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Using internal ribosome entry sites to facilitate engineering of insect cells and used in secretion proteins production

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ABSTRACT

Insect cells have been extensively used for the production of recombinant proteins. The baculovirus expression vector system (BEVS) are routinely used both in laboratories and in industries for the production of recombinant proteins. However, it has been established that secretory proteins can be produced more efficiently in the non-lytic, plasmid-based baculovirus-free insect system (BFIS) than in BEVS. A non-lytic, plasmid based, bi-cistronic insect cell expression system was developed by incorporating the internal ribosomal entry site (IRES) elements derived from *Rhopalosiphum padi* virus (RhPV IRES) or *Perina nuda* virus (PnV_{539} IRES). Both IRESes had been demonstrated to mediate bi-cistronic gene expression in baculovirus-infected Sf21 cell. However, only the PnV_{539} IRES functioned well in Sf21 cells through the plasmid-based baculovirus-free insect cell expression system. Based on this observation, we further combined the PnV_{539} IRES with enhanced green fluorescent protein (EGFP) along with blasticidin-resistant gene to facilitate the isolation of the stably transformed insect cells that expressed the target gene as observed by fluorescence microscopy. A pharmaceutically important secreted protein interferon-γ and secretory alkaline phosphatase (SEAP) was successfully produced in stably transformed Sf21 cells and further proved the application of the novel IRES-based bi-cistronic BFIS.

1. Introduction

The baculovirus expression system (BEVS) are routinely used both in laboratories and in industries for the production of a multitude of diverse types of recombinant proteins for research, medical, agricultural [1] and veterinary applications [2]. The relatively less expensive maintenance and ease of scaling up are the primary advantages of using insect cells over the mammalian cells for the recombinant proteins production. Insect cells can also perform most co-translational and post-translational processes carried out by other eukaryotic cells than the commonly used yeast and bacterial expression systems [3–5].

The BEVS is based on replacing either the baculovirus' *polyhedrin* [6] or *p10* genes [7] with the gene of interest since these two are highly expressed (30–50% of the total protein) during the very late stages of virus infection, and both are non-essential for viral life cycle in insect cells [8]. Among the baculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) and *Bombyx mori* NPV (*Bm*NPV) are the most widely used viral vectors [9–11]. In the recombinant *Ac*MNPV- or *Bm*NPV-infected host cells, the desired genes under the control of *polyhedrin* or *p10* promoters are often expressed in abundance, amounting to about 1–500 mg of protein per liter of insect cell culture. Clearly, the most important benefit of using BEVS is the high level production of recombinant proteins during the late phase of viral infection. However, reports indicated that membrane and secreted proteins were frequently expressed poorly and heterogeneously in the virus-infection dependent, lytic BEVS system [12]. These phenomena suggest that the insect cell secretory pathway may be compromised severely during the later phase of virus infection [3]. Furthermore, the amount of translation initiation factor eIF4E is reduced dramatically during baculovirus infection [13,14]. More importantly, degradation of the recombinant protein is more likely to occur due to the release of intracellular proteases from the

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virus-lysed host cells thus reducing the recovery of the over-expressed protein [15,16]. To overcome these problems, early promoters were used instead of late promoters or the co-expression of molecular chaperones had been employed [3,13,17,18].

In order to resolve intractable problems brought about by the virus infection-induced host cell lysis, the BFIS was developed. In contrast to BEVS which chiefly depends on virus encoded RNA polymerase, BFIS is based on promoters that can be activated by insect cell RNA polymerase II, e. g., *ie1* promoter of *AcMNPV* [19], inducible *Drosophila* gene promoters (such as temperature sensitive *hsp70* promoter and metal induced metallothionine promoter) [20], promoter of the cytoplasmic actin gene of the silk moth *Bombyx mori* [21–23] and *ie1* or *ie2* promoters of *Orgyia pseudotsugata* multiple nucleopolyhedronvirus (*Op*MNPV) [24]. Due to restrictions in the promoter strength, recombinant proteins yielded by BFIS have been considerably lower than those usually obtained from BEVS [16]. However, secretory proteins, such as tissue-plasminogen activator or juvenile hormone esterase, could be produced more efficiently in BFIS than in BEVS [16,17]. Thus, it is suggested that BEVS is suitable for nuclear and cytoplasmic proteins production while BFIS is applicable in the expression of secretory and membrane proteins.

