Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (1): 6-11
Published online March 5, 2014 (http://www.cellmolbiol.com)
Received on December 3, 2013, Accepted on February 17, 2014.
doi : 10.14715/cmb/2013.60.1.2

Cytomegalovirus immediate-early promoter efficiently drives heterogeneous gene expression in *Spodoptera frugiperda* (Sf9) insect cells

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Abstract

Recently, wide attention has been given to the potential of recombinant baculovirus as a gene transfer vehicle for mammalian gene therapy. In this study, we packaged the recombinant baculoviruses with cytomegalovirus immediate-early (CMV-IE) promoter in *Spodoptera frugiperda* (Sf9) insect cells, and found that the CMV-IE promoter could efficiently drive the exogenic gene expression in the cells 12 h post-infection (h.p.i.). The expression level at 72 h.p.i. was only around half of that driven by polyhedrin promoter (Ppolh). However, the biological activity of the reporter proteins at 72 h.p.i. were similar with that driven by Ppolh. In addition, the Sf9 cells transfected with CMV-IE-containing plasmids also expressed foreign genes, suggesting that the CMV-IE-directed heterogeneous gene expression in the Sf9 cells was baculovirus-independent. These results demonstrate that the CMV-IE promoter might be used as a regular promoter in Sf9 cells.

Key words: cytomegalovirus immediate-early promoter, enhanced green fluorescence protein, β-galactosidase, recombinant baculovirus, *Spodoptera frugiperda* (Sf9) insect cells.

Introduction

The baculovirus-insect cell system has been widely used to produce biologically active proteins. In recent years, there have been reports on the independent application of the baculovirus system. With its very-late promoter replaced by mammalian promoter, the recombinant baculovirus can be used as a vector for gene delivery in mammalian cells (1). The very-late promoters such as polyhedrin promoter (Ppolh) and p10 promoter (Pp10) are the most commonly used in driving foreign gene expression in insect cells. However, although the expression of the heterogeneous genes can be high, the production of biologically active fractions is commonly unsatisfactory, especially for secreted or membrane-bound proteins (2). The main reason for this defect might be that these very-late promoters initiate a boost expression of heterogeneous genes at the very late stage of the baculovirus life cycle, so that the production of the recombinant proteins overwhelms the post-translation processing of the cellular organelles and that the ectopic expression happens at the time of ongoing deterioration of the infected cells (3). Therefore, researchers have tried to use other promoters, such as the baculovirus early promoters (4, 5) and insect cell-derived promoters, to solve the problem (6).

In our previous experiment of packaging recombinant baculovirus carrying the cytomegalovirus immediate-early (CMV-IE) promoter in *Spodoptera frugiperda* (Sf9) insect cells, we found that the CMV-IE promoter might drive foreign genes to express in Sf9 cells. To confirm this finding, we conducted the present study. The baculovirus vector-mediated expression of two reporter genes, enhanced green fluorescence protein (EGFP) and β-galactosidase (lacZ), under the control of the CMV-IE promoter in Sf9 cells was tested. The expression of the reporter genes mediated by plasmid carrying CMV-IE promoter was also measured to clarify whether the driving activity of CMV-IE promoter was dependent on the baculovirus infection. Hopefully, the data obtained in this study would shed light on better understanding of the effect of CMV-IE promoter in insect cells.

Materials and methods

Plasmid construction and virus production

Baculovirus constructs were prepared by the Bac-to-Bac® baculovirus expression system (Life Technologies, USA). EGFP coding region isolated from pEGFP-C2 by *Nhe*I and *Xho*I digestion and LacZ coding region isolated from pcDNA3.1/V5-his-lacZ by *Hin*dIII and *Xho*I digestion were inserted into pFastBac ™ HT A to yield recombinant baculoviruses expressing EGFP and lacZ under the control of Ppolh, namely, pFastBac-EGFP and pFastBac-lacZ. The CMV-IE promoter isolated from pCDNA3.1(+) by *Nru*I and *Bam*HI was ligated to the backbone of pFastBac™ HT A digested by *Sna*BI and *Bam*HI to construct a baculovirus donor vector with Ppolh being replaced by CMV-IE, which was named pFastBac-CMV-IE. The lacZ coding region was inserted into pFastBac-CMV-IE to yield pFastBac-CMV-IE-lacZ, while the EGFP coding region was inserted to yield pFastBac-CMV-IE-EGFP.

