

Full-length article

Baculovirus ETL promoter acts as a shuttle promoter between insect cells and mammalian cells¹

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Key words

baculovirus; gene therapy; shuttle promoter; mammalian cell; ETL promoter

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Abstract

Aim: To identify a shuttle promoter that can mediate gene expression in both insect cells and mammalian cells to facilitate the development of a baculovirus vector-based mammalian cell gene delivery vehicle. **Methods:** Recombinant baculoviruses carrying the β -galactosidase reporter gene under the control of an early to late (ETL) promoter of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) or a cytomegalovirus immediate early promoter (CMV promoter) were constructed. COS1, HeLa, CHO-K1, hFob1.19, and MCF-7 mammalian cells were tested for the expression of β -galactosidase. **Results:** ETL promoter activity was higher in bone-derived hFob1.19 than in COS1, HeLa, CHO-K1, or MCF-7 mammalian cells. The transient plasmid transfection assay indicated that ETL promoter activity in mammalian cells was dependent on baculovirus gene expression. **Conclusion:** ETL promoter activity in mammalian cells is baculovirus gene expression-dependent, and the shuttle promoter will facilitate the application of baculovirus expression vectors in mammalian cell expression systems and for gene therapy.

Introduction

The baculovirus-insect expression system is recognized as an excellent tool for the production of recombinant proteins^[1,2]. Since Smith *et al* successfully used the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) as an expression vector to produce human β -interferon in insect cells in 1983^[3], hundreds of foreign genes have been expressed by using baculovirus expression vector systems (BEVS) to date^[4]. When placed under the control of the baculovirus polyhedrin promoter (*polh* promoter) and by replacing the viral polyhedrin gene, recombinant proteins can be expressed at high levels in infected insect cells and larvae, typically at levels ranging between 30% and 50% of the total insect cell proteins during the very late stages of infection^[5].

Baculovirus host specificity has long been considered to be restricted to cells derived from arthropods. However, Hofmann *et al* first demonstrated that baculovirus-derived vectors were able to drive the expression of a reporter gene,

the luciferase gene, in human hepatocytes, provided that a cytomegalovirus (CMV) promoter instead of the *polh* promoter controls transgene expression^[6]. Boyce and Bucher also showed that a human liver tumor cell line, HepG2, and primary rat hepatocytes were able to efficiently express a different reporter gene, the *lacZ* gene, under the control of the Rous sarcoma virus (RSV) promoter^[7]. In both of these pioneering studies, high levels of expression were achieved only in hepatocytes, but only low levels of reporter gene expression were found in other cell lines, such as 293, COS-1, and T-47D. Shoji *et al* subsequently demonstrated that a chimeric CAG promoter, containing the CMV IE enhancer, chicken β -actin promoter, and rabbit β -globin polyadenylation signal, can expand gene expression in non-hepatic cells, such as HeLa and COS-7 cells, by baculovirus transduction^[8]. Furthermore, recombinant baculoviruses carrying a reporter gene under the control of the CAG promoter have been reported to efficiently transduce neural cells *in vitro* and *in vivo*^[9]. Thus, a baculovirus containing a mammalian promoter element is also a promising tool for gene transfer into

mammalian cells in order to study the function of foreign genes, to develop gene therapy strategies, or to express proteins in mammalian cells.

Traditional mammalian promoters, such the simian virus 40 (SV 40) and cytomegalovirus early promoters, do not function well in insect cell systems^[10,11]. However, the AcMNPV transactivator IE1 is functional in mammalian cells^[12]. Because IE1 is able to activate several early, late and very late promoters in the AcMNPV genome, it would be interesting to test whether the baculovirus promoters, including the IE1 promoter, are functional in mammalian cells. Previous studies have indicated that the AcMNPV early to late promoter (ETL promoter) can mediate reporter gene expression in AcMNPV nonpermissive insect cell lines that do not support the virus's replication^[13]. The ETL gene product, proliferating cell nuclear antigen (PCNA), is a protein with a molecular mass of 28 kDa^[14]. In the present study, we demonstrate that the baculovirus ETL promoter can act as a shuttle promoter between insect cells and mammalian cells. We found that ETL promoter activity in mammalian cells was dependent on baculovirus gene expression and that ETL promoter activity was higher in hFob1.19 bone-derived cells than in COS1, HeLa, CHO-K1, and MCF-7 mammalian cells. These results will facilitate the development of baculovirus vector-based mammalian cell gene delivery systems.