We had identified two internal ribosome entry sites (IRESes), the RhPV IRES cloned from *Rhopalosiphum padi* virus [25] and PnV_{539} IRES cloned from *Perina nuda* picorna-like virus [26], that can mediate cap-independent translation in BEVS. Such IRESes can also be combined with selection markers to facilitate the identification and titer determination of recombinant baculoviruses [27]. To explore the IRES-based bi-cistronic expression vector system into the field of BFIS, we constructed plasmids containing the red fluorescent protein (DsRed) from a coral of a *Discosoma* species [28] and an enhanced green fluorescent protein (EGFP, a mutant form of the green fluorescent protein), flanking the RhPV IRES or PnV_{539} IRES to test the expression of dual fluorescent proteins in the transiently-infected Sf21 cells. Fluorescent microscopic observations demonstrated that the PnV_{539} IRES mediated a stronger cap-independent EGFP translational activity than that of RhPV IRES. In addition, we also used the PnV_{539} IRES to generate stably-transformed Sf21 cell lines that can continuously co-express the following pairs of genes: secretory alkaline phosphatase (SEAP) and EGFP; interferon-γ (IFN-γ) and EGFP.

2. Materials and methods

2.1. Construction of plasmids

DNA preparations and manipulations were performed using standard methods as described by Sambrook and Russell [29]. To generate the plasmids list in Fig. 1A-E, the construction of the following vectors pBac-S-PnV_{539}-E and pBac-IFN-γ-PnV_{539}-E were conducted first. The plasmid coded as pBac-S-Rhir-E [25] was digested with *Nhe*I and *Eco*RI to release the fragment of SEAP. SEAP gene fragment were cloned into the *Nhe*I and *Eco*RI sites of the transfer vector pBac-D-PnV_{539}-E [26] and replacing the DsRed gene. The resulting plasmid was re-named pBac-S-PnV_{539}-E. And, pBac-D-PnV_{539}-E was digested with *Eco*RI and *Bam*HI to release PnV_{539}, and the PnV_{539} DNA fragment was subcloned into the *Eco*RI and *Bam*HI sites of the transfer vector pBac-IFN-γ-Rhir-E [30], where Rhir DNA fragment were substituted. The resulting plasmid was named pBac-IFN-γ-PnV_{539}-E.

In the construction of the vectors with dual reporter genes for the monitoring of RhPV or PnV_{539} IRES activity in BFIS, the following plasmids: pBac-D-Rhir-E, pBac-D-PnV_{539}-E, pBac-S-Rhir-E, pBac-S-PnV_{539}-E, and pBac-IFN-γ-PnV_{539}-E were digested enzymatically with *Nhe*I and *Sal*I and sub-cloning of the individual DNA fragments: DsRed-Rhir-EGFP, DsRed-PnV_{539}-EGFP, SEAP-Rhir-EGFP, SEAP-PnV_{539}-EGFP, and IFN-γ-PnV_{539}-EGFP into an *Spe*I and *Xho*I digested pIB vector (Invitrogen, Carlsbad, CA) was done. The resulting plasmids were named pIB-D-Rhir-E, pIB-D-PnV_{539}-E, pIB-S-Rhir-E, pIB-S-PnV_{539}-E, and pIB-IFN-γ-PnV_{539}-E, respectively (Fig. 1A-E).

