Expression of recombinant proteins by BEVS in a macula-like virus-free silkworm cell line

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31 **ABSTRACT**

32 We previously established the first *Bombyx mori* macula-like virus (BmMLV)-free cell
33 line (BmVF cells) from a *B. mori* embryo. In this study, we evaluated the expression of
34 recombinant proteins in BmVF cells using a *B. mori* nucleopolyhedrovirus
35 (BmNPV)-derived expression vector. Our results showed that BmVF cells are
36 susceptible to BmNPV, and both the promoter activity of the *polyhedrin* gene and the
37 post-translated modifications of a recombinant protein are equivalent between
38 BmMLV-negative BmVF and -positive BmN4 cells. These findings indicate that
39 persistent infection with BmMLV has no discernible effect on BmNPV-mediated protein
40 production in *B. mori* cells.

41

1. Introduction

The *Bombyx mori* macula-like virus (BmMLV) is a member of the genus *Maculavirus*, family *Tymoviridae*, and contains a positive-strand RNA genome. Previously, we reported that a *B. mori* ovary-derived cell line, BmN4, was persistently infected with BmMLV (Katsuma et al., 2005). The replication strategy of BmMLV is largely unclear. Interestingly, almost all *B. mori*-derived cell lines have already been contaminated with BmMLV via an unknown infection route (Iwanaga et al., 2012). Recently, we established the first BmMLV-negative *B. mori* cell line, BmVF, derived from a *B. mori* embryo (Iwanaga et al., 2012).

B. mori nucleopolyhedrovirus (BmNPV) is a member of the group I *Alphabaculovirus* and is used for the Baculovirus Expression Vector System (BEVS). Since many of the post-translational modifications present in the eukaryote are performed in baculovirus-infected cells, the BmNPV-based BEVS is frequently used for the expression of recombinant proteins that are biologically active and functional as well as for a native protein (reviewed in Rohrmann, 2008). For the BmNPV-based BEVS, *B. mori*-derived cell lines that are susceptible to BmNPV have become more important tools for the

58 generation and/or propagation of recombinant NPVs. Therefore, the contamination of *B.*
59 *mori* cell lines with BmMLV will cause a serious problem in safety aspects of the
60 production of recombinant proteins. However, it is still unclear whether BmMLV-free
61 BmVF cells are available in the BmNPV-based BEVS.

62 In this study, to assess the effect of BmMLV infection on production of recombinant
63 proteins by the BEVS, properties of recombinant proteins expressed in
64 BmMLV-negative BmVF and -positive BmN4 cells were compared. In addition, we
65 attempted to culture the BmVF cells in a serum-free medium (SFM).

2. Materials and methods

2.1. Cells, virus, and plaque assay

BmVF and BmN4 cell lines were maintained as described previously on IPL-41 and TC-100 media supplemented with 10% fetal bovine serum (FBS), respectively (Iwanaga et al., 2004, 2012). To generate BmMLV-free BmNPV, viral genome DNA was extracted from occlusion bodies (OBs) or budded viruses (BVs) of BmNPV T3 (Maeda et al., 1985) and introduced into BmVF cells by transfection (Iwanaga et al., 2012). At 120 hours posttransfection, a culture medium containing BVs was collected and subjected to a plaque assay on BmN4 cells as appropriate to determine viral titers (Iwanaga et al., 2004). The cells were infected with BmMLV-free BmNPV at a multiplicity of infection (MOI) of 10. The BmMLV-free recombinant BmNPVs expressing a firefly luciferase or mouse interleukin-3 (mIL-3) under the control of a *polyhedrin* promoter, Luc-Bm or IL-Bm (Nakanishi et al., 2010), were generated as described above. Statistical analysis was carried out using an unpaired *t*-test with KaleidaGraph computer software version 4.1 (Synergy Software, Reading, PA, USA).

