Establishment of the BacMam system using silkworm baculovirus

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Abstract

The BacMam system uses modified insect viruses (baculoviruses) as vehicles to efficiently deliver genes for expression in mammalian cells. The technique can be widely applied to large-scale recombinant protein production with appropriate modifications, high-throughput screening platforms for cell-based assays, and the delivery of large genes. The silkworm system is often employed as a rapid and cost-effective approach for recombinant baculovirus generation. Here we have developed the novel BacMam system using silkworm baculovirus, and shown the successful expression of EGFP in mammalian cells. The transduction to mammalian cells via the BacMam system was improved by adding phosphate-buffered saline and sodium butyrate to the culture medium and lowering the temperature after viral infection. This study provides an alternative gene delivery system for mammalian cells, which has various potential applications, including efficient native protein production and gene therapy.

Keywords:

BacMam; silkworm; *Bombyx mori*; baculovirus; BmNPV; mammalian expression.

1. Introduction

Many methods are available for gene delivery into mammalian cells, including the use of plasmid and viral vectors. The plasmid transfection method is widely used [1-3] because it is easy to perform and many kinds of vectors as well as transfection reagents are commercially available. However, the size of the vectors and the transfection efficiency are limited, and transfection reagents are expensive. Viral vectors, such as retroviruses and adenoviruses, may activate oncogenes by the random insertions of foreign genes into the host genome or exhibit cytotoxicity due to the viral proteins produced in the host cells [4, 5].

Baculoviruses, which intrinsically infect insect cells, have a large, double stranded DNA genome (~130 kb) and are capable of carrying a sizable foreign DNA fragment (>15 kb) [6]. Thus, they have been conventionally used for the production of recombinant proteins that are difficult to produce in bacterial expression systems [7, 8]. The baculovirus-insect cell expression system has additional advantages for the expression of eukaryotic proteins, since the posttranslational modifications in insects are similar to those of mammals, and such modifications are sometimes necessary for the biological activities. In addition, baculoviruses are much safer than the other viruses utilized for gene transduction, because they show neither a harmful immune response nor

pathogenicity in mammalian cells, due to the lack of replication and the maintenance of genome integrity in the host cells [9]. Therefore, baculoviruses are an attractive tool for gene transduction.

There are two major types of baculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV). AcMNPV is widely utilized for recombinant protein production, with various options of vectors and systems [10-12]. However, we have previously shown that the combination of BmNPV and silkworm larvae or pupae has advantages for large-scale protein production, in terms of cost, ease of use and safety [6]. To maintain silkworms, only a basic incubator and food are required, and a bio-safety cabinet and expensive culture medium with serum are not needed. The baculovirus-silkworm expression system usually provides a high level of recombinant protein expression, by the high-titer baculoviruses amplified inside the silkworm. Indeed, using the BmNPV-silkworm larvae expression system, we and others have successfully produced G protein coupled receptors (GPCRs), the functionally active human immune cell receptor, KIR2DL1, the human prorenin receptor and sufficient amounts of active protein kinase B (PKB) for structural analysis [13-16]. Notably, the silkworm system is attractive because it enables the generation of high-titer viruses during the preparation of the recombinant proteins, as described above,

and is faster, easier and less costly than the use of the AcMNPV-insect cell expression system. Collectively, the use of BmNPV and silkworm is suitable not only for recombinant protein production but also for the large-scale preparation of recombinant viruses with high-titers.

The BacMam system, baculovirus-mediated gene transduction of mammalian cells, has been developed as a relatively new technique utilizing a modified baculovirus as a gene delivery vehicle to mammalian cells [17-19]. The technique is currently used in various fields, such as recombinant soluble protein production and high-throughput screening platforms. The AcMNPV-mediated BacMam (AcBacMam) system reportedly functions in mammalian host cells and has advantages in terms of efficacy, cost, safety and host ranges [17, 20]. Here we focused on both the BacMam technique and BmNPV, to establish the BmNPV-mediated BacMam (BmBacMam) system. We constructed the recombinant baculovirus BmNPV that encodes EGFP under a mammalian expression promoter (BmNPV-EGFP) using silkworm larvae, and successfully expressed EGFP efficiently in mammalian cells with optimized recombinant BmNPV-EGFP transduction conditions. Our system provides an alternative tool to transduce genes of interest into mammalian cells.

