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Expression of the Gene Encoding Firefly Luciferase Using *Bombyx mori* Nucleopolyhedrovirus Vector

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A cDNA encoding the luciferase of firefly *Luciola lateralis* was cloned downstream from the polyhedrin gene promoter of *Bombyx mori* nucleopolyhedrovirus and expressed in *B. mori* cells (BmN-4). The coding sequence for luciferase was inserted into pBmKSK2 vector, which was reconstructed from the polyhedrin-based transfer vector pBmKSK1 by modifying cloning sites. Recombinant virus, BmK2-LUCDF, containing the luciferase gene was selected and purified in BmN-4 cells. The emission of luminescence by luciferase was only detected in BmK2-LUCDF-infected cell extracts. This result indicates that the cloned new luciferase gene of firefly *L. lateralis* can be expressed efficiently in baculovirus expression system and used as a useful reporter gene.

Key words : Luciferase, Firefly *Luciola lateralis, Bombyx mori* nucleopolyhedrovirus, Expression vector

Introduction

The insect baculovirus has been used as an efficient vector for the expression of foreign genes in established insect cell lines and whole insects (King and Possee, 1992; Luckow and Summers, 1988, 1989; Maeda, 1989; O'Reilly *et al.*, 1992). In the very late phase of baculovirus infected insect cells, polyhedrin that is the major constituent of polyhedra is abundantly synthesized. Since the polyhedrin gene is not essential for viral replication, its promoter has been widely used for expression of foreign genes (King and Possee, 1992, O'Reilly *et al.*, 1992). It has been shown that baculovirus expression vector systems have several advantages over other expression systems (Maeda, 1989, 1994). Both *Autographa californica* nucleopolyhedrovirus (AcNPV) and *Bombyx mori* NPV (BmNPV) have been developed as vectors (Smith *et al.*, 1983; Maeda *et al.*, 1985). For the mass production of foreign gene products, the BmNPV vector has a unique advantage of having an advanced system, the silkworm *B. mori*, that can be used for *in vivo* expression, and this system has several attractive features in comparison with the AcNPV vector system (Maeda, 1989, 1994; Reis *et al.*, 1992).

Firefly luciferases catalyze a light-producing reaction using ATP, O_2 and D-luciferin as substrate (Deluca and McElory, 1974). The best known light-producing enzyme is the luciferase of the firefly, *Photinus pyralis* (Seliger and McElroy, 1964). The gene encoding this luciferase has been cloned, sequenced and expressed in plants as well as animal cells (deWet *et al*, 1985, 1987; Ow *et al.*, 1986; Keller *et al.*, 1987). As few as 2000 molecules of luciferase are detectable by amplification using photomultipliers, and the negligible background activity in most cells keeps the signal-to-noise ratio extremely high (Wood, 1991). The gene encoding firefly luciferase has recently been successfully expressed in insect cell cultures as well as in caterpillars by a recombinant baculovirus (Hasnain and Nakhai, 1990; Jha *et al.*, 1990). Now, the luciferase gene has been used widely as the reporter gene because the assay of the enzyme is very sensitive, rapid, nonradioactive and can be quantitated in a noninvasive manner.

A number of new luciferase genes from Jamaican click beetles, *Pyrophorus plagiophthalamus*, have been isolated and expressed in *E. coli* (Wood *et al.*, 1989a). Although these luciferases differ by less than five percent from each other with respect to their amino acid sequences, they are capable of emitting light at different wavelengths ranging from green (546 nm) to orange (594 nm), enabling the simultaneous monitoring of different gene constructs in

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the same cell (Wood *et al.*, 1989b).

Recently, we reported the new luciferase gene from *Luciola lateralis*, which is the most abundant firefly in Korea (Cho *et al.*, 1999). To investigate the activity and usefulness of this luciferase gene in BmNPV vector system, we constructed a new transfer vector pBmKSK2 by improvement of cloning sites of pBmKSK1 (Woo *et al.*, 1995), and expressed luciferase in *B. mori* cells.

Materials and Methods

Cell culture and virus

B. mori BmN-4 cells was maintained at 27 in TC-100 medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, USA). The BmNPV-K1 was used as wild-type virus. Routine cell culture maintenance and virus production procedures were carried out according to published procedure (Summers and Smith, 1987).