Materials and methods

Cells, plasmids and viruses The *Spodoptera frugiperda* IPBL-Sf21 (Sf21) cell line was cultured in TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum^[15]. The recombinant viruses used in the present study were vGS-EV71 and vAcR-IR-G. vGS-EV71 was generated by cotransfection of linearized viral DNA, Bakpak6 (Clontech, Mountain View, CA USA), with transfer vector pGS-EV71 in Sf21 cells. pGS-EV71 contains the internal ribosomal entry site (IRES) element derived from enterovirus 71 (EV71) flanked by the β -galactosidase and secreted alkaline phosphatase (SEAP) reporter genes^[16] (Figure 1A). vAcR-IR-G was generated by cotransfection of linearized viral DNA, Bac-N-Blue (Invitrogen), with transfer vector pBacDIRE in Sf21 cells. pBacDIRE contains the IRES element of the encephalomyocarditis virus (EMCV) flanked by the red fluorescent protein (DsRed) and enhanced green fluorescent protein (EGFP) genes^[17] (Figure 1B). vAcR-IR-G also contains the β -galactosidase gene under the control of the ETL promoter (Figure 1). Sf21 monolayers were used for virus propagation, and all viral stocks were prepared and titers determined according to the standard protocols described by O'Reilly *et al*^[1]. All mammalian cell lines (African green monkey kidney fibro-

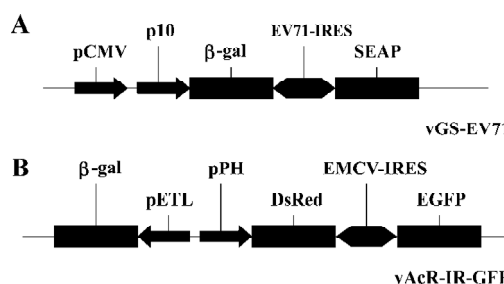


Figure 1. Construction of the recombinant virus. (A) vGS-71, with dual reporter genes, β -gal and SEAP, flanking the EV-71 IRES under the control of the CMV promoter (pCMV) and baculovirus p10 promoter (p10). (B) vAcR-IR-G, with dual fluorescent protein reporter genes. pPH, polyhedrin promoter; DsRed, red fluorescent protein gene; EMCV-IRES, element of encephalomyocarditis virus internal ribosome entry site; EGFP, enhanced green fluorescent protein gene; β -gal, β -galactosidase reporter gene under the control of the ETL promoter, pETL.

blast-like cell line, COS1; human breast cancer cell line, MCF-7; human cervix carcinoma cell line, HeLa; Chinese hamster ovary cells, CHO-K1; and human fetal osteoblastic cells, hFob1.19) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum. Sodium pyruvate containing 1 and 0.1 mmol/L non-essential amino acids were added to the culture medium of MCF-7.

Infection of insect cells and transduction of mammalian cells Cells were seeded in 24-well plates at 1×10^5 – 2×10^5 cells/well. Culture medium was removed and replaced with virus inocula, and the mixture was centrifuged at 2000 rpm for 1 h. Then the supernatant was removed, and fresh medium was added, and the cells were cultured at 27 °C (for insect cells) or 37 °C (for mammalian cells). Forty-eight hours after transduction or infection, the reporter gene assays were carried out.

Transfection of mammalian cells Transfections of mammalian cells were performed using the Lipofectin reagent (Invitrogen). Cells (1×10^5 – 2×10^5) were plated onto 24-well plates. Before transfection, cells were repeatedly washed with serum-free media to remove all traces of sera. One microgram of plasmid was diluted in 200 μ L of serum-free medium, and 2 μ L of Lipofectin reagent was added. The DNA-Lipofectin mix was incubated for 15 min for DNA-Lipofectin complex formation. Then, the DNA-Lipofectin complex solution was transferred to the cells, and serum-free medium was added to adjust the total volume to 0.5 mL. After 12 h, the medium was removed, and 1 mL of fresh medium with 10% fetal bovine serum was added.

β -Galactosidase assay Cells (1×10^5 per well) were infected with vAcR-IR-G or vGSEV-71 at multiplicity of infec-

tion (MOI) values of 10 or 100, respectively. Cell extracts were prepared 48 h after infection. Levels of β -galactosidase activity were measured using a Luminescent β -galactosidase Detection Kit II (BD Biosciences) with a Luminator (Mithras LB940; Berthold Technologies). β -Galactosidase activity was expressed as relative light units (RLU).