A baculovirus donor vector carrying the simian virus 40 (SV40) promoter, pFastBac-SV40, was also constructed by subcloning SV40 fragment in between the *Sna*BI and *Bam*HI sites of pFastBac-1. Full-length cDNA of

lacZ was inserted between the *Bam*HI and *Xho*I sites of pFastBac-SV40 to creat pFastBac-SV40-lacZ, whereas the EGFP coding region isolated from pEGFP-1 by *Bam*HI and *Not*I digestion was inserted to create pFastBac-SV40-EGFP.

The recombinant pFastBac constructs were individually transformed into *Escherichia coli* DH10Bac cells to generate the corresponding recombinant bacmids. PCR was performed using M13 primers to verify correct recombination.

The *Spodoptera frugiperda* (Sf9) insect cells (American Type Culture Collection, USA) were cultured with SFM 900 II medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin G and 100 U/ml of streptomycin at 28°C. Transfection of Sf9 cells with the recombinant bacmids was performed using Cellfectin™ (Life Technologies, USA), and the recombinant baculovirus vectors were amplified by four-round passages. The obtained viral stocks were stored at 4°C in the dark. The viruses were titrated in Sf9 cells using the BD BacPAK baculovirus rapid titer kit (BD Biosciences Pharmingen, USA) and quantified as focus forming units (FFU).

Sf9 cell infection

Sf9 cells were cultured as described above in 6-well plates. Baculovirus infection was performed at an MOI of 10. Expressions of EGFP and lacZ were detected 48 h after infection.

Sf9 cell transfection

Sf9 cells were incubated in 6-well plates at 2×10^6/well and cultured at 28°C for 2 h, and were then transfected with pFastBac-EGFP, pFastBac-CMV-IE-EGFP, and pEGFP-C2, respectively, using Cellfectin™. Upon transfection, 2 μg plasmid was mixed with 4 μl Cellfectin™ in SFM 900 II, and the mixture was added to the wells 30 min later. Twelve hours after transfection, the cells were infected with an empty baculovirus without a recombinant expression cassette, bacV-Δ-p. The expressions of the reporter genes were analyzed 72 h after the infection.

β-Gal staining

For *in situ* detection of lacZ expression, the cells were washed with phosphate-buffered saline (PBS) two times and fixed with 0.2% glutaraldehyde for 15 min. Then, freshly prepared β-Gal staining solution composed of 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$ and 40 mM citrate (titrated to pH 6.0 with NaH_2PO_4) was added, and the staining was carried out at 37°C for 4 h. Stained cells were observed under a microscope.

β-galactosidase assay

To quantitatively determine the activity of β-galactosidase, the cells were inoculated on 96-well plates at 1×10^5/well and cultured for 12 h at 28°C, followed by baculovirus infection at an MOI of 1. β-galactosidase activity was measured at 0, 6, 12, 18, 24, 36, 48 and 72 h.p.i. using a colorimetric β-Galactosidase Reporter Gene Activity Detection Kit (Sigma-Aldrich, Product Code GAL-A, USA). At each given time point, the medium was removed from the infected wells and 100 μl of cell lysis buffer (1×) was added to each well. After 20-minute incubation at 37°C, 100 ml of β-gal assay buffer (2×) was added to each well, followed by another 30 min incubation. A plate reader was used to determine the absorbance values at a wavelength of 405 nm. The readings were applied to the tissue culture 50% infectious dose (TCID50) equation to obtain quantitative data. β-galactosidase activity was expressed as relative light units (RLU).

Detection of EGFP expression

Expression of EGFP was detected under a fluorescence microscope with a fluorescent intensity manager. The quantitation of EGFP fluorescence was carried out with an FL600 Fluorescence Microplate Reader (BioTek Instruments, Winooski, Vermont, USA) with KC4 data reduction software on an external PC controlling reader function and data capture (BioTek Instruments, Winooski, VT, USA) using a 480/30 nm bandpass excitation filter and a 508/20 nm bandpass emission filter.