2. Materials and Methods

2.1. Insects, cells and materials

The fifth instar silkworm larvae were purchased from Ehime-Sanshu (Japan) and were grown at 25 °C with moderate humidity in the incubator. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂, unless particular growth conditions are mentioned. The pFastBacTMDual vector was purchased from Invitrogen, and pCA7-EGFP [21] was kindly donated by Prof. Yanagi.

2.2. Construction of the donor plasmid, recombinant bacmid and virus

To develop the BacMam system, we constructed the donor plasmid, designated as pFastBacDual-PCAG-EGFP. The fragment encoding the CAG promoter sequence [22] and enhanced green fluorescent protein (EGFP) was digested from pCA7-EGFP [21] for insertion next to the p10 promoter and the following multiple cloning site in pFastBacTMDual (Invitrogen) (Fig. 1). The polyhedrin promoter was amplified by PCR. The amplified fragment and the digested PCAG-EGFP fragment were ligated into

pFastBacTMDual. The DNA sequence was confirmed with an ABI3100 sequencer (Applied Biosystems). Plasmid preparation was performed with a Nucleo Bond Xtra Midi kit (TaKaRa), according to the manufacturer's instructions.

We then transformed *Escherichia coli* (*E. coli*) BmDH10Bac [6], which contains the viral cysteine protease- and chitinase-deficient BmNPV bacmid, with pFastBacDual-PCAG-EGFP. The positive transformants were monitored by blue-white selection and colony PCR using M13 forward (-40) and M13 reverse (-20) primers. Appropriate transformants were grown and the recombinant bacmid DNA was extracted using the same protocol as for plasmid preparation.

One µg of the recombinant bacmid DNA, bacmid-EGFP, was diluted in 50 µL of ultra-pure water, and 3 µL of DMRIE-C (Invitrogen) were added per silkworm larva. After an incubation at room temperature for 45 min, the bacmid - DMRIE-C mixture was injected into the silkworm larva. Six days after the bacmid injection, the silkworm hemolymph was collected, filtered through a 0.45 µm PVDF filter (Millipore), and precipitated by centrifugation at 45,000 x g for 30 min. The resultant pellet was resuspended in an appropriate volume of PBS and stored at 4 °C, as the virus (BmNPV-EGFP) stock solution.

2.3. Virus titer determination by Q-PCR

The recombinant BmNPV titer was determined by quantitative real-time PCR (Q-PCR), as previously described [15]. Briefly, the virus stock solution was diluted with an equal volume of resuspension buffer (10 mM Tris-HCl, pH 7.5, 10 μ M EDTA, 0.25% SDS), and then the virus genome DNA was extracted with phenol-chloroform. Using the virus genome DNA as the template and the *ie-1* gene specific primer set [15], Q-PCR was performed with KAPA SYBR FAST (Kapa Biosystems) and a CFX96 Touch™ real-time PCR detection system (Bio-Rad). Recombinant AcMNPV, with a titer determined by a plaque assay, was used to make the standard curve. PCR amplifications and melting curves were analyzed with the CFX Manager version 2.1 software (Bio-Rad).

2.4. Introduction of the EGFP gene into mammalian cells via BmNPV

HEK293T cells (4×10^4) were seeded in a 24-well plate one day prior to the virus infection. The culture medium was changed to the transduction medium containing PBS and additives, as indicated in the Figures or their legends, and BmNPV-EGFP was added to the cells at various multiplicity of infection (MOI) values, as indicated in the Results section or the Figure legends. EGFP expression was observed with a fluorescent microscope, IX71N-23 IFL/DIC (OLYMPUS).

2.5. Flow cytometric analysis

At different time points after infection, the transduced cells were harvested, washed with PBS and subsequently stained with propidium iodide (PI). The cells were subjected to flow cytometry, and the percentages of EGFP expressing cells were evaluated. Flow cytometry experiments were performed using a FACS Calibur flow cytometer (BD Biosciences), and the obtained data were analyzed with the CellQuest software (BD Biosciences). In total, 10,000 cells were counted. Each single cell with appropriate size and granularity was gated, and GFP and PI fluorescent emissions were detected by FL1-H and FL3-H detectors, respectively. The PI stained cells were excluded from the GFP expressing cells, and the rest of the cells were regarded as GFP positive cells.