2.2. Western blot analysis

For Western blotting, proteins were electrophoresed, transferred on a Hybond-ECL membrane (GE Healthcare, Buckinghamshire, UK), and probed with antibodies of BmMLV COAT PROTEIN (CP) (Iwanaga et al., 2012), BmNPV POLYHEDRIN (Shimada et al., 1994), β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or FLAG tag (Sigma-Aldrich, St. Louis, MO, USA). The anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (KPL, Gaithersburg, MD, USA) and Western blotting substrate plus (Thermo Fisher Scientific, Waltham, MA, USA) were used. Signal detection was carried out using the LightCapture II system (ATTO, Tokyo, Japan).

2.3. Luciferase assay

The cells were inoculated with the recombinant BmNPV (Luc-Bm), which expresses a firefly luciferase under the control of a *polyhedrin* promoter (Nakanishi et al., 2010). After inoculation, the cells were collected at designated times postinfection. A luciferase reporter assay was carried out using a Bright-Glo luciferase assay system (Promega, Madison, WI, USA), and luciferase activity was measured using a Wallac ARVO SX

1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA), as described previously
(Iwanaga et al., 2014a).

2.4. Expression of *mIL-3*

The cells were inoculated with the recombinant BmNPV (IL-Bm), which expresses a
mIL-3 under the control of a *polyhedrin* promoter (Nakanishi et al., 2010). After
inoculation, the cells were treated with tunicamycin (10 µg/ml, Wako Pure Chemical,
Osaka, Japan) or dimethylsulfoxide (DMSO). The cells and the cultured media were
collected at 72 hours postinfection (hpi) and subjected to Western blot analysis, as
described previously (Iwanaga et al., 2014a).

2.5. Adaptation of *BmVF* cells to serum-free medium

BmVF cells were adapted to an SFM in a stepwise manner, as described previously
(Iwanaga et al., 2014b). At the beginning of the adaptation to the SFM, 20% of the culture
medium was transferred every four days to four kinds of SFMs (insect serum-free media
PB, OM, BY, Shima Laboratory, Tokyo, Japan; Sf900-II, Life Technologies, Carlsbad,

114 CA, USA) to progressively remove serum from the culture. After 10 passages, all of the
115 culture medium was transferred to the SFMs, and FBS in IPL-41 was eliminated
116 completely. At this time, the PB medium was selected as a suitable SFM to grow BmVF
117 cells. Then 90% of the culture medium was transferred to the PB every four days.
118

3. Results and discussion

To elucidate the susceptibility to BmNPV infection, BmVF cells were inoculated with BmNPV. As shown in Fig. 1A, a typical formation of occlusion bodies (OBs) was observed in BmNPV-infected BmVF cells. Additionally, Western blot analysis revealed that viral POLYHEDRIN was expressed abundantly from 48 hpi in virus-infected BmVF cells, whereas the expression of host ACTIN declined as the infection proceeded (Fig. 1B). On the other hand, there was no signal of BmMLV coat protein (CP) in BmMLV-negative BmVF cells, while strong signals were detected in BmMLV-positive BmN4 throughout BmNPV infection (Fig. 1B). Furthermore, to compare the ability of virus multiplication between BmVF and BmN4 cells, the titers of BV were measured by plaque assay. As shown in Fig. 1C, the BV titer in the culture media of BmVF cells increased rapidly from 24 hpi and reached a plateau between 72 and 120 hpi. There was no significant difference for BV titers between these cell lines. Collectively, the present results indicate that BmMLV-negative BmVF cells are permissive to BmNPV, and virus propagation in BmVF cells is comparable to that in BmMLV-positive BmN4 cells.

To compare the promoter activity of the *polyhedrin* gene in BmVF and BmN4 cells, cells were inoculated with Luc-Bm, which expresses a firefly luciferase (Nakanishi et al.,

2010). As shown in Fig. 2A, the *luciferase* gene was expressed rapidly at 72 hpi in Luc-Bm-infected BmVF cells. The maximum luminescence was observed at 96 hpi and maintained throughout virus infection. The expression of luciferase was almost the same between these cell lines (Fig. 2A), indicating that the activity of the *polyhedrin* promoter that is most used in the BEVS is comparable in these two cell lines.