Plasmid and PCR

Standard plasmid manipulation techniques were used throughout these experiments (Sambrook *et al.*, 1989). In order to amplify the polyhedrin gene region, a 20-mer oligonucleotide, 5'-TAAGTATTTTACTGTTTTCG-3' corresponding to the 5' mRNA transcription region, and 19-mer oligonucleotide, 5'-GTGATATGTAACATATATC-3' corresponding to the 3' flanking region, were synthesized for polyhedrin gene. The reaction was carried out using PreMix^{TM}-Top (Bioneer), 50 ng of viral DNA and 1 μl of each primer in a total volume of 20 μl. Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer). Following amplification, the PCR products were analyzed by 0.9% agarose gel electrophoresis.

Construction of transfer vector

In order to improve cloning sites of a pBmKSK1, pUC19 was cleaved with *Sac*I and *Kpn*I and then cloned into pBmKSK1 digested with the same restriction endonucleases. The cloned transfer vector, pBmKSK1-pUC19, was cleaved with *Pst*I and *Sac*I, blunted with Klenow fragment and then self-ligated to make the transfer vector pBmKSK2 (Fig. 1A). Previously, we cloned the luciferase gene of firefly *L. lateralis* that was isolated from Korea. A 1.7 kb *Xba*I-*Kpn*I fragment containing entire cDNA of luciferase gene was isolated from the plasmid pLUCDF and ligated into the same restriction endonuclease sites of the transfer vector pBmKSK2, under the control of polyhedrin promoter, creating pBmKSK2-LUCDF (Fig. 2A).

Transfection and selection

BmN-4 cells in 60 mm tissue culture plate were cotransfected with wild-type BmNPV DNA and transfer vector by liposome-mediated method (O'Reilly *et al.*, 1992) using transfection reagent DOTAP (Boehringer-Mannheim, Germany). After incubation for 5 days at 27°C, the transfection supernatants were subjected to an end-point dilution (O'Reilly *et al.*, 1992) in order to purify recombinant virus. End-point dilution was performed three times to clone the viruses genetically. The recombinant viruses were propagated and stored at -20°C.

Virus infection and luciferase assay

The cells were infected with BmK2-LUCDF at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell in 25 cm^2 culture flask containing 5.0×10^6 cells. Following the 6 hr virus adsorption period, the cells were washed, supplied with fresh medium, and incubated at 27°C. At 3 days after inoculation, the culture supernatant was harvested and pelleted. The cell pellet was washed with phosphate-buffered saline (pH 6.2), and disrupted by repeated freeze thawing. After cellular debris was pelleted, the extracts were assayed for luciferase activity by adding measuring buffer (25 mM glycyl glycine, pH 7.8; 15 mM $MgSO_4$; 5 mM ATP) and 250 mM luciferin solution in the dark condition.

Results and Discussion

Construction of polyhedrin-based transfer vector

Previously constructed BmNPV polyhedrin-based transfer vector pBmKSK1 has three restriction endonuclease sites for the cloning of foreign gene (Woo *et al.*, 1995). To improve its usefulness, we modified the cloning sites of pBmKSK1 by insertion of pUC19 plasmid (Fig. 1A). Although the resulting transfer vector, pBmKSK1-pUC 19, has multiple cloning sites for foreign gene, this vector has disadvantages that must be cleaved with two restriction enzymes and has large size. Therefore we constructed pBmKSK2 by deletion of pUC19 plasmid from pBm KSK1-pUC19 (Fig. 1A). The restriction enzyme digestion pattern showed four unique cloning sites in pBmKSK2 (Fig. 1B). The polyhedrin gene promoter and multiple cloning site sequences in pBmKSK2 were confirmed by nucleotide sequence determination (Fig. 1C). As a result, transfer vector pBmKSK2 has a unique *Eco*RI, *Xba*I, *Sma*I and *Kpn*I site for insertion of foreign genes immediately downstream of the BmNPV polyhedrin gene promoter. According to the desired restriction enzyme sites for foreign genes, these vectors will be used usefully to construct recombinant vectors.

