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Full Length Research Paper

Quantitative analysis of three commonly-used insect cell-specific promoters' activities by transient and baculovirus-mediated expression

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The activities of three insect cell-specific promoters were compared and analyzed here, including the cytoplasmic Actin 3 (A3) promoter from *Bombox mori* (silkworm), immediately early 2 (ie2) promoter from *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) and the very late polyhedrin (*polh*) promoter from *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). Results of transient expression assays showed the activity of ie2 promoter was approximately forty fold higher in *Spodoptera frugiperda* 9 (Sf9) cells and thirty fold higher in ovarian cell line of *Bombyx mori* (BmN) than that of A3 promoter. However, it showed reverse effect in baculovirus system that the activity of A3 promoter became approximately ten fold higher than that of ie2 promoter in the late stage of baculovirus infection. We constructed recombinant baculoviruses with enhanced green fluorescent protein gene (EGFP) reporter gene controlled by individual A3, ie2 and polh promoter. The analysis result of EGFP expression level showed the activity of polh promoter was nearly 50 fold higher than that of ie2, and 5 times higher than that of A3 at 72 h post infection in Sf9 cells. The systemic studies on the activities of the three promoters indicate they are ideal drivers for the construction of a time sequential baculovirus expression system.

Key words: Promoter activity, immediately early promoter, very late promoter, transient expression, recombinant baculovirus.

INTRODUCTION

Insect cell expression system is a valuable tool for the high-throughput production of soluble, active proteins with a relatively low cost (Ikonomou et al., 2003; Pfeifer, 1998). Insect cell culture was normally performed in basal media supplemented with 10% fetal bovine serum (FBS), however, current trends show that serum-free culture for insect cells has been available by using media Sf-900II or HyQ SFX-Insect from Hyclone, which is more efficent and economic to purify target proteins (Weiss et al., 1990).

Insect cell expression involves three types of vector systems, including transient, stable and baculovirus expression vector systems (BEVS). Immediate-early promoters (ie1) from *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and immediately early 2 (ie2) from *Orgyia pseudotsugata* multicapsid nucleo-polyhedrovirus (OpMNPV) have been widely used to construct transient and stable expression vectors. The product encoded by ie1 (called IE-1) is the principal transcriptional regulator of baculovirus, which can also markedly stimulate promoter activity through binding to the palindrome units. The introduction of homologous region (hr) sequence linking in cis to ie1 promoter provides up to two fold enhancement of transcriptional

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efficiency, whereas ie2 promoter without linking hr sequence has been as efficient as ie1-hr construct (Pfeifer, 1998; Pfeifer et al., 1997).

In addition to the immediate-early promoters, the very late p10 and polyhedrin (polh) promoters are powerful candicates for over-expression in the very-late stages of virus infection. Actin 3 (A3) promoter from *Bombyx mori* is also another widely-used driver for expressing foreign genes in lepidopteran cells (Johnson et al., 1992; Coulon-Bublex et al., 1993), whose transcriptional ability can be greatly improved with the help of the constitutive enhancer formed by hr sequence and IE-1 protein of *B. mori* nucleopolyhedrovirus (BmNPV) (Lin et al., 2010; Lu, et al., 1997). Recently, A3 promoter has been used to develop piggyBac system for transgenic silkworm or transgenic insect cell lines (Tamura et al., 2000; Yamamoto et al., 2004).

Interestingly, baculovirus is not only used for recombinant protein expression, but also popular for its application of constructing genetically-engineered baculovirus insecticides. Pest will quickly incapacitate after viral infection resulting from the enhancement of expressing foreign toxin genes under the immediate-early or early gene promoters (Tomalski and Miller, 1991). It also showed that baculovirus could invade into mammalian cells without replication, indicating the possibility of being one kind of gene transfer vector system of mammalian cells (Boyce and Bucher, 1996; Ghosh et al., 2002). Considering these great potential advantages of baculovirus system, it is important to estimate these cell-specific promoters, such as their different transcriptional efficiency at different expression environment.

The activity of A3 promoter from *B. mori* and polh promoter from BmNPV has been compared in *B. mori* larvae by hemocoelic injections of recombinant BmNPV (Johnson et al., 1992). The activity of the A3 promoter and ie1 promoter from AcMNPV has been compared in *B. mori* larvae by hemocoelic injections of recombinant AcMNPV, too (Zhang et al., 2008). With the development of multiple genes expression system based on recombinant baculovrius (for example MultiBac), it is exremely necessary to test and analyze the acitivity of these insect-specific promoters before introducing them into insect expression system (Berger et al., 2004). In this study, we cloned a novel A3 promoter and compared its activity with the other two commonly-used promoters, ie2 and polh promoters. The outcomings indicate that the three promoters display distinct activity in transient expression and baculovirus-mediated expression system, which benefit the selection of suitable promoters for different purpose.