To evaluate the post-translational modification of the recombinant protein produced by the BEVS in BmMLV-free BmVF cells, cells were inoculated with IL-Bm, which expresses mIL-3 (Nakanishi et al., 2010). As shown in Fig. 2B, a lower molecular weight form of mIL-3 was observed in the lysate of BmVF cells treated with tunicamycin, indicating that BmVF cells glycosylated mIL-3. This result was consistent with the data for BmN4, which has been reported previously (Miyajima et al., 1987). In addition, similar signals were detected clearly from not only the cell lysate but also the culture media of both *B. mori* cell lines (Fig. 2B), indicating that BmVF cells secreted mIL-3 efficiently, as observed in BmN4 cells. These results indicate that the presence or the absence of BmMLV does not affect proper post-translational modification or secretion in BmNPV-infected *B. mori* cells.

Previously, we reported that several *B. mori* cell lines were maintainable in an SFM (Imanishi et al., 2012; Iwanaga et al., 2014b). SFM-adapted cells offer a solution to a wide variety of problems, including high cost; risk of contamination by mycoplasma, prion, or virus; low reproducibility; poorly defined composition; and difficulties in downstream processing in the use of FBS. Therefore, to adapt BmVF cells to an SFM, the cell culture medium containing 10% FBS was transferred to four kinds of commercial SFMs in a stepwise manner. During cell adaptation to the SFM, the induced cell aggregation was observed in Sf900-II- or OM-adapted BmVF cells (Fig. 2C). On the other hand, there was no morphological change in PB- or BY-adapted cells. After 100 passages, the BmVF cells showed reproducible growth in the PB medium, and thus we designated them as BmVF-SFM. BmVF-SFM is suitable for a BmNPV-based BEVS with serum-free conditions.

B. mori cell lines that are susceptible to BmNPV are important for the generation and/or propagation of recombinant BmNPV. However, almost all *B. mori* cell lines have been contaminated with BmMLV (Iwanaga et al., 2012). Our findings indicated that BmMLV-free BmVF cells are permissive to BmNPV, and BmVF cells would be

applicable for a novel BmMLV-free BmNPV-based BEVS. In particular, BmNPV-based BEVS is utilized not only in *B. mori* cell lines but also in *B. mori* larvae. As BmMLV has not been detected in *B. mori* larvae to date, the recombinant BmNPVs produced in BmVF cells could be subjected to large-scale protein expression using *B. mori* larvae under a BmMLV-free condition.

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180 **References**

- 181 Katsuma, S., Tanaka, S., Omuro, N., Takabuchi, L., Daimon, T., Imanishi, S., Yamashita,
182 S., Iwanaga, M., Mita, K., Maeda, S., Kobayashi, M., Shimada, T., 2005. Novel
183 macula-like virus identified in *Bombyx mori* cultured cells. J. Virol. 79, 5577-5584.
- 184 Imanishi, S., Kobayashi, J., Sekine, T., 2012. Serum-free culture of an embryonic cell line
185 from *Bombyx mori* and reinforcement of susceptibility of a recombinant BmNPV by
186 cooling. In Vitro Cell. Dev. Biol. Animal 48, 137-142.
- 187 Iwanaga, M., Takaya, K., Katsuma, S., Ote, M., Tanaka, S., Kamita, S.G., Kang W.,
188 Shimada, T., Kobayashi, M., 2004. Expression profiling of baculovirus genes in
189 permissive and nonpermissive cell lines. Biochem. Biophys. Res. Commun. 323,
190 599-614.
- 191 Iwanaga, M., Hitotsuyama, T., Katsuma, S., Ishihara, G., Daimon, T., Shimada, T.,
192 Imanishi, S., Kawasaki, H., 2012. Infection study of *Bombyx mori* macula-like virus
193 (BmMLV) using a BmMLV-negative cell line and an infectious cDNA clone. J. Virol.
194 Methods 179, 316-324.
- 195 Iwanaga, M., Shibano, Y., Ohsawa, T., Fujita, T., Katsuma, S., Kawasaki, H., 2014a.

196 Involvement of HSC70-4 and other inducible HSPs in *Bombyx mori*
 197 nucleopolyhedrovirus infection. Virus Res. 179, 113-118.