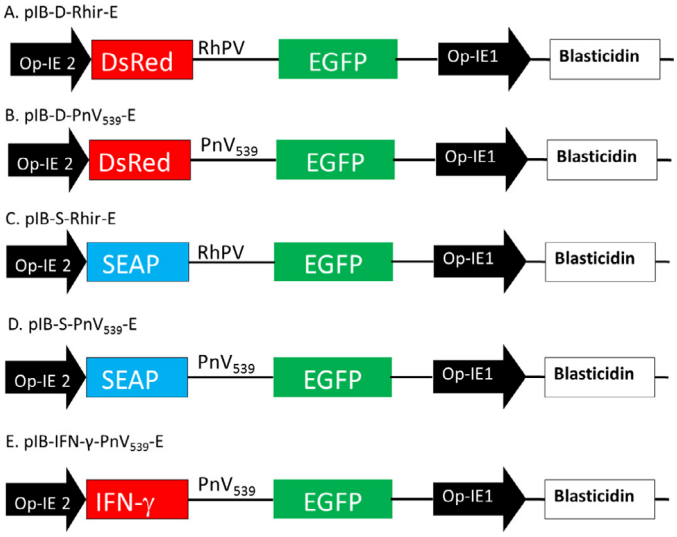

Fig. 1. Plasmids constructs used in this study. The plasmids contained the DsRed gene (A-B) or SEAP gene (C–D) as the first cistron and the EGFP as the second cistron under the control of either the RhPV or the PnV_{539} IRES, respectively. The reporter vectors used containing the respective fragment: Op-IE 2, IE 2 promoter from *Orgyia pseudotsugata* multiple nucleopolyhedronvirus (OpMNPV). DsRed, red fluorescent protein from *Discosoma sp*. SEAP, secreted alkaline phosphatase gene. RhPV IRES, an element from *Rhopalosiphum padi* 5′ IRES. PnV_{539}, 5′ internal ribosomal entry site from *Perina nuda* virus. EGFP, enhanced green fluorescent protein gene. Op-IE 1, IE-1 promoter from OpMNV. Blasticidin, resistant gene for the antibiotic blasticidin. Blasticidin was used during the selection of stably transformed insect cells. (E) The plasmid used for the expression of the pharmaceutically important protein, interferon-γ (IFN$-\gamma$) as the first cistron and the EGFP as the second cistron under the control of the PnV_{539} IRES. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2.2. Transfection of reporter vectors into insect cells and establishment of stably transformed cell lines

The *S. frugiperda* IPBL-Sf21 (Sf21) was cultured in TNM-FH (Invitrogen) insect medium containing 8% heat-inactivated fetal bovine serum (Gibco, N.Y., USA) [31]. For transient and stable transfection studies, corresponding insect cell with a density of about 1×10^5–2×10^5 cells/well was seeded onto a 24-well plate. Cells were then transfected with corresponding individual plasmid DNA using Lipofectin reagent (Invitrogen) following the manufacturer's protocol.

In the establishment of a stable Sf21 cell line, cells were selected by the addition of 50 µg Blasticidin (BSD)/ml (Invitrogen) at 48 h after transfection, then, the transfectants were isolated by dilution method. Two weeks later, the cells were monitored for distinct "islands" of surviving cells through the dilution method. Individual clones of stable Sf21 cells were then transferred to T75-flask and maintained in a medium containing 0.1 ug/ml blasticidin.

2.3. SEAP activity assay

The medium of Sf21 cells (2×10^5/well in a 24-well plate) transfected with the respective plasmids namely pIB-S-Rhir-E, pIB-S-PnV539-E, pIB-D-Rhir-SE and pIB-D-PnV_{539}-SE were as collected. The medium was centrifuged (12,000 g) for 10 s and then preserved in a −20 °C freezer until the SEAP activity assay was performed. The SEAP activity in culture medium was measured using a BD Great EscApe™ SEAP detection kit (Clontech, Mountain

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