3. Results and Discussion

To establish the BmBacMam system, we modified our BmNPV-silkworm expression system [6, 14] by replacing the authentic insect promoter with the eukaryotic promoter.

We first constructed the donor plasmid, which encodes the EGFP gene under the CAG promoter cassette in pFastBacTMDual (Fig. 1). Using this donor vector, the recombinant bacmid was generated in *E. coli* BmDH10Bac, which harbors the BmNPV bacmid DNA lacking cysteine protease and chitinase to avoid liquefaction of silkworm larvae [23, 24]. The CAG promoter and EGFP gene were transposed into the BmNPV genome in the *E. coli* BmDH10Bac cells. The recombinant bacmid was purified and injected into silkworm larvae (Fig. 1). Approximately 0.5 mL of hemolymph containing the recombinant baculovirus, named BmNPV-EGFP, was extracted from one silkworm larva. The baculoviruses were purified by ultracentrifugation and resuspended in a volume of PBS equal to that of the harvested hemolymph.

We applied the Q-PCR method to determine the titer of BmNPV-EGFP [15]. We used AcMNPV, with a titer of 8.6×10^6 pfu/mL previously determined by a conventional plaque assay, for the standard curve generation (Fig. 2A). The standard curve was obtained in a good linear correlation between PCR cycles and titers, with a correlation efficiency of 0.99 (Fig. 2B). The diluted BmNPV genome DNA was subjected to the Q-PCR reaction, and the titer of BmNPV-EGFP was determined to be 1.0×10^9 pfu/mL (Fig. 2C). The quality of the Q-PCR reaction was validated by the melting curve as well as the agarose gel electrophoresis analyses of the Q-PCR products (Fig. 2 D and E). The

titer of BmNPV was equivalent to or even higher than that of the P3 virus of AcMNPV.

We successfully obtained the high titer BmNPV in only 6 days from silkworm larvae.

At first, BmNPV-EGFP was simply added at an MOI of 100 to the HEK293T cells, and then the cells were cultured in conventional medium, 10%FCS-DMEM. However, the EGFP expression was hardly observed even 6 days after the addition of the BmNPV-EGFP (Fig. 3A). We then increased the MOIs to 250, 500 and 1,250. More EGFP expressing cells were observed as the MOI increased (Fig. 3B). However, the EGFP expression level was still low, even at an MOI of 500. Therefore, we tried to optimize the BmNPV transduction by changing the culture conditions. The use of PBS, instead the normal medium DMEM, reportedly improved the efficacy of AcBacMam transduction to some types of cells, including HEK293 cells [25-27]. Thus, we first tested various concentrations of DMEM and PBS at an MOI of 500 (Fig. 3C). As the PBS proportion increased (DMEM and serum concentrations decreased), higher EGFP expression was observed, especially in 80% PBS, 18% DMEM, and 2% FCS (Fig. 3C, image). At different time points, we quantified the GFP positive cells by flow cytometry, which revealed that the percentage of GFP positive cells was the highest in 80% PBS, 18% DMEM, and 2% FCS, as observed by microscopy, and the EGFP positive cells reached a saturated level around 3 to 6 days after virus infection (Fig. 3C, graph). The

saturation level decreased as the PBS concentration decreased. The trend of the GFP expression level against the different PBS concentrations was similar to that of the different MOI conditions (MOI = 100), although the proportion of GFP positive cells decreased as the MOI decreased (Fig. 3C, graph). Although the addition of PBS improved the EGFP expression level, it is important to determine which step was the rate-determining step for BmNPV-mediated gene expression, the viral entry into the host cells or the translation in the host cells. To address this, we examined the effect of the exposure time of the baculovirus on the EGFP expression. For this, BmNPV-EGFP was added at an MOI = 250, and at several time points (2, 6, 12, 24, 48, 72, and 144 hours) after infection, the medium was exchanged to either normal medium or that containing 80% PBS without any viruses. The EGFP expression was observed at 6 days (144 hours) after the addition of BmNPV. If the viral entry was the rate-determining step, then the EGFP expression would be observed only after long-term exposure of the virus. In contrast, EGFP expression would be detected even after a short exposure time if the protein expression was rate-determining. As shown in Figure 3D, the cells should be exposed to BmNPV for at least 3 days for efficient EGFP expression. This suggested that the viral entry is likely to be the rate-determining step for BmNPV transduction. The addition of PBS did not influence the EGFP expression at all under these conditions (Fig.