Construction of recombinant virus expressing luciferase

The strategy for construction of the recombinant transfer

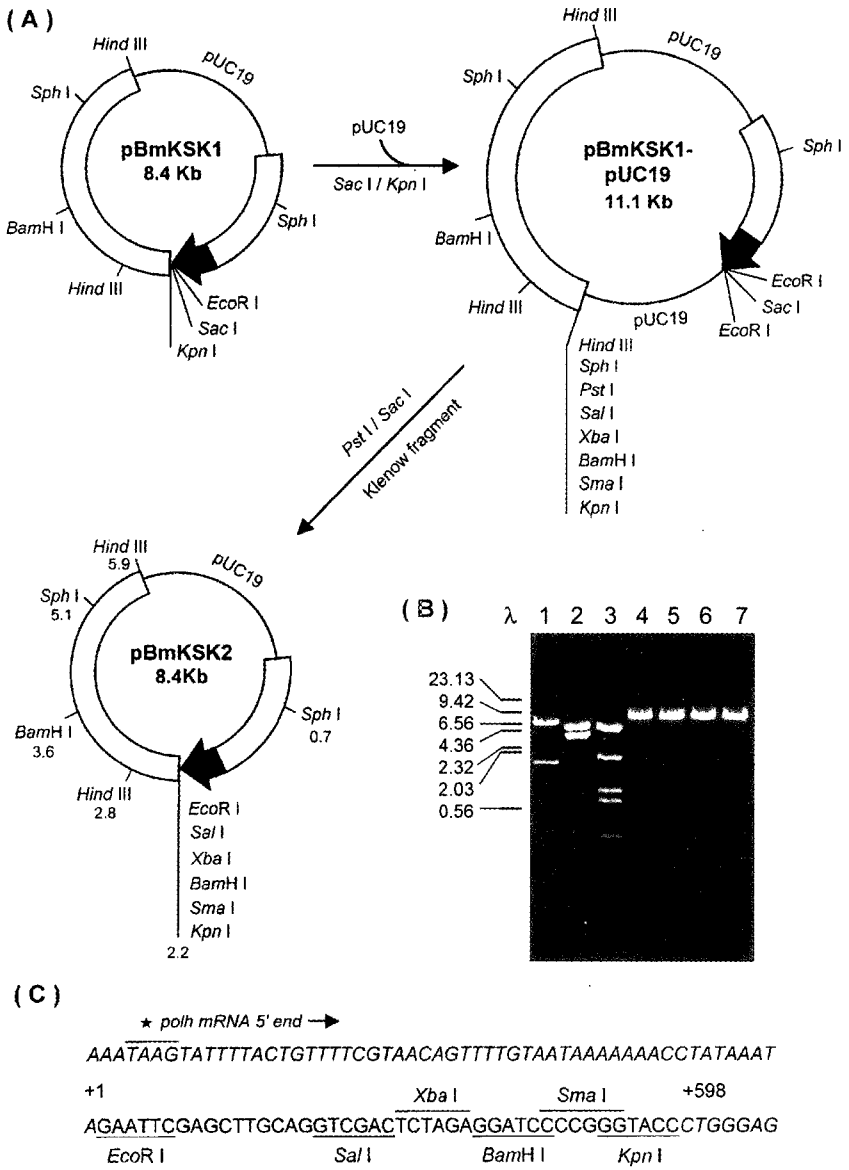

Fig. 1. Schematic diagram of the transfer vector construction using the polyhedrin promoter. (A) The insertion of pUC19 plasmid into transfer vector pBmKSK1 (Woo *et al.*, 1995) resulted the transfer vector pBmKSK1-pUC19. The transfer vector pBmKSK2 was constructed by deletion of pUC19 plasmid from pBmKSK1-pUC19. (B) Restriction endonuclease digestion patterns of pBmKSK2. A cleaved DNA fragments were electrophoresed on a 0.8 % agarose gel. Lane: λ, *Hind*III-digested λ DNA were used molecular weight marker; 1, *Bam*HI; 2, *Hind*III; 3, *Sal*I; 4, *Eco*RI; 5, *Kpn*I; 6, *Sma*I; 7, *Xba*I. (C) The nucleotide sequences of promoter region in pBmKSK2. The pBmKSK2 contained unique restriction endonuclease sites, *Eco*RI, *Kpn*I, *Sma*I and *Xba*I for the cloning of foreign gene. The first nucleotide of the initiator ATG codon of polyhedrin has been designated +1.