MATERIALS AND METHODS

Bacterial strains, plasmids, viral Bacmid, reagents and insect cell lines

Escherichia coli DH10Bacmid-gfp containing Bacmid-gfp and pHelper was constructed previously, in which an enhanced green fluorescent protein (EGFP) gene expression cassette was introduced into the p74 locus of Bacmid (Yao et al., 2006). Plasmid pFBDM was given by Prof. Richmond and Dr. Berger (Berger et al., 2004). *E. coli* DH10Bac, plasmids pIZT/v5-His and pBAD/D/*lac*Z, as well as Cellfectin, Grace medium and fetal bovine serum (FBS) were purchased from Invitrogen (USA). Universal genomic DNA extraction kit, pMD18-T simple vector, ExTaq, restriction enzymes and T4 DNA ligase were bought from Takara (Japan). BCA protein quantification kit was purchased from Solarbio (China). AP-GAPDH and AP-GFP polyclonal antibodies were purchased from Proteintech Group Inc. (USA). β-galactosidase standard sample was purchased from Wako (Japan), while ONPG (O-nitrophenyl beta-D-glucoside) was purchased from Amresco (USA). Sf9 and BmN cells were maintained at 27°C in Grace medium supplemented with 10% FBS.

Construction of transient expression vectors and recombinant baculoviruses

The genomic DNA of BmN cells was extracted by using genomic DNA extraction kit (Takara). The A3 promoter (GenBank accession number: HQ918291) was amplified from BmN genomic DNA by polymerase chain reaction (PCR) using the primers A3-F [5'-AAAGTATACGGAGTCGGGGAGAGGTTACA-3', *Bst*Z17 I underlined] and A3-R [5'- AAACTCGAGCCCGGGCTTGAATT-AGTCTGCAAG-3', *Xma* I underlined]. The PCR product was digested with *Bst*Z17 I and *Xma* I, and then was ligated to the same sites of pFBDM to replace p10 promoter, forming a transfer vector pFA3 (Figure 1A, upper). The 572 bp ie2 promoter of OpMNPV was amplified from pIZT/v5-His using ie2-F [5'-AAAGATATCTACGTAGGATCATGATGATAAACA-3', *Sna*B I underlined] and ie2-R [5'- AAACCCGGGCTTTAAATTCGAACAGAT -3', *Xma* I underlined]. The PCR product was digested with *Sna*B I and *Xma* I, which was then ligated into the *Bst*Z17 I and *Xma* I sites of pFBDM to form another transfer vector pFIE2 (Figure 2A, bottom). The 3.5 kb fragment containing lacZ gene from pBAD/D/*lac*Z was first digested with *Pme* I and *Nco* I, and ligated into the *Pvu* II and *Nco* I sites of pFA3 and pFIE2 to generate the transient expression vectors pFA3/*lac*Z and pFIE2/*lac*Z, respectively (Figure 1A).

The plasmid pIRES-GFP (Clontech) was digested with *Xma* I and *Xho* I to release *egfp* cDNA. Then the egfp fragment was cloned into the same sites of pFA3 and pFIE2 to make donor vectors pFA3EGFP and pFIE2EGFP, respectively. pIRES-GFP was digested with *Bam*H I and *Xho* I to release egfp, and the fragment was cloned into pFBDM via *Bam*H I and *Sal* I to construct donor vector pFpolhEGFP.

Donor plasmids pFA3/*lac*Z and pFIE2/*lac*Z were respectively transferred into the *E. coli* DH10Bacmid-GFP to generate two kinds of Bacmids, including BacmidA3/*lac*Z and Bacmidie2lacZ as previous study (Yao et al., 2010). Similarly, pFA3EGFP, pFIE2EGFP, pFpolhEGFP were separately transferred into the *E. coli* DH10Bac to construct the other three kinds of recombinant Bacmids, BacA3EGFP, Bacie2EGFP and BacpolhEGFP. The positive recombinant Bacmids were identified by white-blue screening and PCR amplication. The purified Bacmids were then respectively transfected into Sf9 cells using Cellfectin to produce five kinds of recombinant viruses named with AcNPV-A3/*lac*Z, AcNPV-ie2/*lac*Z, AcNPV-A3EGFP, AcNPV-ie2EGFP and AcNPV-polhEGFP. The plaque assay technique was used to determine the recombinant virus titer (Roldao et al., 2009).