3D).

Next, we tested the addition of sodium butyrate, a histone deacetylase inhibitor (HDACI), since HDACIs reportedly enhanced protein expression in mammalian cells transduced by the AcMNPV-mediated BacMam, as well as by transient transfection [17, 20, 28]. We tested various concentrations of sodium butyrate and PBS in the media at an MOI = 100. Images were obtained after 6 days, and revealed that the EGFP intensity was increased when more than 2 mM sodium butyrate was added under the 40%, 60%, and 80% PBS conditions, while concentrations of sodium butyrate less than 1 mM did not alter the EGFP expression level (Fig. 4A). It is noteworthy, however, that the cells tended to detach and die under the 10 mM sodium butyrate and 80% PBS conditions. Quantitative analyses indicated that the conditions with 4 mM sodium butyrate and 60% PBS were the best for EGFP expression (Fig. 4A).

Finally, we tested the temperature for transduction, since BacMam transduction was reportedly improved by lowering the temperature [17]. After the addition of BmNPV-EGFP at an MOI = 100, the cells were incubated at either 30 °C or 37 °C under the indicated medium conditions. After a seven day incubation, we found that the EGFP expression was higher at 30 °C than at 37 °C under almost all of the conditions tested (Fig. 4B), indicating that the decreased temperature during transduction is effective. Taken

together, unfavorable conditions for the cells, such as poor nutrition and low temperature, may facilitate BmBacMam transduction. This is essentially similar to the case for AcBacMam, as reported previously [16].

To compare the EGFP expression efficiency and the time between plasmid transfection and BmNPV transduction, the time courses of EGFP expression were examined between HEK293T cells transfected with pFastBacDual-PCAG-EGFP or transduced by BmNPV-EGFP. In this experiment, after BmNPV-EGFP (MOI = 500) or the EGFP plasmid (0.5 μ g) was introduced into the HEK293T cells, the cells were maintained for 6 days for sufficient gene expression. The culture medium containing either the viral or plasmid vector was then replaced with fresh medium (normal DMEM medium with 10% FBS) without any vectors, 6 days after the addition of BmNPV-EGFP or transfection. At this time point, we started to observe EGFP expression as day 0 and continued our observations with different time points, as indicated in Figure 4C. The EGFP expression level was evaluated relative to that at day 0. The EGFP expression lasted at a similar level for at least 6 days for the BmNPV transduction, whereas it gradually attenuated for the plasmid transfection and was roughly half after 6 days (Fig. 4C). These data suggested that BmBacMam may be applicable to the long-term expression of a transgene.

Although many virus vector systems, such as retrovirus and adenovirus vectors, have been used for gene delivery to mammalian cells, the BmBacMam system has advantages in terms of safety, gene size capacity and cost. Baculoviruses can carry large foreign genes, unlike most of the other viral vectors, including Adenovirus associated virus (AAV) and Sendai virus. Thus, the BmBacMam system is applicable for the production of proteins with large sizes, multiple subunits or polycistronic systems. In addition, the baculovirus genome DNA does not integrate into the host cell genome, and thus there is little possibility of the activation of host oncogenes, unlike retroviruses.

The AcMNPV-mediated BacMam system efficiently functions in mammalian host cells [17, 18, 20]. However, the preparation of a high titer AcMNPV stock usually takes a few weeks or a month. In contrast, only 6 days were needed to obtain BmNPV with a high titer (10^9 pfu/mL), equivalent to that of AcMNPV P3 virus. As we expected, the BmNPV-silkworm combination for the virus amplification allowed us to shorten this time consuming step for the AcMNPV-insect cell combination. Moreover, in the case of HEK293T cells, BmBacMam efficiently expressed EGFP under low nutrition conditions, indicating that less medium and serum are required for transduction, as compared to the normal inoculation of cells.