vector pBmKSK2-LUCDF is shown in Fig. 2. A 1.7 kb *Xba*I-*Kpn*I fragment containing the luciferase gene was isolated from the plasmid pLUCDF and cloned into the transfer vector pBmKSK2 which was digested with the same restriction enzymes (Fig. 2A). The exact insertion of luciferase gene into the pBmKSK2 was confirmed by sequencing of pBmKSK2-LUCDF (Fig. 2B). The pBm KSK2-LUCDF, then, was cotransfected with the genomic DNA of BmNPV into BmN-4 cells to construct the recombinant virus, BmK2-LUCDF, where the polyhedrin gene was replaced with the luciferase gene. The recombinant virus producing luciferase was screened by observation of polyhedrin-minus virus produced well. Follo-wing purification, the purity of the BmK2-LUCDF was confirmed by an analysis of the viral genomic DNA using PCR with primers specific to polyhedrin gene. The result of PCR amplification showed that recombinant virus BmK2-LUCDF had the luciferase gene instead of polyhedrin gene (Fig. 3).

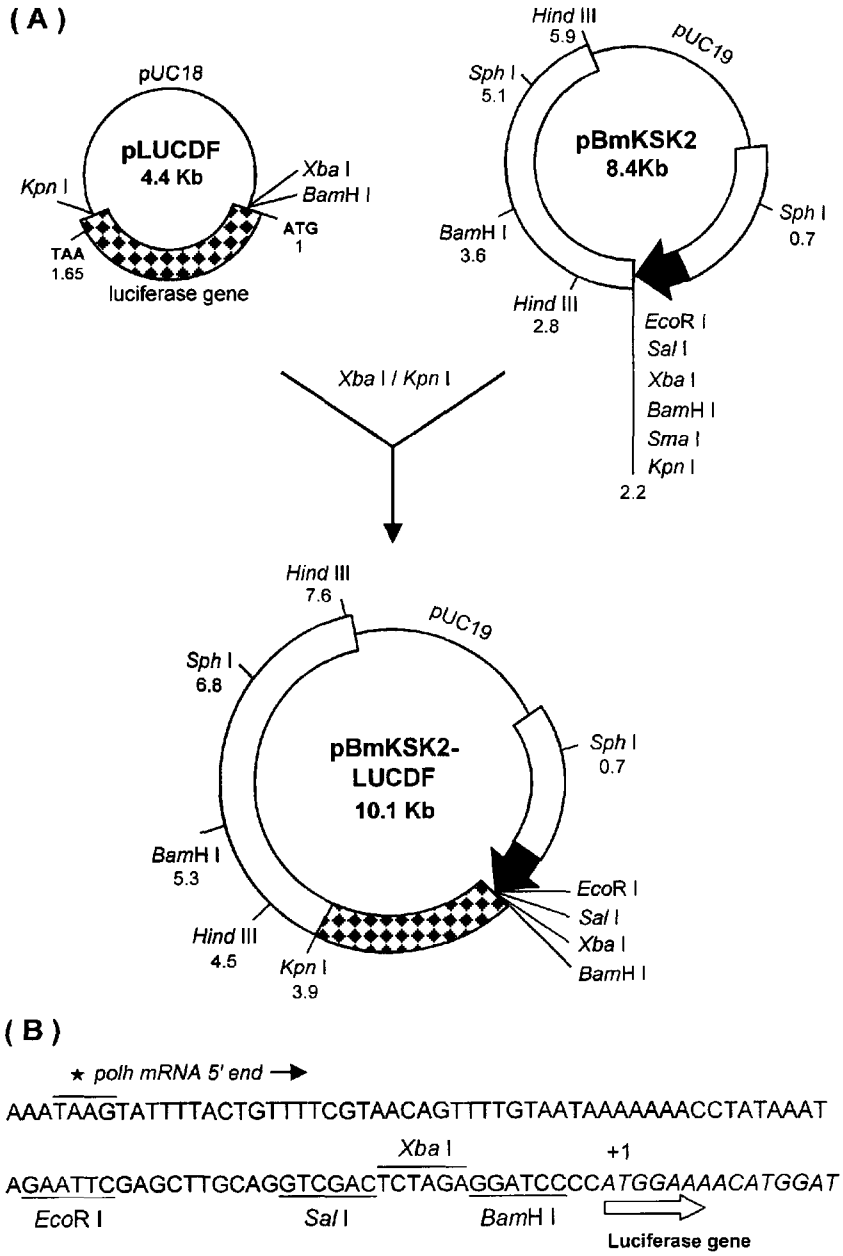

Fig. 2. Schematic diagram of the construction of transfer vector pBmKSK2-LUCDF. (A) A DNA fragment of pLUCDF containing luciferase gene was ligated to the transfer vector pBmKSK2 to construct pBmKSK2-LUCDF. (B) The nucleotide sequences of polyhedrin promoter and the 5 end of luciferase gene in pBmKSK2-LUCDF. The first nucleotide of the initiator ATG codon of luciferase has been designated +1.

