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Short Communication

# Baculovirus-mediated gene transfer systems in silkworm larvae using constitutive host promoters

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ABSTRACT

Baculoviruses serve as efficient viral vectors for gene delivery into vertebrate and invertebrate cells. The identification and characterization of the functional promoters in different baculovirus-infected hosts are essential for the efficient gene expression. To establish a baculovirus-mediated gene transfer system in the silkworm, *Bombyx mori*, we investigated the activities of silkworm-derived *TCTP*, *ACTIN3*, and *HSC70-4* promoters delivered by AcNPV or BmNPV in various tissues of silkworm. In many of the tested silkworm tissues, the *BmHSC70-4* promoter exhibited a higher transcription activity than those of *BmTCTP* or *BmACTIN3* promoters when delivered by AcNPV, which is reported to be incapable of replicating in silkworms. In contrast, the *BmACTIN3* promoter was found to be the strongest promoters when delivered by BmNPV. The present results indicate that the *BmHSC70-4* promoter is potentially useful for the stable gene expression by the non-replicating AcNPV vector for gene function analysis in the silkworm.



## Introduction

The silkworm *Bombyx mori* is one of several model insects for genetic, biochemical and physiological studies; the situation of silkworm is the next to the fruit fly, *Drosophila melanogaster*, but the first one in Lepidoptera (Goldsmith et al., 2005). An efficient transgene delivery system with wide variety is absolutely imperative for the future development of insect models. In any experiment testing the expression of an exogenous gene introduced into silkworm cell lines or larvae, there are a few choices of promoters to transcribe the target gene and relatively few kinds of plasmid vectors which can be expected to express exogenous genes in all organs/tissues. Furthermore, there is no ubiquitous promoter with a strong transcription potency in silkworm vector system. To address these issues, we characterized two constitutive genes, heat shock cognate protein 70-4 (*HSC70-4*) and translationally controlled tumor protein (*TCTP*), in previous studies (Lee et al., 2003, 2004). Transcripts from both genes are widely and abundantly found in all silkworm tissues. Further studies also showed that a series of constitutive gene expression vectors utilizing the *BmTCTP* and *BmHSC70-4* promoters were active in a wide range of cells including lepidopteran and dipteran cell lines (Lee et al., 2008). Thus, we hypothesized that these promoters might be among the most promising candidates for achieving strong and ubiquitous gene expression in different tissues of silkworm.

Virus-mediated gene transfer is a powerful method for delivering exogenous DNAs into tissues that often prove difficult, such as the nerve system (Ruitenberg et al., 2002; Watanabe et al., 2006). In the case of the silkworm, two types of baculovirus vector system, *Autographa californica* nucleopolyhedrovirus (AcNPV) and *B. mori* nucleopolyhedrovirus (BmNPV), are available for gene delivery (Iatrou and Meidinger, 1990; Mori et al., 1995; Yamamoto et al., 2004). Indeed, Moto et al. (2003) utilized these two systems for the *in vivo* introduction of exogenous genes and demonstrated that baculovirus can serve as an efficient system that permits gene delivery into neural tissues in the silkworm. Due to the widespread use of baculoviruses for high-level expression of recombinant proteins (Maeda et al., 1985; Higashihashi et al., 1991; Kadonookuda et al., 1995; Shi et al., 1996; Murakami et al., 2001; Wu et al., 2001), several simple and easy methods for manipulating baculoviral genomes have been developed (Noad et al., 2009; Hitchman et al., 2011, 2012; Yao et al., 2012), suggesting that viral vector systems have high potential for *in vivo* gene transfers into silkworm tissues. Interestingly, besides its application in *in vivo* gene delivery system, baculovirus can also be used for generating recombinant baculovirus insecticides with enhanced foreign toxin gene expressions, which could incapacitate pest insects rapidly after viral infection (Michael et al., 1991). Considering the development of viral vector systems, it is necessary to analyze the

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activity of different novel promoters, especially their different transcriptional efficiencies in different silkworm tissues and the potential influence on the expression of both viral and host genes. To date, however, there have been only few reports concerning the application of different promoters in baculovirus vector systems.

In the present study, we constructed recombinant viruses with luciferase reporter under the control of *BmTCTP* or *BmHSC70-4* promoters, and further compared their relative activities with *BmActin3* promoter. Our results indicated that *BmHSC70-4* promoter is a fine candidate for the efficient baculovirus-mediated gene transfer system in silkworm individuals and cells.

## Materials and methods

### *Cells and media*

*Spodoptera frugiperda* cell line (Sf9) was grown in Grace's insect medium (GIBCO Invitrogen Co) containing 10% fetal bovine serum (GIBCO Invitrogen Co) and used for propagation of recombinant AcNPVs according to the manufacturer's instruction for the Bac-to-Bac® Baculovirus Expression System instruction (Invitrogen). Bme21 (e21-12: Embryo derived) cell line was maintained in IPL-41 insect medium (Sigma Chemical Co) supplemented with 10% fetal bovine serum and used for propagation of recombinant BmNPVs (Lee et al., 2012a). All cell lines were maintained at 27 °C.