For *in vitro* staining of β -galactosidase activity, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 0.25% glutaraldehyde at 48 h post-infection or transduction. After they were rinsed twice with PBS, cells were stained with a solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium hexacyanoferrate, and 2 mmol/L $MgCl_2$ in PBS.

Results

ETL promoter of AcMNPV is a shuttle promoter Because the bicistronic plasmid pGS-EV71 was based on pTriEX-4 (Norvegon, Madison, WI), a baculovirus transfer vector^[16], we were able to generate the recombinant virus, vGS-EV71 (Figure 1A). vGS-EV71 contained the β -galactosidase and SEAP reporter genes under the control of the baculovirus p10 promoter and CMV promoter, so we tried to monitor the expression of β -galactosidase and SEAP in vGS-EV71-infected insect Sf21 cells and in vGS-EV71-transduced COS-1 cells to test whether the EV71-IRES could still mediate cap-independent function in the baculovirus genome. In this experiment, we used vAcR-IR-G (Figure 1B), which contained the green and red fluorescence genes flanking the EMCV IRES sequence under the *polh* promoter, as the control. vAcR-IR-G-infected Sf21 cells appeared blue after X-Gal staining as expected (Figure 2A), because the baculovirus ETL promoter can function well in Sf21 cells. However, the COS1 cells transduced with vAcR-IR-G were also blue after X-Gal staining, which was unexpected (Figure 2B), and vAcR-IR-G-infected hFob1.19 cells also appeared blue after X-Gal staining. To rule out the possibility that the X-Gal staining signal stemmed from baculovirus infection, we infected the Sf21 cells and transduced the COS1 and hFob1.19 mammalian cells with a β -galactosidase gene-deficient recombinant virus, vABhRps, which contained the red fluorescent protein gene under the control of a heat shock promoter. Figure 2A and 2B show that neither the vABhRps-infected Sf21 cells nor transduced mammalian cells had blue staining. These results suggest that the baculovirus ETL promoter may act as a shuttle promoter between insect cells and mammalian cells. Furthermore, ETL promoter activity should be immediately early gene-dependent^[14]; thus, we

hypothesize that COS1 cells and hFob1.19 cells must support baculovirus gene expression at least to the delayed early stage.

Activity of the ETL promoter in different mammalian cells To analyze the cell tropism of the ETL promoter, we tried to transduce COS1, HeLa, CHO-K1, hFob1.19, and MCF-7 mammalian cells with vAcR-IR-G, and then quantified ETL promoter activity by using the β -galactosidase activity assay. We detected significant levels of β -galactosidase expression in CHO-K1, hFob1.19, and HeLa cells (Figure 3). The bone-derived cell line hFob1.19 had the highest expression level of β -galactosidase in the tested mammalian cell lines. This result was similar to that noted for the CAG promoter-luciferase gene cassette-based baculovirus transfer vector^[8]. However, low-level expression of β -galactosidase was found in COS1 and MCF-7 cells. Figure 3 also shows that all of the tested mammalian cell lines infected with vAcR-IR-G at an MOI of 100 had higher β -galactosidase activity than those infected at an MOI of 10; this is consistent with previous studies, which reported that highly efficient transduction of mammalian cells required baculovirus with a high MOI^[6-8].

Activity of the ETL promoter in mammalian cells is baculovirus gene expression-dependent To explore whether the baculovirus ETL promoter is transcribed from the mammalian host RNA polymerase or if its transcription is dependent on the baculovirus immediate early gene, we transiently transfected COS-1, HeLa, and CHO-K1 cells with the pBacDIRE and pGSEV-71 plasmids. Figure 4 shows that the CMV promoter in the pGSEV-71 plasmid mediated β -galactosidase gene expression in these cell lines, but that the ETL promoter in pBacDIRE could not mediate β -galactosidase gene expression in mammalian cells. This result implies that ETL promoter activity in baculovirus-infected mammalian cells may be baculovirus early gene (eg IE0, IE1+, or IE2)-dependent^[14]. It will be interesting to test the baculoviral early promoters in mammalian cells, and this result also suggests that early genes and the ETL gene may be transcribed within mammalian cells transduced with recombinant baculoviruses, although the viral DNA did not replicate and no apparent cytopathic effects (CPE) were reported^[8].