Western blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% nonidet P-40 and 0.5% sodium deoxycholate) on ice-bath, followed by 10 min centrifugation at 12,000 rpm at 4°C. The concentration of total cell proteins in the supernatant was determined by Bradford assay. Equal amounts (100 μg) of the total proteins were loaded in 8% SDS-PAGE and electroblotted to a nitrocellulose (NC) membrane (Millipore, USA). Non-specific binding was blocked with 5% nonfat milk in PBST for 1 h at room temperature. Then, the membrane was incubated with anti-EGFP rabbit polyclonal antibody (Abcam ab616, 1: 5000 in blocking solution, USA), anti-lacZ rabbit polyclonal antibody (Abcam ab111258, 1:1000, USA) or anti-GAPDH antibody (Abcam 125247, 1: 2000, USA) overnight at 4°C, rinsed with PBST three times, followed by incubation with HRP-labeled goat anti-rabbit IgG for 1 h. After washing with PBST three times, the blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, USA) reagent for 5 min.

Baculoviral genome extraction and polymerase chain reaction

To avoid the baculoviruses carrying CMV-IE promoter from being contaminated by baculoviruses carrying Ppolh, PCR was utilized to amplify polyhedrin gene and CMV-IE enhancer fragment. Baculovirus genome DNA was extracted after 10 h protease K digestion (10 mM Tris-HCl, pH 8.0, 1 mg/ml protease K, and 0.5% SDS) in 56°C, followed by phenol-chloroform extraction and ethanol sedimentation. The primers used to detect CMV-IE promoter were 5'-GCGTTACATAACTTACGG-3' (forward) and 5'-CGTCAATGGGAGTTTGTTTTG-3' (reverse), and that used to detect Ppolh were 5'-GCGTTACATAACTTACGG-3' (forward) and 5'-CGTCAATGGGAGTTTGTTTTG-3' (reverse). The PCR cycling conditions were 25 cycles of 94°C for 40 s, 47°C for 40 s and 72°C for 40 s. The PCR products were electrophoresed in a 1.5% agarose gel and observed in an ultraviolet lamp.

Results

CMV-IE promoter efficiently drove foreign gene expression

In our initial study, we modified the commercially available baculovirus shuttle plasmid pFastBac™ HT A by replacing Ppolh with CMV-IE or SV40 promoter and prepared recombinant baculoviruses with reporter genes EGFP and lacZ. As reported elsewhere (7), these baculoviruses could efficiently mediate the expression of either EGFP or lacZ in HEK293 cells (data not shown). During the packaging of the recombinant baculoviruses in Sf9 insect cells, we found that cells transfected with the bacmids carrying CMV-IE promoter exhibited EGFP fluorescence and β-galactosidase activity, suggesting that CMV-IE promoter could drive foreign genes to express in Sf9 cells. To confirm this finding, we conducted the present study. The baculovirus stocks were used to infect Sf9 cells at an MOI of 1. EGFP fluorescence and β-galactosidase activity were observed at 72 h.p.i.. As shown in Fig. 1, strong fluorescence is present in the cells infected with bacV-CMV-IE-EGFP, whereas strong β-galactosidase activity is observed in those infected with bacV-CMV-IE-lacZ. These results demonstrated that CMV-IE promoter could efficiently drive the expression of foreign genes in Sf9 cells. Although SV40 promoter was another commonly used promoter to direct the ectopic expression of exogenous genes in