Sample preparation for the analysis of β-gal activity

2 µg of purified plasmids, pFA3/*lac*Z and pFIE2/*lac*Z were separately

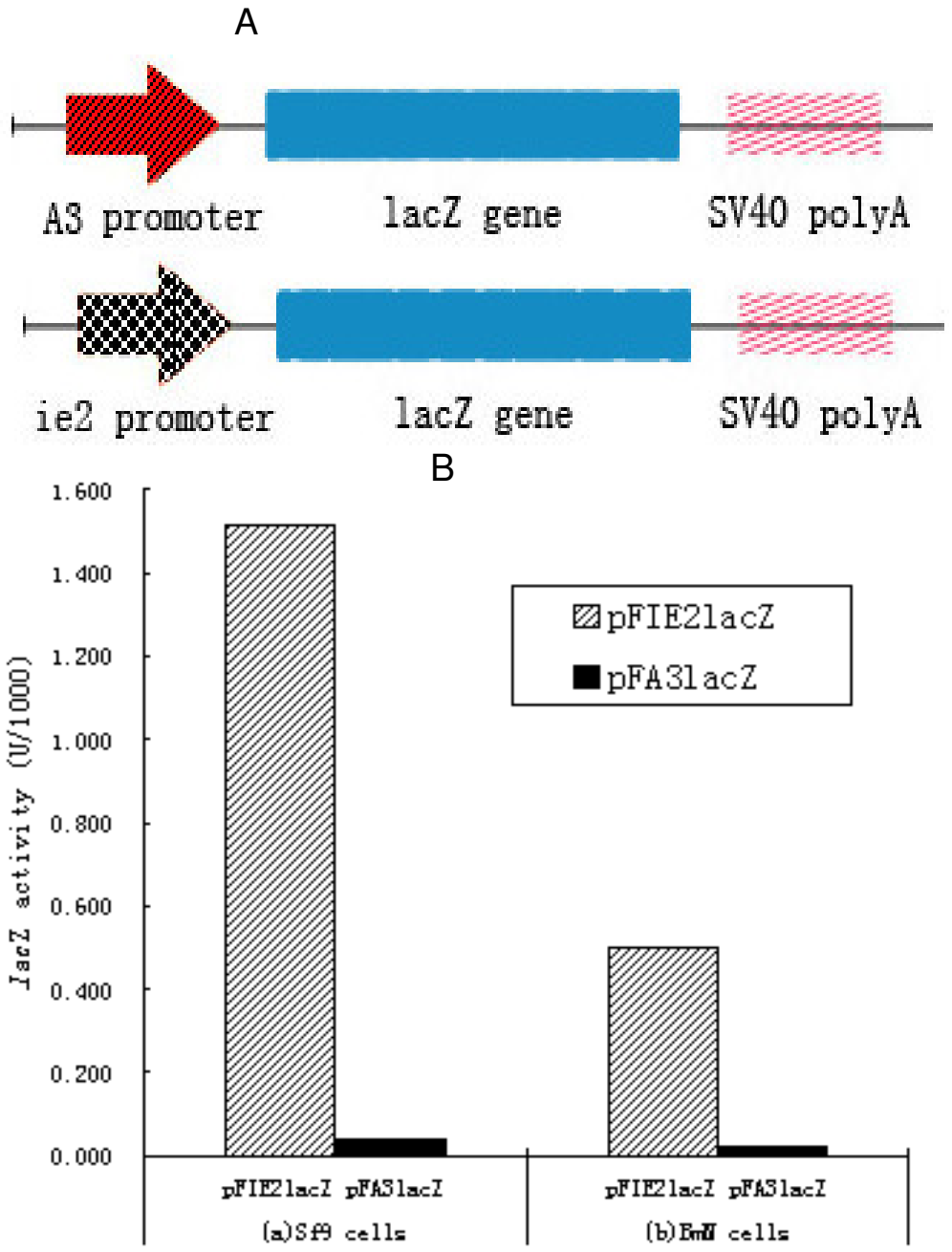

Figure 1. The construction of transient expression vectors and the transient activity analysis of *A3* and ie2 promoters. Diagram (A) indicated *lac*Z gene was respectively constructed into plasmid pFA3 and pFIE2 under the control of A3 and ie2 promoters. (B) showed the normalized ß-gal transient activity under the control of A3 and ie2 promoters in Sf9 (a) and BmN (b) cells.

transfected into Sf9 or BmN cells. 48 h post-transfection (h.p.i), cells were washed three times with phosphate buffered saline (PBS), and then suspended in Tris EDTA sodium chloride (TEN) buffer. The activity of β-galactosidase expressed in insect cells was measured by the standard o-nitrophenyl beta-D-glucoside (ONPG) test according to the Promega's protocol (Schenborn and Goiffon, 1993). When the β-galactosidase cleaves ONPG, the released o-nitrophenol has a yellow color, absorbing 420 nm light. The tests for all samples were repeated at least three times. Test and calculation methods for β-gal quantitative activity assay were in full accordance with the Promega's protocol. Recombinant viruses AcNPV-A3*lac*Z and AcNPV-ie2*lac*Z were used to infect Sf9 cells (MOI=1). Infected cells were collected at the following time post infection: 0, 6, 12, 24, 48 and 72 h.p.i. Samples preparation and β-gal activity assay were performed as described previously. Similarly, all experiments were repeated at least three times.