Butyrate did not enhance the activity of the ETL promoter in mammalian cells Several studies have shown that histone deacetylase (HDAC) inhibitors, such as butyrate, can effectively enhance protein expression in mammalian cells^[18,19]. To clarify whether the baculovirus early gene-dependent ETL promoter can also be enhanced by butyrate, we added 10 mmol/L butyrate to the COS-1 cell culture medium after transduction with vAcR-IR-G or vGS-EV71 (Figure 5). We found that the addition of 10 mmol/L butyrate did

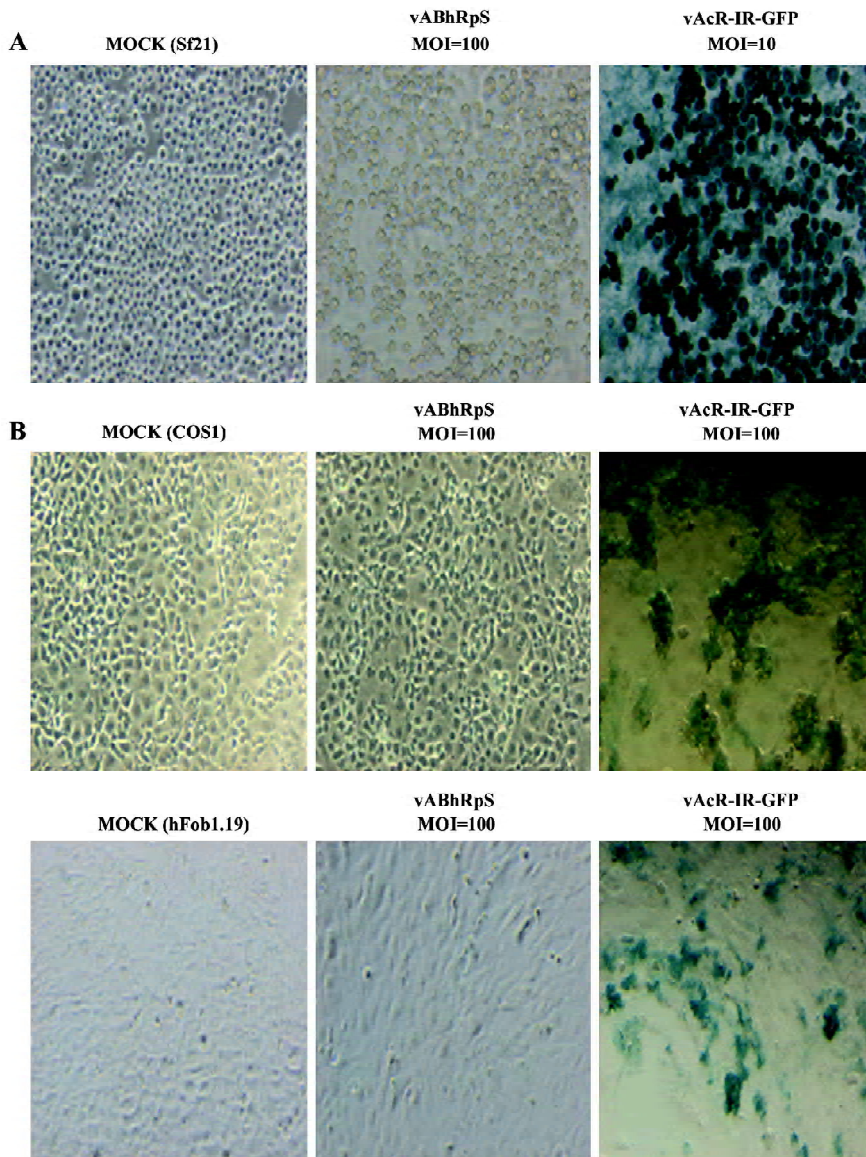


Figure 2. X-Gal staining of cells infected with the recombinant baculovirus vAcR-IR-G. (A) Sf21 cells were stained with X-Gal 48 h after infection with recombinant baculovirus vAcR-IR-G and a control recombinant baculovirus that did not carry the β -galactosidase reporter gene, vABhRpS. (B) COS1 and hFob1.19 cells were stained with X-Gal 48 h after being transduced with either the vAcR-IR-G or vABhRpS recombinant baculovirus. Photographs ($\times 100$) were taken under fluorescence microscopy (Nikon).

enhance β -galactosidase gene expression in COS-1 cells transduced with vGS-EV71, with a nearly 10-fold enhancement (Figure 5A). This result is consistent with the results of previous studies, in which butyrate was found to enhance protein expression in baculovirus-transduced mammalian cells^[9,20]. However, the addition of 10 mmol/L butyrate did not enhance β -galactosidase gene expression in COS-1 cells transduced with vAcR-IR-G (Figure 5A). We further tested the effect of butyrate on COS-1 cells transduced with vAcR-IR-G at different MOI. Figure 5B shows that the addition of butyrate did not enhance β -galactosidase gene expression controlled by the ETL promoter. Thus, these results revealed that blockage of HDAC activity and then prevention of histone deacetylation could not promote the baculovirus ETL

promoter, although blockage of the activity of HDAC often upregulates gene expression.