### *Generation of recombinant AcNPVs and BmNPVs*

To construct recombinant AcNPV and BmNPV bacmids expressing luciferase (kindly provided by Professor Hisanori Bando of Hokkaido University Graduate School; GENBANK Accession Number: AF434924) as a reporter gene, we used the expression cassettes driven by the silkworm constitutive promoters (CPs) constructed in the previous study (Lee et al., 2008) (Fig. 1). In order to create the recombinant donor plasmid pFBBmCP-Luc (pFBBmTCTPhscin-Luc, pFBBmActin3-Luc and pFBBmHSC70-4-Luc), the expression cassette was double-digested with *Apa*I/*Sac*II, blunt-ended by T4 DNA polymerase and cloned into a *Sna*BI/*Bcl*I blunt-ended site of pFastBac 1 (Invitrogen), from which polyhedrin promoter region and SV40 polyadenylation signal have been removed. The recombinant donor plasmid pFBBmCP-Luc was transformed both into *E. coli* DH10Bac cells for AcNPV (Invitrogen) and for BmNPV (Motohashi et al., 2005) to generate recombinant viral DNAs. The viral DNA was isolated using the FlexiPrep kit (Amersham Pharmacia Biotech) and then transfected into Sf9 or Bme21 cell lines using CellFectin transfection reagent (GIBCO Invitrogen). The cells were incubated for 5 days, after which the supernatant was collected as P1 viral solution. The P1 viral stocks were used to infect Sf9 and Bme21 cells to generate P2 stock and further used for high-titer P3 stock preparation. The resulting recombinant P3 virus stocks were stored at 4 °C and titer of these viruses was determined on Sf9 and Bme21 cell lines, respectively, according to the standard protocols (O'Reilly et al., 1992).

### *Silkworm strain, inoculation, dissection and luciferase assay*

A commercially available hybrid race of the silkworm, Kinshu (Japanese race) × Showa (Chinese race), was used in this study. Larvae were reared on an artificial diet, Silkmate (Nihon Nosan Kogyo, Japan), at 24–26 °C.

The recombinant AcNPV/BmCP-Luc and BmNPV/BmCP-Luc baculoviruses were carefully injected into the hemocoel of 2-day-old fifth instar silkworm larvae using a microliter™ syringe with a 30-gauge needle (Hamilton Co, USA). Four days after injection, larvae were dissected and hemocytes, silk glands, fat body, mid-gut, Malpighian tubules, ovaries, and testes were collected. After rinsed in phosphate buffered saline (PBS, pH 7.5), the specimens were homogenized to prepare cell extracts using lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol and 1% Triton X-100) for 30 min. After brief centrifugation, the resulting solutions were assayed for luciferase activity by luciferase substrate solution (20 mM Tricine–NaOH, pH 7.8, 1.07 mM basic magnesium carbonate, 2.67 mM magnesium sulfate, 3.3 mM ethylendiaminetetraacetic acid, 33.3 mM dithiothreitol, 270 mM coenzyme A, 470 mM luciferin D and 530 mM ATP in 1 × PBS) using a 1253 Luminometer (Bio-Orbit). Results were expressed in relative light units (RLU) per microgram of tissue protein with a standard deviation. The total protein content of each sample was determined as described previously (Wang and

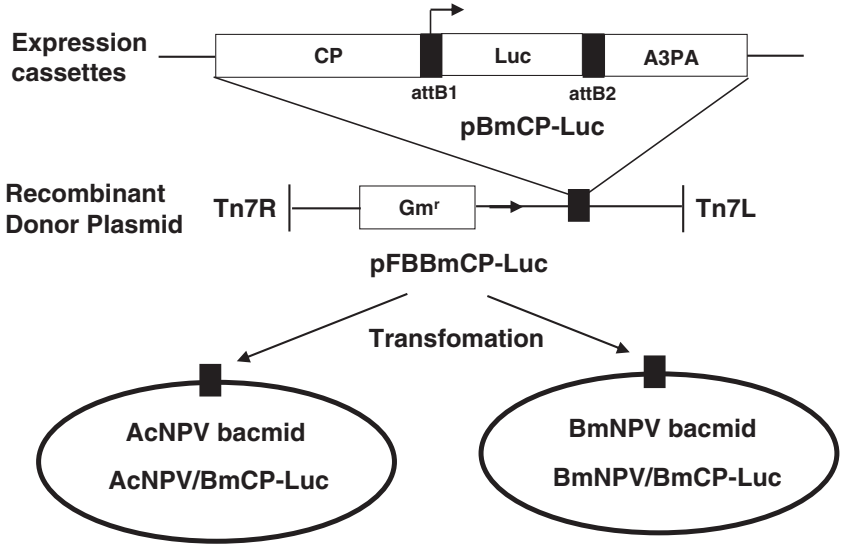


**Fig. 1.** Experimental strategy for generation of recombinant AcNPV/BmCP-Luc and BmNPV/BmCP-Luc bacmid. All recombinant donor plasmids constructed by using the pFastBac 1 (Invitrogen), which contains the constitutive promoter (CP), luciferase ORF and BmActin3 polyadenylation signal (A3PA). Through transposition in bacterial cells, expression cassettes were transferred into a mini-attTn7 target site (indicated by the right and left insertion sites, Tn7R and Tn7L) in the *polyhedrin* locus of the bacmid viral genome.