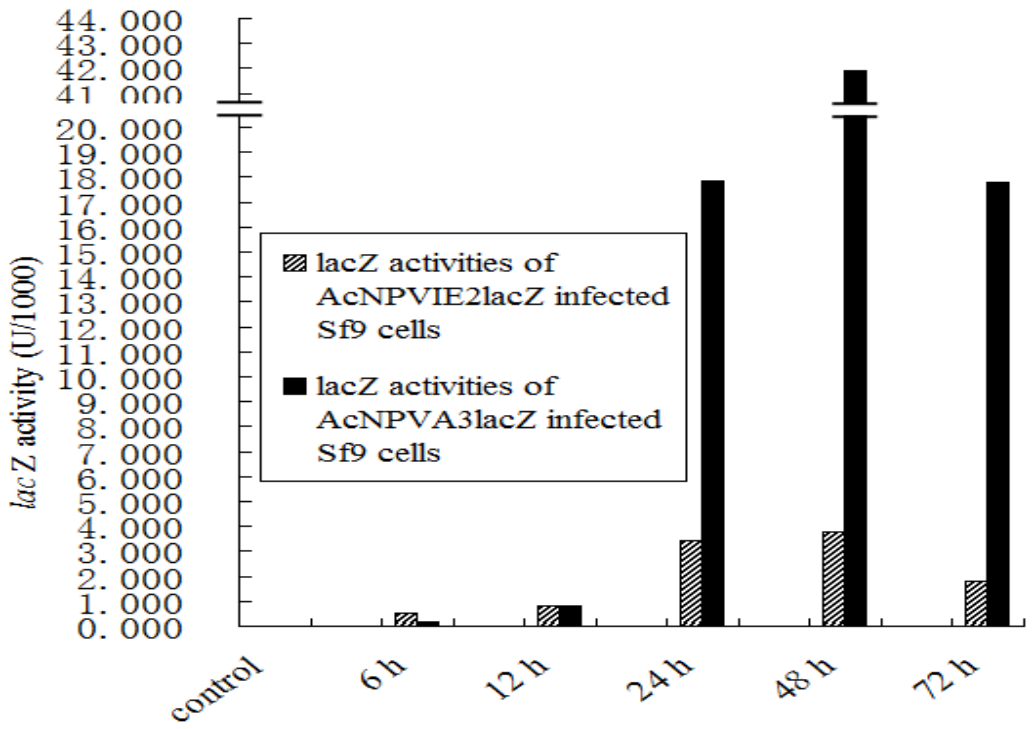

Figure 2. Kinetic activity analysis of β-gal at different stages of infection by recombinant baculovirus. ▨ represents the activity of β-gal in Sf9 cells infected with AcNPV-ie2*lac*Z at different stages of infection. ■ represents the activity of β-gal in Sf9 cells infected with AcNPV-A3*lac*Z.

Sample preparation for the analysis of EGFP expression

Recombinant viruses AcNPV-A3EGFP, AcNPV-ie2EGFP and AcNPV-polhEGFP were used to infect Sf9 cells (MOI = 1). Green cells were observed under a confocal laser scanning microscope (Nikon, Japan) at 0, 6, 12, 24, 48 and 72 h.p.i. respectively. Samples at 72 h.p.i were collected for fluorescence detection and western blot assay. The infected cells were collected using a cell scraper, which was followed by disrupting these collected cells with ultrasonic treatment. After removing the cell debris by centrifugation, the supernatant was used for fluorescence analysis using a fluorescence spectrophotometer (Varian, USA). The fluorescence spectrophotometer was set up as the following parameters: 488 nm for excitation and 500 to 600 nm for emission wavelength. And the intensity at 510 nm (peak) was recorded. EGFP concentration of each sample was calculated by the number of average spectrum peak and EGFP standard curve.

For performing the standard EGFP fluorescence density curve, EGFP was expressed and purified from *E. coli*. In practice, EGFP gene from pIRES-EGFP digested by *Bam*H Ⅰ and *Xho* Ⅰ was cloned into the same sites of pET32b (Novagen) in frame with the His tag at N-terminus to form recombinant pET32b expression vector, which was then transformed into *E. coli* BL21 (DE3) competent cells. EGFP was expressed, isolated and affinity-purified following the instructions in the His-tag fusion protein purification manual (fast protein liquid chromatography (FPLC) of GE Health). The total protein content of purifeid EGFP was determined by using bicinchoninic acid (BCA) protein quantification kit. The fluorescence density of different known concentration of purified EGFP was measured and recorded for making standard fluorescence density curve.

Cell lysates were used for western blotting with alkaline phosphatase-conjugated EGFP and GAPDH polyclonal antibodies according to the manufacturer's protocol, following the running of 10% SDS-PAGE.

RESULTS

Analysis of the transient expression activity of A3 and ie2 promoters in insect cells

The BmA3 promoter was cloned and sequenced (GenBank ID: HQ918291). The sequence analysis indicates that it is a typical eukaryotic promoter and it contains the key elements reported in other actin promoter (Mange et al., 1997). LacZ reporter gene was cloned into the plasmids pFA3 and pFIE2 to create two transient expression vectors pFA3*lac*Z and pFIE2*lac*Z, in which the lacZ was driven by A3 and ie2 promoters, respectively (Figure 1A). Following that, the vectors pFA3*lac*Z and pFIE2*lac*Z were respectively transfected into Sf9 cells that were collected for β-gal activity assay using ONPG test at 48 h.p.i. The same processing method is used to perform the β-gal activity assay for the BmN cells transfected with pFA3*lac*Z or pFIE2*lac*Z. Sf9 and BmN cells transfected with vector pFBDM were used as their negative control.

The results revealed that the transient expression activity of the ie2 promoter was approximately 38-fold and 28-fold higher than that of A3 promoter in Sf9 cells and in

BmN cells, respectively (Figure 1B), indicating ie2 promoter is a remarkably stronger promoter in this case. The results also showed that the transient expression activity of the two promoters in Sf9 cells was approximately three fold higher than that in BmN cells, proving Sf9 cells are more suitable for expressing foreign genes than BmN cells.

Kinetic expression of lacZ gene driven by A3 and ie2 promoters mediated by recombinant baculovirus

The β-gal activity has been determined in those Sf9 cells infected with recombinant baculovirus AcNPV-ie2lacZ or AcNPV-A3*lac*Z at different stages post infection. The kinetic analysis results showed that the activity of ie2 promoter in baculovirus was higher than that of A3 promoter within 12 h.p.i, particularly at 6 h.p.i. and the β-gal activity in cells infected with AcNPV-ie2*lac*Z was approximately equal to that of AcNPV-A3*lac*Z-infected cells at 12 h.p.i (Figure 2).