198 Iwanaga, M., Adachi, Y., Uchiyama, K., Tsukui, K., Katsuma, S., Kawasaki, H., 2014b.
 199 Long-term adaptation of the *Bombyx mori* BmN4 cell line to grow in serum-free
 200 culture. In Vitro Cell. Dev. Biol. Anim., DOI: 10.1007/s11626-014-9781-y, June 17,
 201 2014.

202 Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Furusawa, M.,
 203 1985. Production of human interferon in silkworm using a baculovirus vector. Nature
 204 315, 592-594.

205 Miyajima, A., Schreurs, J., Otsu, K., Kondo, A., Arai, K., Maeda, S., 1987. Use of the
 206 silkworm, *Bombyx mori*, and an insect baculovirus vector for high-level expression
 207 and secretion of biologically active mouse interleukin-3. Gene 58, 273-281.

208 Nakanishi, T., Goto, C., Kobayashi, M., Kang, W.K., Suzuki, T., Dohmae, N., Matsumoto,
 209 S., Shimada, T., Katsuma, S., 2010. Comparative studies of lepidopteran
 210 baculovirus-specific protein FP25K: development of a novel *Bombyx mori*
 211 nucleopolyhedrovirus-based vector with a modified fp25K gene. J. Virol. 84,

212 5191-5200.

213 Rohrmann, G.F., 2008. Baculovirus Molecular Biology. National Library of Medicine
214 (US), National Center for Biotechnology Information, Bethesda (MD).

215 Shimada, T., Chan, I.C., Noguchi, Y., Nagata, M., Kobayashi, M., 1994. Structural and
216 functional abnormality of the polyhedrin gene in a polyhedrin-deficient mutant of the
217 *Bombyx mori* nuclear polyhedrosis virus. J. Seric. Sci. Jpn. 63, 353-360.

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Figure legends

Figure 1

Susceptibility of BmVF cells to BmNPV. (A) The morphology of BmMLV-negative BmVF and BmMLV-positive BmN4 cells at 84 hours post-BmNPV-infection. The scale bar is 50 μ m. (B) Expression of *B. mori*, BmMLV, and BmNPV proteins. BmVF and BmN4 cells were inoculated with BmNPV. Extracts were prepared from 1×10^5 mock- or BmNPV-infected cells harvested at the indicated times postinfection and were subjected to SDS-PAGE followed by Western blotting. Arrowheads indicate BmACTIN (ACTIN), BmMLV CP (CP), and BmNPV POLYHEDRIN (POLH). (C) Growth curves of BmNPV in *B. mori* cell lines. BmVF (open circles) and BmN4 (closed circles) cells were inoculated with BmNPV at an MOI of 10. At 2, 6, 12, 18, 24, 36, 48, 72, 96, and 120 hpi, a culture supernatant was collected and subjected to a plaque assay on BmN cells. The results represent the average of three independent experiments; standard errors are indicated.

Figure 2

235 Expression of recombinant proteins by BmNPV-based BEVS. (A) *Polyhedrin* promoter
236 activity in *B. mori* cell lines. BmVF (open circle) and BmN4 (closed circle) cells were
237 infected with Luc-Bm, which expresses the *luciferase* gene under the control of the
238 *polyhedrin* promoter. At the indicated time postinfection, virus-infected cells were
239 harvested and subjected to a luciferase assay. The results represent the average of three
240 independent experiments; standard errors are indicated. (B) Expression of mIL-3 in *B.*
241 *mori* cell lines. BmVF and BmN4 cells were inoculated with IL-Bm, which expresses the
242 *mIL-3* gene under the control of the *polyhedrin* promoter. After inoculation, the cells
243 were treated with tunicamycin (10 µg/ml) or DMSO and harvested for 72 hours. The
244 extracts, prepared from 1×10^5 BmNPV-infected cells, and the culture media were
245 collected and subjected to SDS-PAGE followed by Western blotting using an anti-FLAG
246 antibody. Size markers are indicated on the right side of the panel. (C) Adaptation of
247 BmVF cells to the SFM. The morphology of BmVF cells after 10 passages using four
248 commercial insect serum-free media, SF900-II, BY, OM, and PB. The scale bar is 50 µm.

Figure

Fig. 1

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Fig. 1

Fig. 2

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Fig. 2