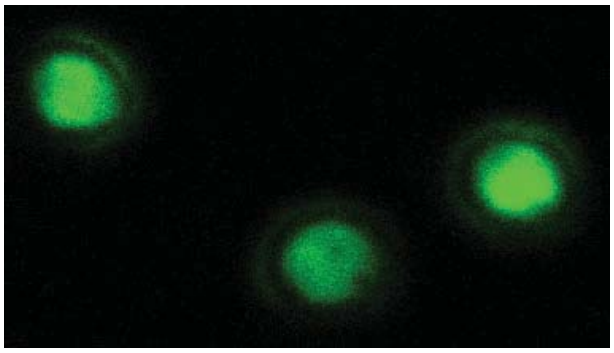

Figure 3. Subcellular localization of Bm4-EGFP fusion protein. Using a confocal scanning microscope, **g**reen fluorescent signal was found to be primarily localized in the nucleus of BmN cells, however, a weaker signal was also observed in the membrane.

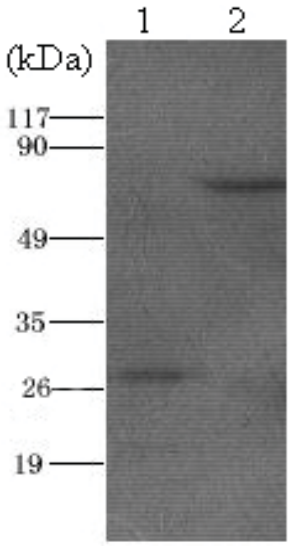

Figure 4. Western blot analysis of total proteins of BmN cells transfected with recombinant bacmid using antibody against EGFP. Lane 1, total proteins of BmN cells transfected with recombinant bacmid expressing EGFP; Lane 2, total proteins of BmN cells transfected with recombinant bacmid expressing Bm4-EGFP fusion protein.

DISCUSSION

GFP is fluorescent and can be monitored non-invasively by external illumination under fluorescent microscope without adding external substrates. Therefore, GFP can be used to localize GFP fusion proteins in living cells, thus eliminating potential fixation artifacts. Moreover, due to its low molecular weight, GFP is easy to fuse with other genes without influencing their own respective structure and function, thus making it a widely used fluorescent tag for studying the localization in living cells [9]. Accordingly, EGFP, which is mutated from GFP, was used as the reporter gene in our study.

As mentioned in the introduction, BmNPV bacmid is infectious to Bm cell lines and silkworm, and therefore, to study the subcellular localization of Bm4-EGFP fusion protein in Bm cells in the context of BmNPV infection, we used BmNPV bacmid to express Bm4-EGFP fusion protein under the control of polyhedrin promoter. By using confocal laser scanning microscope, we found that fluorescent signal was localized primarily in the nucleus of infected BmN cells, however, a weaker signal was also observed in the membrane (Figure.3). According to Iwanaga et al. [5], the expression level of *Bm4* reaches 40.5 % at 2 h post infection, and peaks at 6 h post infection, suggesting that *Bm4* may be involved in activities preceding or during virus DNA replication, which is consistent with its nuclear localization. By using "PredictNLS server (http://cubic.bioc.columbia.edu/predictNLS/)", we found no nuclear localization signal (NLS) in the sequence of Bm4, indicating that Bm4-EGFP fusion protein may be colocalized with host- or virus-encoded factors in the nucleus. The presence of Bm4-EGFP fusion protein in the membrane suggests that Bm4 may play a role in the membrane; however, TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) finds no transmembrane helices (TMHs) in the sequence of Bm4, indicating that Bm4 may have the ability to bind to certain protein located in the membrane. Some baculorvirus proteins, such as ODV-EC27, P35 and VLF-1, have shown to be multifunctional [10, 11, 12], and the localization of Bm4-EGFP fusion protein in the nucleus and membrane implies that Bm4 may have two different functions. To unravel the biological function of *Bm4*, a *Bm4* knockout mutant of BmNPV is being constructed.

Acknowledgements

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