At the late stages of infection, the activity of A3 promoter rapidly increased to a higher level, and reached the peak at 48 h.p.i., which was approximately ten fold higher than that of ie2 promoter. In comparison with the activity of A3 and ie2 promoters at 48 h.p.i., their activity became lower at 72 h.p.i. At the very late stages of infection (such as 72 h.p.i.), some cells lysed and released the expressed β-gal protein into medium, resulting in a decrease of β-gal activity. Thus, the activity distinction between different stages might be caused by the used method of cell treatment rather than the self-degradation or self-inactivation of the β-gal protein. Certainly, the measurement of β-gal activity is accurate and sensitive before the very late stages of baculovirus infection.

Activity comparison among A3 promoter, ie2 promoter and polh promoter in recombinant baculovirus at the very late stages of infection

EGFP was used as the reporter gene for the activity comparison of A3 promoter, ie2 promoter and polh promoter at the very late stages during baculovirus infection, because the quantitative analysis of β-gal activity is not so authentic at the very late stages due to the cell lysis (72 h.p.i). The polh promoter was strongly activated during the very late stage of infection. EGFP under the control of polh, A3 and ie2 promoters was respectively introduced into Bacmid to construct three types of recombinant baculoviruses and the Sf9 cells infected with the recombinant virus AcNPV-A3EGFP, AcNPV-ie2EGFP and AcNPV-polhEGFP were monitored by confocal laser scanning microscope at different stages of infection (Figure 3).

It was unable to observe obvious fluorescence in Sf9 cells infected by AcNPV-A3EGFP within the 12 h.p.i., while it could detect weak fluorescence in Sf9 cells infected with AcNPV-ie2EGFP at the same time. From 12 to 24 h.p.i., the cells expressing EGFP driven by ie2 and A3 promoters displayed approximately equal fluorescence intensity (Figure 3, lane A and B). However, the fluorescence intensity in Sf9 cells infected by AcNPV-A3EGFP increased rapidly accompanying the elongation of time, whereas that of AcNPV-ie2EGFP enhanced slowly. According to the two kinds of kinetic experiments used to measure the activity of A3 promoter and ie2 promoter, the results using egfp as a reporter gene were consistent with that of using *lac*Z.

The polh promoter was strongly activated during the very late stage of infection as a very late promoter. The cells showed strong fluorescence at 48 h.p.i., and the fluorescence intensity reached to maximum at 72 h.p.i. The cells expressing EGFP were collected and detected by a fluorescence spectrophotometer at 72 h.p.i. (Figure 3, lane C), the data of which was normalized by peak intensity number and standard curve. According to the results, polh promoter exhibited the highest level of activity when mediated by recombinant baculovirus at 72 h.p.i. The activity of polh promoter was approximately five fold than that of A3 promoter and the activity of A3 promoter was approximately ten fold higher than that of ie2 promoter during virus infection (Figure 4 and Table 1), which was also consistent with the result of western blotting analysis (Figure 5). All these results indicated that A3 promoter is another excellent driver for BEVS to complement polh promoter.

DISCUSSION

Our study showed that the transient expression activity of ie2 promoter was higher than that of A3 promoter in both Sf9 and BmN cells. However, the active level of A3 promoter at the late stage of infection was approximately ten fold higher than that of ie2 promoter in baculovirus-mediated expression. As the ie2 promoter from OpMNPV is an immediate early promoter, only host transcriptional factors (such as host RNA polymerase II) are required for its efficient expression (Theilmann and Stewart, 1992). It proved ie2 promoter is more suitable for efficiently producing foreign protein in transient expression and stable expression system.

However, ie2 promoter only performed well before the late stage of infection in BEVS according to the comparison of kinetic activity mediated by baculovirus between ie2 and A3 promoters. As described previously, IE1 protein of baculovirus is a trans-regulator, and it can inhibit the expression level of IE2 protein by controlling ie2 promoter at the late stage of infection (Theilmann and Stewart, 1993).

As we know, it is not easy to express foreign proteins with potential N-linked glycosylation sites in a normal