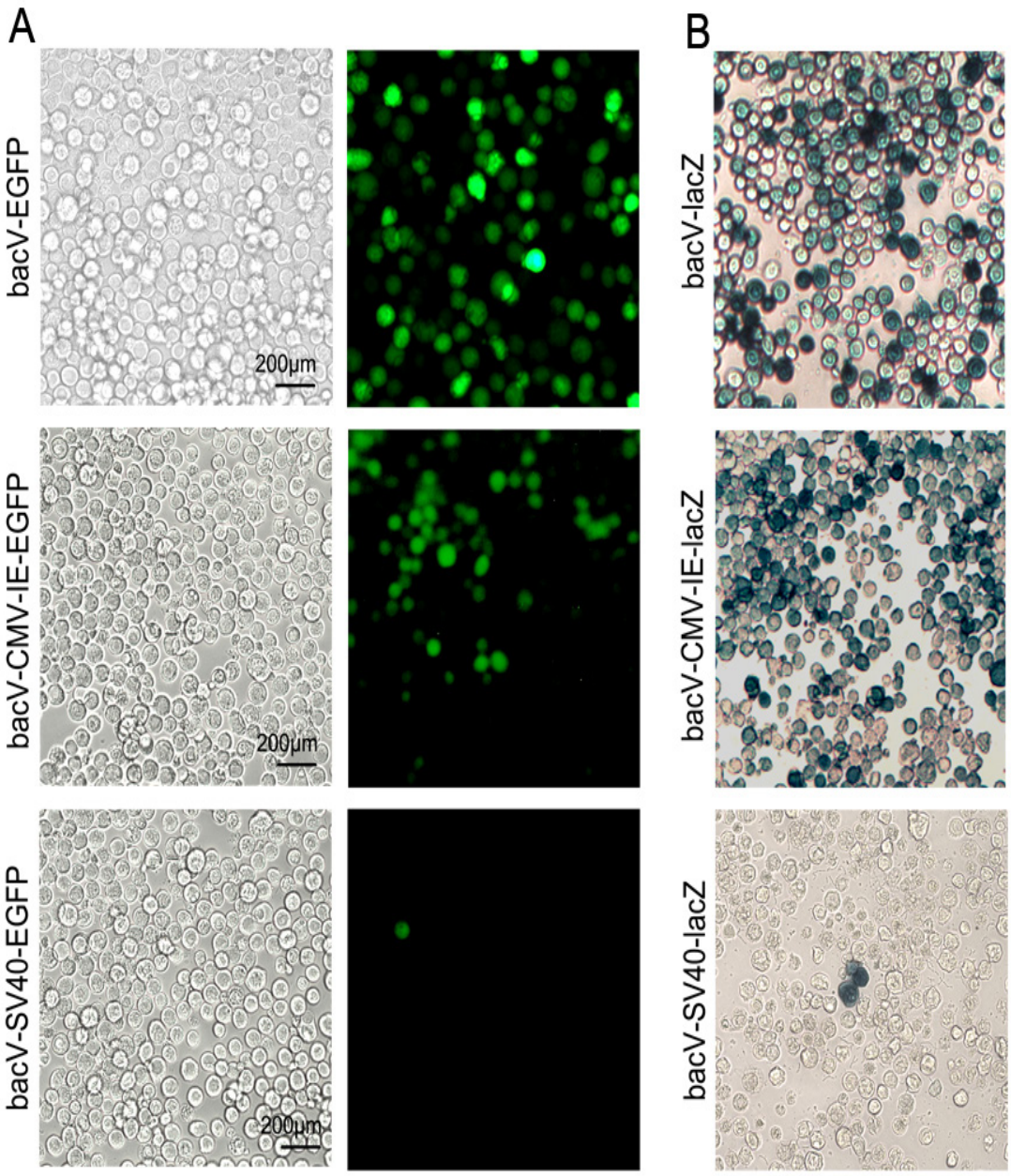

Figure 1. The expressions of reporter genes in the Sf9 cells infected by recombinant baculovirus were observed at 72 h post-infection (h.p.i.). (A) Sf9 cells infected by bacV-EGFP, bacV-CMV-IE-EGFP or bacV-SV40-EGFP (MOI=1) were observed under fluorescent microscopy, respectively. Green fluorescent picture was taken with the FITC channel (green) with a 450/490 nm filter set. BacV-EGFP and bacV-CMV-IE-EGFP both induced the expression of EGFP, and the expression level induced by bacV-CMV-IE-EGFP was lower than that induced by bacV-EGFP. BacV-SV40-EGFP scarcely induced EGFP expression. (B) Sf9 cells infected by bacV-lacZ, bacV-CMV-IE-lacZ or bacV-SV40-lacZ were stained with X-gal at 72 h.p.i. β-galactosidase activity was detected in cells infected by bacV-lacZ or bacV-CMV-IE-lacZ, but minimal activity was seen in cells infected by bacV-SV40-lacZ.

vertebrate cells, EGFP fluorescence or β-galactosidase activity were not detected in Sf9 cells infected with bacV-SV40-EGFP or bacV-SV40-lacZ even when the cells showed obvious lesion (Figure 1).

To exclude the possible contamination of the baculovirus stocks, PCR amplification of CMV-IE promoter and polyhedrin gene was performed. The results confirmed that the CMV-IE-driven expression was not due to the contamination of the baculoviruses carrying Ppolh (data not shown).