Discussion

Since recombinant baculoviruses became established as efficient gene transduction vehicles for mammalian cells, recombinant baculoviruses have also become attractive vectors for *in vitro* and *in vivo* gene therapy. One of the advantages of using these insect viruses for delivery of therapeutic genes to mammalian cells is that unlike other mammalian viruses, they do not replicate in mammalian cells. Thus, a baculovirus-based vector can be much safer than current viral vectors^[21]. In addition, as gene therapy vectors,

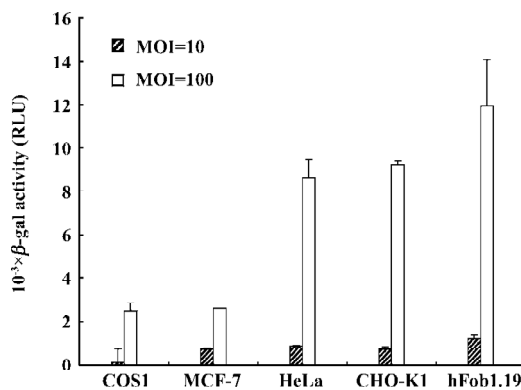


Figure 3. Expression of the β -galactosidase gene in various mammalian cells transduced with vAcR-IR-G. Cells were transduced with vAcR-IR-G at an MOI of 10 or 100 and harvested 48 h after inoculation. β -Galactosidase activity was measured as described in the text. Values for relative light units refer to extracts from 10^5 cells. The data shown are representative averages of 3 independent experiments.

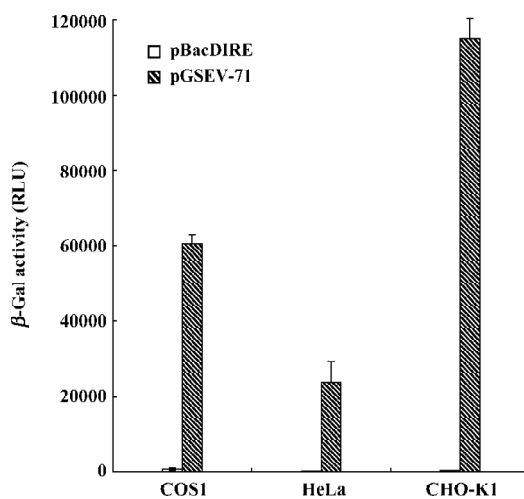


Figure 4. Transient transfection of pBacDIRE and pGSEV-71 in COS1, CHO-K1, and HeLa cells. Cells were transfected with pBacDIRE or pGSEV-71 (1 μ g) as described in the text and harvested 48 h after transfection. β -galactosidase activity was measured as described in the text. Values for relative light units refer to extracts from 1×10^5 cells. The data shown are representative averages of 3 independent experiments.

baculoviruses also possess the following advantages^[8,22]: (1) given the flexibility of the baculovirus envelope, large DNA insertions (>20 kb) can be accommodated in the dsDNA genome; (2) recombinant baculoviruses have the ability to reach high titers (> 10^{10} pfu/mL); and (3) recombinant baculoviruses are easily prepared. Recently, studies have demonstrated that baculoviruses can mediate gene transfer