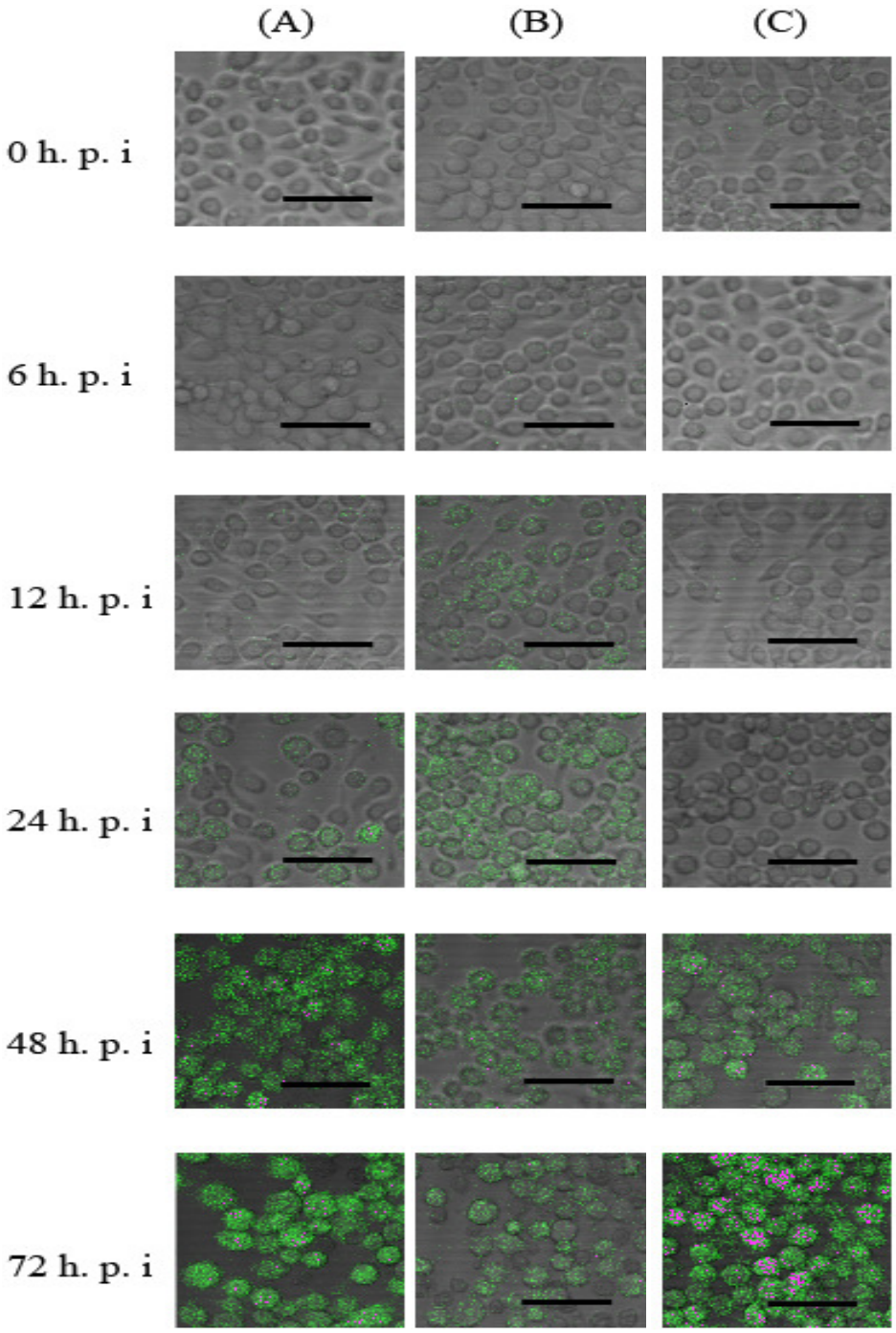

Figure 3. Fluorescence images of Sf9 cells infected by recombinant baculovirus AcNPV-A3EGFP (lane a), AcNPV-ie2EGFP (lane b) and AcNPV-polhEGFP (lane c) at different stages post infection. Bar = 50 μm.

insect cell expression system because not all of them are fully glycosylated. β-1, 4-galactosyltransferase gene under the immediate early promoters can be integrated into chromosome of insect cells or recombinant baculovirus (Hollister et al.,1998). Therefore, it becomes available to produce N-linked glycoproteins in insect cells through expressing mammalian β-1, 4-galactosyltransferase under the control of ie2 promoter by stable expression system and BEVS.

Actin is one type of highly conserved house-keeping protein in all eukaryotic cells, participating in many complex cellular processes, such as muscle contraction, cell motility, cell division and cytokinesis. Similarly, the regulation of A3 promoter is also complicated and