CMV-IE promoter drove an early onset, continuous and low-level foreign gene expression

To explore the possible differences in the dynamics between Ppolh- and CMV-IE-driven expressions of foreign genes in Sf9 cells, cells were infected with bacV-lacZ, bacV-CMV-IE-lacZ, bacV-EGFP and bacV-CMV-IE-EGFP, respectively, at the same MOI. The expression levels of β-galactosidase and EGFP were determined by Western blotting at 6, 12, 18, 24, 36, 48 and 72 h.p.i. and the biological activities were quantitated by β-galactosidase assay and fluorometry, respectively. Results showed that the expressions of β-galactosidase and EGFP mediated by bacV-CMV-IE-lacZ and bacV-CMV-IE-EGFP could be detected at 12 h.p.i., and the expression levels increased continuously until 72 h.p.i. when the cells exhibited obvious signs of viral infection. By contrast, the expressions mediated by bacV-lacZ and bacV-EGFP were detected until 24 h.p.i., and increased sharply in the next 48 h. The CMV-IE-driven expressions at 72 h.p.i. were only around half of that driven by Ppolh. In contrast, the biological activities of the reporter proteins at 72 h.p.i. were slightly higher in the cells transduced by the baculoviruses carrying CMV-IE than in the cells transduced by the baculoviruses carrying Ppolh (Figure 2).

The driving activity of CMV-IE promoter is independent of baculovirus infection

Cells were divided into two groups and transfected with plasmids pFastBac-CMV-IE-EGFP, pEGFP-C2 and pFastBac-EGFP. The expression of EGFP in the first group was observed and was measured after 72 h. While, the cells in the second group were infected with an empty baculovirus without a recombinant expression cassette (bacV-Δ-p) 12 h after transfection, and the expression of EGFP was then measured at 72 h.p.i. In the first group, about 10% of the cells transfected with pFastBac-CMV-IE-EGFP or pEGFP-C2 exhibited fluorescence after 72 h, while no fluorescence was observed in the cells transfected with pFastBac-EGFP. In the second group, a similar percent of cells transfected with pFastBac-CMV-IE-EGFP or pEGFP-C2 exhibited fluorescence, but more than 30 percent of the cells transfected with pFastBac-EGFP exhibited fluorescence (Figure 3). These results showed no significant difference in