Expression of luciferase in cells

Expression of firefly luciferase by recombinant virus BmK2-LUCDF was observed in BmN-4 cells. The infected cell extracts were assayed for luciferase activity by detection of light production in the dark condition. BmK2-LUCDF infected cell extracts only emitted luminescence by luciferase (Fig. 4). Mock- or wild-type Bm NPV-infected cell extracts did not produce any light. This result indicated that the cloned new luciferase gene of firefly *L. lateralis* has intact coding gene structure and newly constructed transfer vectors, pBmKSK1 and pBmKSK2, can express foreign genes of eucaryote as well as procaryote (Woo *et al.*, 1996).

The luciferase gene is specifically expressed in the firefly light organ and its expression changes with the development of the organ as the firefly grows from egg to an adult fly (Strause *et al.*, 1979; Lee and Boo, 1991). Previously the several firefly luciferase genes, from *P. pyralis* (Vikas *et al.*, 1995), *P. plagiophthalamus* (Karp *et al.*, 1992) and *Vibrio harveyi* (Richardson *et al.*,

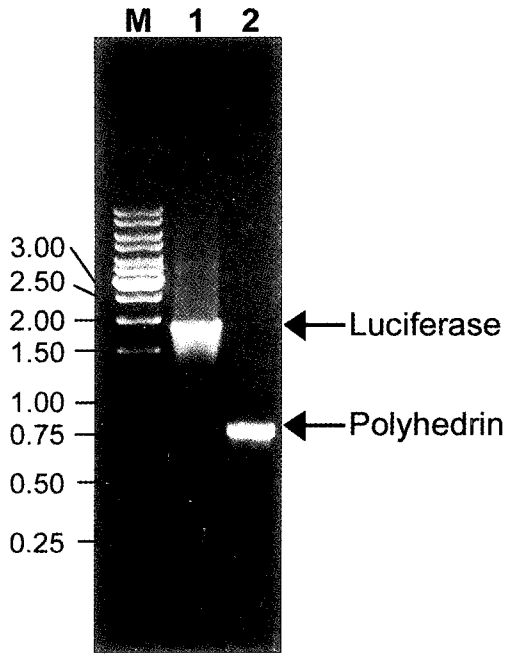

Fig. 3. Specific PCR amplification of the polyhedrin gene region of BmK2-LUCDF and wild-type BmNPV. Lane: M, 1 Kb DNA ladder marker; 1, BmK2-LUCDF; 2, wild-type BmNPV.

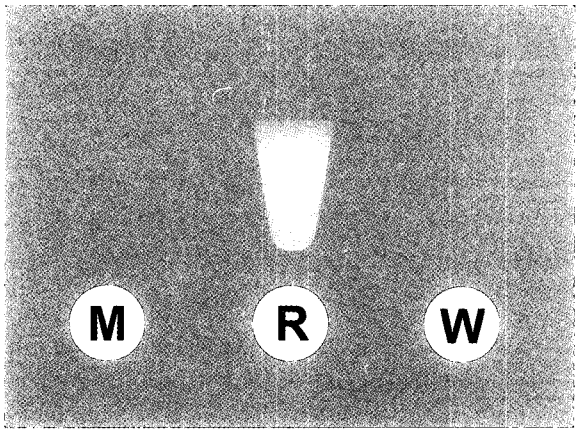

Fig. 4. The assay of luciferase activity in BmN-4 cells infected with recombinant virus BmK2-LUCDF. Mock- (M), BmNPV- (W) and BmK2-LUCDF-(R) infected cells were harvested at 3 days post infection and treated with measuring buffer containing luciferin as a substrate in the dark condition.

1992), were successfully expressed under the control of polyhedrin or p10 promoters of nucleopolyhedroviruses. In addition, high sensitivity and noninvasive nature of the enzymatic assay qualify luciferase gene as useful reporter for monitoring the temporal regulation of baculovirus gene expression, virus dissemination, the screening and purification of recombinant baculovirus. Therefore, *L. lateralis* luciferase in this study should be useful as a reporter enzyme for baculovirus expression vector system.

Acknowledgments

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