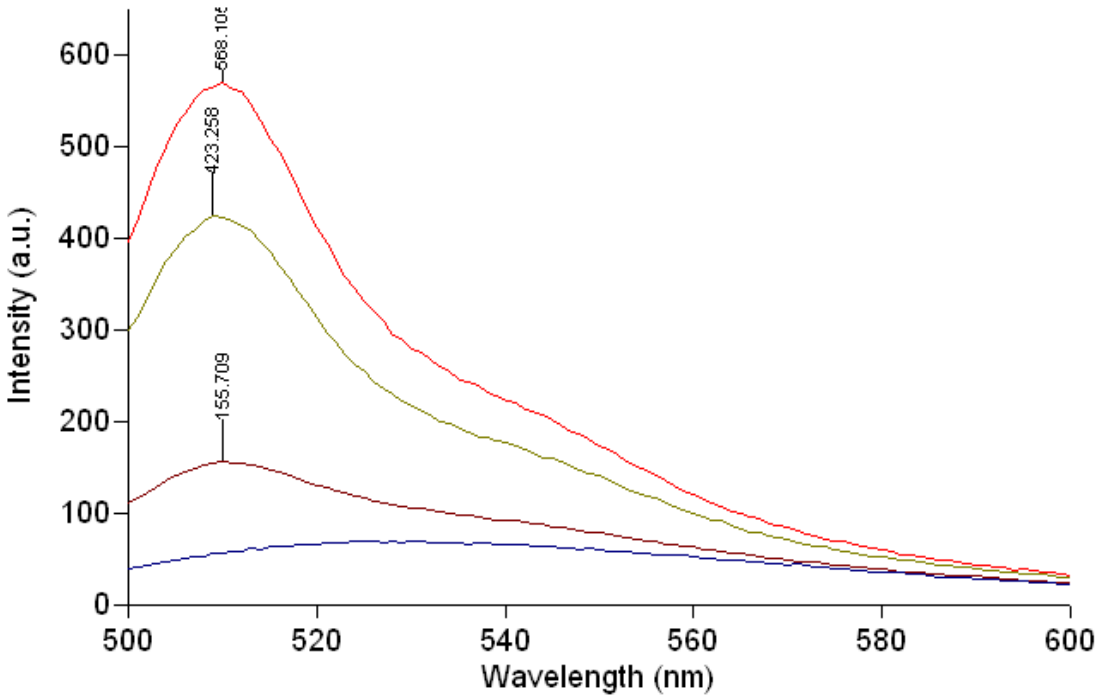

Figure 4. Excitation optical intensity curves of EGFP expression under the control of *A3*, *ie2* and *polh* promoters. Curves respectively represent the fluorescence intensity of *Sf*9 cells infected with recombinant baculovirus AcNPV-A3EGFP (red), AcNPV-polhEGFP (green), AcNPV-ie2EGFP (purple) at 72 h.p.i and mock infection control (blue). To ensure that peak optical intensity within threshold (the standard curve), the sample from Sf9 cells infected with AcNPV-polhEGFP was diluted 5 times before the measurement.

Table 1. Quantitative analysis of the three promoters' activity at the very late stages of infection (72 h.p.i).

Parameter	AcNPV-A3EGFP	AcNPV-ie2EGFP	AcNPV-polhEGFP	Control
Intensity (a.u)	568.105	155.709	423.258	No peak at 510nm
EGFP content (μg/ml)	24.101	2.336	101.86	/

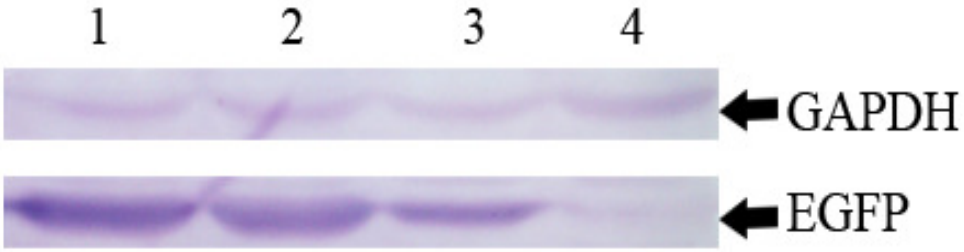

Figure 5. Western blotting analysis of EGFP expression in *Sf9* cells infected by different baculovirus containing ie2, polh and A3 promoter. 1, cells infected with AcNPV-polhEGFP; 2, cells infected with AcNPV-A3EGFP; 3, cells infected with AcNPV-ie2EGFP; 4, mock infection control.

tissue-specific, which might result in its low transient activity in Sf9 and BmN cells. However, the expression efficiency of A3 promoter was improved greatly after the introduction of recombinant baculovirus. The change might be caused by the mass replication of virus vector and the extremely high expression of viral factors that affect the cellular protein synthesis machinery. Previous studies have proved that the transcriptional activator IE1 protein from baculovirus can function as a co-activator of A3 promoter in transfected cells, and enhance the trans-

criptional level (Lu et al., 1996). Then it is possible to stimulate the activity of A3 promoter by adding hr genetic elements into expression vectors (Lu et al., 1997).

In addition, Baculoviruses have been engineered genetically to produce insect selective toxins for developing safe, effective and pest-oriented biocontrol agents for pests, so that pest insects are quickly incapacitated after infection. The continued-advantage from 24 to 48 h.p.i in expression of a passenger gene in infected cell indicates that the actin promoter based expression cassette could be used for generating recombinant baculovirus insecticides which could incapacitate pest insects more quickly than viruses employing the polh or other late viral promoters (Johnson et al., 1992).

Overall, quantitative analysis of the insect cell-specific promoters is important to select proper promoters for high level expression of functional genes in insect cells. Here we compared the total content and kinetics of expressing reporter genes under the control of A3, ie2 and polh promoters. The results indicated that the very late promoters polh can be used for over-expression of foreign genes at the very late stage of infection with the baculovirus expression system. In contrast, the immediate early promoter ie2 is more suitable for the immediate early and early stage of infection. A3 promoter shows great advantages for over-expression at the middle and late stages of infection. The expression level at different virus infection stage of the three promoters indicated that the ie2, A3 and polh promoters are ideal elements for the construction of a time sequential baculovirus multiple genes expression system. For example, in MultiBac system, ie2 promoter is used to express glycosyltransferases at immediate early and early stage for protein modification, while A3 promoter is used to express the secreted polypeptides at middle and late stage, and polh promoter is used to over-express nonsecreted protein. It is possible to combine the three promoters to express viral capsid proteins at different time for improving the productivity of virus-like particles (VLPs) in insect cells. The systemic studies of the insect-specific promoters' activity will improve the insect cell expression system greatly.

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Abbreviations:

AcMNPV, *Autographa californica* multicapsid nucleopolyhedro-virus; **BmNPV**, *Bombyx mori* nucleopolyhedrovirus; ***Op*MNPV**, *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus; ***B. mori*,** *Bombyx mori*/silkworm; **egfp**, enhanced green fluorescent protein gene; **A3,** Actin 3; ie-2, immediately early 2; **ie-1**, immediately early; polh, polyhedron; hr, homologous region; ***E. coli, Escherichia coli***; **h.p.i,** hours post infection; **MOI**, multiplicity of infection; **PCR**, polymerase chain reaction; **PFU**, plaque forming unit; **Sf9**, *Spodoptera frugiperda* 9; **ONPG**, o-Nitrophenyl beta-D-glucoside; **PBS,** phosphate buffered saline; **TEN** buffer, Tris **EDTA** Sodium chloride buffer; β-gal/*lac*Z, β-galactosidase.