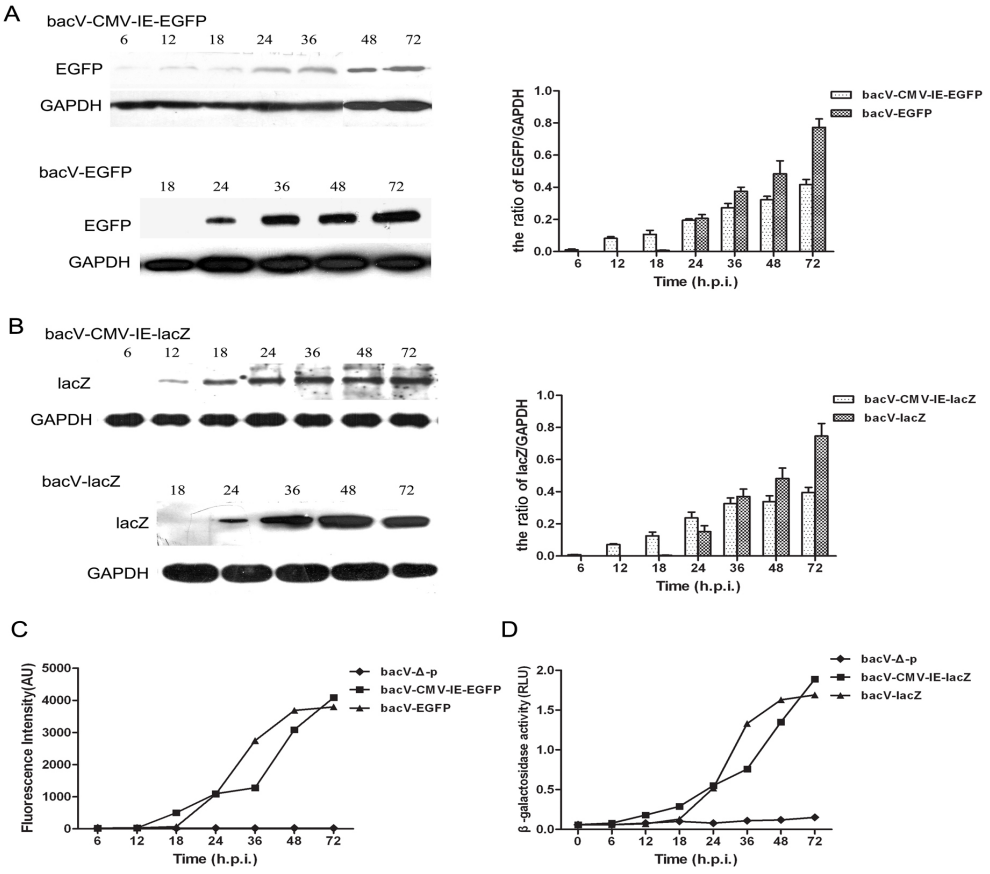

Figure 2. The differences in the dynamics between Ppolh- and CMV-IE-driven expressions of foreign genes in Sf9 cells. (A) Sf9 cells were infected with bacV-CMV-IE-EGFP or bacV-EGFP. EGFP expression driven by CMV-IE promoter was determined by Western blotting at 6, 12, 18, 24, 36, 48 and 72 h.p.i., and that driven by Ppolh was determined at 18, 24, 36, 48 and 72 h.p.i.. (B) Sf9 cells were infected with bacV-CMV-IE-lacZ or bacV-lacZ. LacZ expression driven by CMV-IE promoter was determined at 6, 12, 18, 24, 36, 48 and 72 h.p.i., and that driven by Ppolh was determined at 18, 24, 36, 48 and 72 h.p.i.. (C) The fluorescence intensities of bacV-CMV-IE-EGFP and bacV-EGFP at 6, 12, 18, 24, 36, 48 and 72 h.p.i. were analyzed. bacV-Δ-p without exogenous genes acted as control. (D) The biological activities of β-galactosidase at 6, 12, 18, 24, 36, 48 and 72 h.p.i. were quantitatively determined.