We have shown, for the first time, that BmNPV could transduce a gene of

interest into mammalian cells, and we have established the optimum transduction conditions. As discussed above, the BmBacMam system has several advantages over the conventional viral and non-viral transgene methods. The transfection system described in this study may provide an excellent tool for the scientific research as well as the industrial and medical fields.

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Figure legends

Figure 1: Strategy of the BmBacMam system. Construction and preparation of the recombinant BmNPV-EGFP using silkworm larvae, and the strategy for the BacMac transduction to mammalian cells.

Figure 2: Determination of BmNPV titer by Q-PCR. (A) The fluorescent signals during Q-PCR cycles using serially diluted samples of the AcMNPV stock. The titer of the AcMNPV used for the reaction was determined to be 8.6×10^6 pfu/mL by a conventional plaque assay. (B) Standard curve of the cross points versus the serially diluted titer of the AcMNPV determined in (A). (C) The fluorescent signals during Q-PCR cycles using serially diluted samples of the BmNPV stock. The titer of the BmNPV stock was determined using the cross points data and the standard curve obtained in (B). Representative curves of the fluorescent signals are shown. (D) Melting curve analysis of the Q-PCR products. The black line represents the products from the BmNPV genome, and the gray line represents those from the AcMNPV genome. (E) Q-PCR products from the BmNPV and AcMNPV genomes were analyzed by agarose gel electrophoresis. Lane 1: Q-PCR products from AcMNPV, lane 2: Q-PCR products from

BmNPV, lane 3: 100 bp DNA Ladder.

Figure 3: BmBacMam-mediated EGFP expression in HEK293T cells. (A) Time course of the BmNPV-EGFP transduction. EGFP expression was observed at 1, 3, and 6 days after the addition of BmNPV-EGFP. (B) The effect of MOI on the BmNPV-EGFP transduction. EGFP expression was observed 6 days after the infection with BmNPV-EGFP. Cells were maintained under the normal conditions, DMEM with 10% FCS, at 37 °C and with 5% CO₂. (C) The effect of PBS on the BmNPV-EGFP transduction. Representative images of the EGFP expression in HEK293T cells at an MOI = 500. HEK293T cells were transduced with BmNPV-EGFP at an MOI = 500 for EGFP expression under different medium conditions (medium containing 0, 20, 40, 60 and 80% PBS). The images were captured at the indicated time points. Quantitative analysis of the percentages of EGFP expressing cells. The percentages of EGFP positive cells were plotted against the different time points post-infection. The filled symbols and lines indicate the data of MOI = 500, and the open symbols and dashed lines are the data of MOI = 100. (D) The effect of the viral exposure time on the BmNPV-EGFP transduction. HEK293T cells were transduced with BmNPV-EGFP at an MOI = 250, and at the indicated times, the virus containing medium was exchanged to fresh medium

containing the indicated percentage of PBS. The images were taken 144 hours (6 days) after viral infection.

Figure 4: Investigation of the BmBacMam transduction conditions and time course

comparison between plasmid- and baculovirus-mediated transductions. (A) The

effect of different concentrations of sodium butyrate, a histone deacetylase inhibitor, on the BmNPV-EGFP transduction. The experiments were performed with different PBS concentrations. Representative images were obtained 6 days after transduction. The data

were quantified using CellQuest. (B) The effect of the temperature after viral infection on the BmNPV-EGFP transduction. Comparison of the EGFP expression levels at different

growth temperatures. The cells were incubated at either 30 or 37 °C after the baculoviruses were added to the cells. The images were captured by fluorescent

microscopy 7 days after transduction, and representative images are shown. (C)

Persistence of the EGFP expression by the different transduction methods. EGFP

expression in HEK293T cells was induced by plasmid or baculovirus. Six days after transduction, the DNA containing medium was exchanged to fresh medium without any

vectors (day 0). Cells were maintained in medium containing either 0% or 80% PBS. The

images were taken at the different time points indicated in the figure, and representative

images are shown. EGFP positive cells were counted by a FACS Calibur flow cytometer.

The quantified data were normalized relative to the data on day 0.

Figure 1

Figure 2

Figure 3

Figure 4