REFERENCES

Berger I, Fitzgerald DJ, Richmond TJ (2004). Baculovirus expression system for heterologous multiprotein complexes. Nat. Biotechnol. 22: 1583-1587.

Boyce FM, Bucher NL (1996). Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. USA, 93: 2348-2352.

Coulon-Bublex M, Mounier N, Couble P (1993). Cytoplasmic actin A3 gene promoter injected as supercoiled plasmid is transiently active in *Bombyx mori* embryonic ritellophages. Roux's Arch. Dev. Biol. 202: 123-127.

Ghosh S, Parvez MK, Banerjee K, Sarin SK, Hasnain SE (2002). Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. Mol. Ther. 6: 5-11.

Hollister JR, Shaper JH, Jarvis DL (1998). Stable expression of mammalian beta 1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. Glycobiology, 8: 473-480.

Ikonomou L, Schneider YJ, Agathos SN (2003). Insect cell culture for industrial production of recombinant proteins. Appl. Microbiol. Biotechnol. 62: 1-20.

Johnson R, Meidinger RG, Iatrou K (1992). Cellular promoter-based expression cassette for generating recombinant baculoviruses directing rapid expression of passenger genes in infected insects. Virology, 190: 815-823.

Lin X, Chen Y, Yi Y, Zhang Z (2010). Baculovirus immediately early 1, a mediator for homologous regions enhancer function in trans. Virol. J. 7: 32.

Lu M, Farrell PJ, Johnson R, Iatrou K (1997). A baculovirus (Bombyx mori nuclear polyhedrosis virus) repeat element functions as a powerful constitutive enhancer in transfected insect cells. J. Biol. Chem. 272: 30724-30728.

Lu M, Johnson R R, Iatrou K (1996). Trans-activation of a cell housekeeping gene promoter by the IE1 gene product of baculoviruses. Virology, 218: 103-113.

Mange A, Julien E, Prudhomme JC (1997). A strong inhibitory element down-regulates SRE-stimulated transcription of the A3 cytoplasmic actin gene of Bombyx mori. J. Mol. Biol. 265: 266-274

Pfeifer TA (1998). Expression of heterologous proteins in stable insect cell culture. Curr. Opin. Biotechnol. 9: 518-521.

Pfeifer TA, Hegedus DD, Grigliatti TA, Theilmann DA (1997). Baculovirus immediate-early promoter-mediated expression of the Zeocin resistance gene for use as a dominant selectable marker in dipteran and lepidopteran insect cell lines. Gene. 188: 183-190.

Roldao A, Oliveira R, Carrondo MJ, Alves PM (2009). Error assessment in recombinant baculovirus titration: evaluation of different methods. J. Virol. Methods, 159: 69-80.

Schenborn E, Goiffon V (1993). A new lysis buffer for luciferase, CAT and β-galactosidase reporter gene co-transfections Promega, Notes, 41: 11-14.

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas J L, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P (2000). Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. Nat. Biotechnol. 18: 81-84.

Theilmann DA, Stewart S (1992). Molecular analysis of the trans-activating IE-2 gene of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus. Virology, 187: 84-96.

Theilmann DA, Stewart S (1993). Analysis of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus trans-activators IE-1 and IE-2 using monoclonal antibodies. J. Gen. Virol. 74: 1819-1826.

Tomalski MD, Miller LK (1991). Hitchman RB, Possee RD, King LAB Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature, 352: 82-85.

Weiss S, Grefrath P, Whitford W. (1990). Growth of insect cells in a serum-free medium and production of recombinant proteins using various bioreactors. *In Vitro* Cell. Dev. Biol. 30A.

Yamamoto M, Yamao M, Nishiyama H, Sugihara S, Nagaoka S, Tomita M, Yoshizato K, Tamura T, Mori H (2004). New and highly efficient method for silkworm transgenesis using Autographa californica nucleopolyhedrovirus and piggyBac transposable elements. Biotechnol. Bioeng. 88: 849-853.

Yao LG, Sun JC, Xu H, Kan YC, Zhang XM, Yan HC (2010). A novel economic method for high throughput production of recombinant baculovirus by infecting insect cells with Bacmid-containing diaminopimelate-auxotrophic *Escherichia coli*. J. Biotechnol. 145: 23-29.

Yao N, Yao LG, Kan YC, Zhou WK, QI YP (2006). Improvement of Baculovirus Expression System and Purification of IL-6 Protein Expressed in Insect Cells. Chin. J. Biotechnol. 22: 572-580.

Zhang Y, Zhang X, Xia H, Xue Y, Wang J, Tian B, Wei Z, Lu C (2008). Quantitative analysis of cytoplasmic actin gene promoter and nuclear polyhedrosis virus immediate-early promoter activities in various tissues of silkworm *Bombyx mori* using recombinant Autographa californica nuclear polyhedrosis virus as vector. Acta Biochim. Biophys. Sin. 40: 533-538.