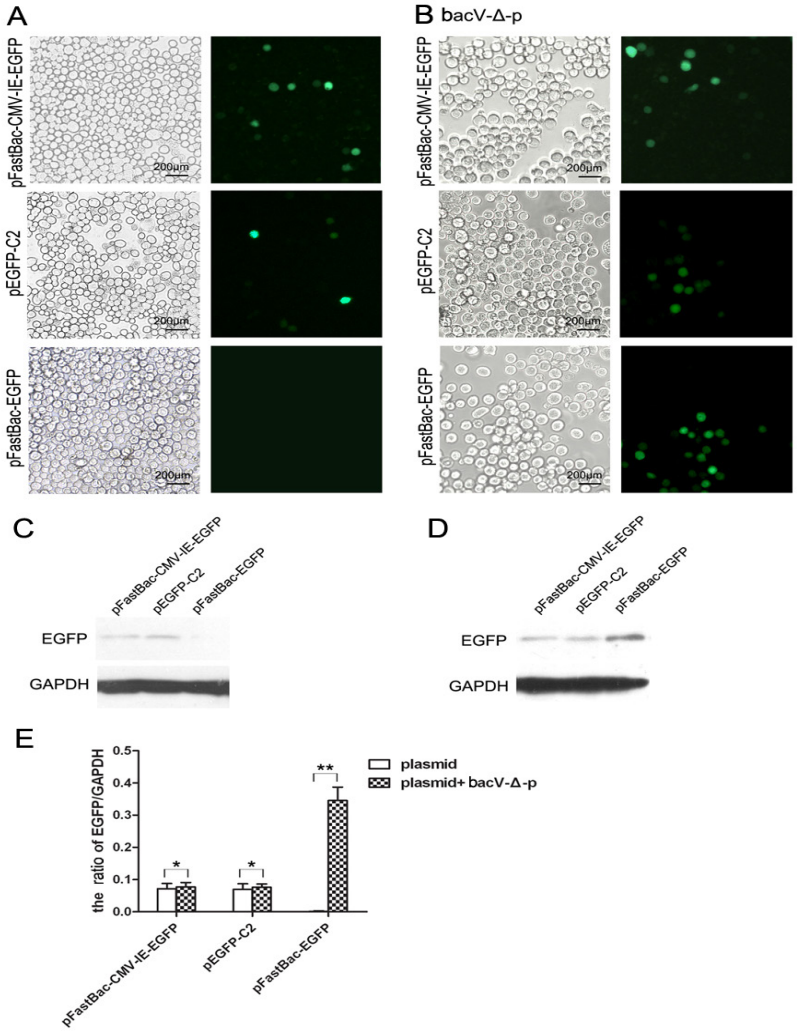

Figure 3. The driving activity of CMV-IE promoter is independent of baculovirus infection, as shown by plasmid transfection with or without baculovirus infection. (A) Sf9 cells were transfected with plasmids pFastBac-CMV-IE-EGFP, pEGFP-C2 or pFastBac-EGFP. The expressions of EGFP in the Sf9 cells transfected were detected after 72 h. (B) Sf9 cells were infected with an empty baculovirus without a recombinant expression cassette (bacV-Δ-p) 12 h after transfection, and the expressions of EGFP were detected at 72 h.p.i.. (C) Sf9 cells were transfected with plasmids pFastBac-CMV-IE-EGFP, pEGFP-C2 or pFastBac-EGFP. (D) Sf9 cells were transfected with plasmids pFastBac-CMV-IE-EGFP, pEGFP-C2 or pFastBac-EGFP, followed by bacV-Δ-p infection. (E) In the group of pFastBac-CMV-IE-EGFP and pEGFP-C2, western blotting did not demonstrate a significant difference in expression levels between the transfected Sf9 cells and the transfection plus baculovirus infected Sf9 cells ($^{*}P > 0.05$). In contrast, subsequent infection of bacV-Δ-p led to an obvious expression of EGFP in pFastBac-EGFP-transfected Sf9 cells ($^{**}P < 0.05$).

the EGFP expression between the transfected cells with or without baculovirus infection. It was indicated that CMV-IE promoter mediated the foreign gene expression in Sf9 cells is independent of baculovirus.

Discussion

CMV-IE promoter can direct the expression of foreign genes in a lot of cells of various origins. Till now, CMV-IE promoter has been reported to be functional in vertebrate cells, *Saccharomyces cerevisiae* (8) and *Escherichia coli* (9). However, its function in insect cells remains controversial. The present study demonstrates that CMV-IE promoter can efficiently drive the foreign genes to express in Sf9 insect cells either transfected with plasmid containing CMV-IE promoter or infected with recombinant baculovirus containing CMV-IE promoter. Further, CMV-IE promoter, compared with Ppolh, drives an early-onset and low-level expression of foreign genes in Sf9 cells, but a similar level of expression of biologically active protein. In addition, the driving activity of CMV-IE promoter in Sf9 cells is independent of baculovirus infection, indicating that Sf9 cells might provide elements for CMV-IE promoter driving, such as RNA polymerase which could bind to CMV-IE promoter and open up the DNA double helix locally to form the transcription bubble.

Our findings imply that CMV-IE promoter might be an alternative choice apart from the baculovirus- or cell-derived promoters in baculovirus-insect cell system. CMV-IE-directed early-onset and low-level foreign gene expression might be beneficial for the post-translational processing, which in turn increases the production of biologically active proteins. For example, the three elements of recombinant adeno-associated virus (AAV) can be expressed by baculovirus carrying CMV-IE promoter at low level from the early period to the very late period of insect cell infection, which improves the efficiency of packaging. Another implication of our study is that the plasmids carrying CMV-IE promoter could be used to transfect insect cells for research purposes, such as displaying cell-surface (10) or observing the protein-protein interaction (11). Further, cells permanently expressing exogenous proteins could be selected

from the transfected cells (12). In addition, insect cells require simpler culture condition and lower cost, and can be suspended at a higher density compare with the mammalian cells. As a result, insect cells may be more suitable to be used in biological research and producing viruses such as AAV (13) and lentivirus (14).

Additionally, the SV40 promoter is a commonly used viral promoter to direct foreign gene expression in mammalian cells, and is reportedly functional in *E. coli* (9). However, our experiment shows that SV40 promoter cannot drive the expression of exogenous genes in Sf9 cells. During the package of baculovirus carrying CMV-IE promoter with some downstream toxic genes, the expression of the toxic genes might influence the viability of insect cells and lead to the failure of producing high-titer recombinant baculoviruses. In this case, SV40 promoter might be a better choice.

Baculovirus-insect cell system is generally jointly used for the production of biologically active proteins. In recent years, the recombinant baculovirus and insect cells in the system are independently applied in some fields. Baculovirus carrying different promoters exhibits various functions (4, 5, 15). CMV-IE promoter overcomes the shortcomings of very late promoters such as Ppolh and Pp10. This is exemplified by our study results that CMV-IE promoter, compared with Ppolh, mediated an earlier-onset and lower-level expression of exogenous genes but a similar level of expression of biologically active proteins in Sf9 cells. Baculovirus design will serve to further enhance the value of baculovirus technology.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No.81070351) and the National Natural Science Foundation of China (No. 81101755).

References

1. Kost, T.A., Condreay, J.P. and Jarvis, D.L., Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol.* 2005, **23**: 567-75. doi: 10.1038/nbt1095.

2. King, L.A., Kaur, K., Mann, S.G., Lawrie, A.M., Steven, J. and Ogden, J.E., Secretion of single-chain urokinase-type plasminogen activator from insect cells. *Gene.* 1991, **106**: 151-157. doi: 10.1016/0378-1119(91)90194-G.

3. Vlak, J.M., Klinkenberg, F.A., Zaal, K.J., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B., Roosien, J. and van Lent, J.W., Functional studies on the p10 gene of Autographa californica nuclear polyhedrosis virus using a recombinant expressing a p10-beta-galactosidase fusion gene. *J. Gen. Virol*. 1988, **69**: 765-776. doi: 10.1099/0022-1317-69-4-765.

4. Jarvis, D.L., Fleming, J.A., Kovacs, G.R., Summers, M.D. and Guarino, L.A., Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Biotechnology (N Y).* 1990, **8**: 950-955. doi: 10.1038/nbt1090-950.

5. Ishiyama, S. and Ikeda, M., High-level expression and improved folding of proteins by using the vp39 late promoter enhanced with homologous DNA regions. *Biotechnol. Lett.* 2010, **32**: 1637-1647. doi: 10.1007/s10529-010-0340-7.

6. Yao, L., Jin, P., Su, S., Xu, H., He, J., Peng, L. and Sun, J., Quantitative analysis of three commonly-used insect cell-specific promoters' activities by transient and baculovirus-mediated expression. *Afr. J. Biotechnol.* 2012, **11**: 1037-1045. doi: 10.5897/AJB11.2662.

7. Jardin, B.A., Zhao, Y., Selvaraj, M., Montes, J., Tran, R., Prakash, S. and Elias, C.B., Expression of SEAP (secreted alkaline phosphatase) by baculovirus mediated transduction of HEK 293 cells in a hollow fiber bioreactor system. *J Biotechnol*. 2008, **135**:272-280. doi: 10.1016/j.jbiotec.2008.04.006.

8. Romero-Santacreu, L., Orozco, H., Garre, E. and Alepuz, P., The bidirectional cytomegalovirus immediate/early promoter is regulated by Hog1 and the stress transcription factors Sko1 and Hot1 in yeast. *Mol. Genet. Genomics.* 2010, **283**: 511-518. doi: 10.1007/s00438-010-0537-4.

9. Lewin, A., Mayer, M., Chusainow, J., Jacob, D. and Appel, B., Viral promoters can initiate expression of toxin genes introduced into Escherichia coli. *BMC Biotechnol.* 2005, **5**: 19. doi: 10.1186/1472-6750-5-19.

10. Borg, J., Nevsten, P., Wallenberg, R., Stenstrom, M., Cardell, S., Falkenberg, C. and Holm, C., Amino-terminal anchored surface display in insect cells and budded baculovirus using the amino-terminal end of neuraminidase. *J. Biotechnol.* 2004, **114**: 21-30. doi: 10.1016/j.jbiotec.2004.05.014.

11. Mon, H., Sugahara, R., Hong, S.M., Lee, J.M., Kamachi, Y., Kawaguchi, Y. and Kusakabe, T., Analysis of protein interactions with two-hybrid system in cultured insect cells. *Anal. Biochem.* 2009, **392**: 180-182. doi: 10.1016/j.ab.2009.05.033.

12. Sonoda, H., Kumada, Y., Katsuda, T. and Yamajia, H., Production of single-chain Fv-Fc fusion protein in stably transformed insect cells. *Biochem. Eng. J.* 2012, **67**: 77-82. doi: 10.1016/j.bej.2012.05.005.

13. Smith, R.H., Levy, J.R. and Kotin, R.M., A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. *Mol. Ther.* 2009, **17**: 1888-1896. doi: 10.1038/mt.2009.128.

14. Lesch, H.P., Turpeinen, S., Niskanen, E.A., Mähönen, A.J., Airenne, K.J. and Ylä-Herttuala, S., Generation of lentivirus vectors using recombinant baculoviruses. *Gene Ther.* 2008, **15**: 1280-1286. doi: 10.1038/gt.2008.76.

15. He, F., Ho, Y., Yu, L. and Kwang, J., WSSV ie1 promoter is more efficient than CMV promoter to express H5 hemagglutinin from influenza virus in baculovirus as a chicken vaccine. *BMC Microbiol.* 2008, **8**: 238. doi: 10.1186/1471-2180-8-238.