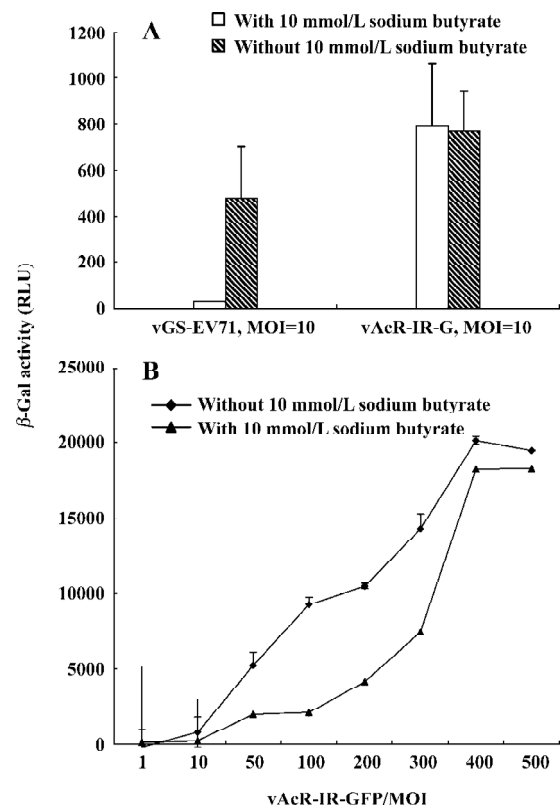


Figure 5. Effect of sodium butyrate on the activity of the ETL promoter in COS1 cells. (A) COS1 cells (1×10^5 per well) were transduced with vAcR-IR-G or vGS-71 at an MOI of 10 in the presence of 10 mmol/L sodium butyrate and harvested at 48 h after inoculation. β -galactosidase activity was measured as described in the text. The data shown are representative averages of 3 independent experiments. (B) Dose-response curve to vAcR-IR-G transduction in the presence of 10 mmol/L sodium butyrate. The data shown are representative averages of 3 independent experiments.

into articular chondrocytes^[23] and human mesenchymal stem cells^[24]. These results will expand the application of baculovirus vector systems into the field of cartilage tissue engineering^[22]. In the present study, we demonstrated that the baculovirus ETL promoter could function as a shuttle promoter between insect cells, the hosts of baculoviruses, and mammalian cells (Figures 2 and 3). Neither the traditional CMV promoter nor the CAG promoter appears to function well, if at all, in insect systems^[10,11,25]. So the expression of recombinant baculoviruses containing the desired gene under the control of the CMV promoter or CAG promoter cannot be detected until transduction into mammalian cells. In contrast, the desired gene expression under the control of the ETL promoter can be monitored and checked in insect cells before performing mammalian cell transduction experiments. Therefore, the finding that the ETL promoter

can act as a shuttle promoter will facilitate the development of baculovirus vector-based mammalian cell gene delivery vehicles.

The susceptibility of various mammalian cell lines to the ETL promoter was monitored by the β -galactosidase reporter assay (Figure 3). We detected significant levels of β -galactosidase expression in CHO-K1, hFob1.19, and HeLa cells. This result was similar to that found for a CAG promoter-luciferase gene cassette-based baculovirus transfer vector^[8]. The bone-derived cell line hFob1.19 had the highest expression level of β -galactosidase among the tested mammalian cell lines (Figure 3). Interestingly, previous studies also found that a human bone-derived sarcoma cell line, osteogenic sarcoma cell line (Saos-2), had higher susceptibility than any other known permissive mammalian cells to infection by baculoviruses^[21]. However, low-level expression of β -galactosidase was found in COS1 and MCF-7 cells. Previous studies showed that the monkey kidney cell line, like COS1 and COS7, exhibited high-level gene expression through CAG promoter-based baculovirus transfer vectors. So, the transcription factors that are required for the ETL promoter may be restricted in COS1 cells. In addition, we also found that sodium butyrate enhanced CMV promoter-mediated β -galactosidase gene expression, but did not enhance ETL promoter-mediated β -galactosidase gene expression in COS-1 cells (Figure 5). A plasmid-based transient transfection assay also revealed that activation of the ETL promoter in mammalian cells was baculovirus gene expression-dependent (Figure 4). These results indicate that transcription initiation from the ETL promoter differs from that of the CMV promoter.

To study the expression of foreign genes *in vitro* or *in vivo*, viral vectors are generally useful. However, the replication or gene expression of viral vectors in the desired host may sometimes cause complex effects. The present study also raised a question about baculovirus gene expression in mammalian cells when recombinant baculoviruses are employed as a gene delivery vehicles. Although previous studies indicated that baculovirus viral DNA would not replicate in mammalian cells, no apparent CPE was observed even when cells were transduced with an MOI as high as 100^[8,22]. Our experiments demonstrated that the baculovirus gene will be transcribed by the mammalian RNA polymerase, and that the transcriptional factors required for ETL promoter activity should be activated in mammalian cells. For example, it has been reported that the baculovirus transactivator IE1 is functional in mammalian cells^[12]. To investigate the expression profile of AcMNPV genes in mammalian cells, a baculovirus-specific DNA microarray may be critical.

Recently, a baculovirus-specific DNA microarray was used to investigate the expression profile of AcMNPV genes in permissive and nonpermissive insect cell lines^[26]. Surprisingly, nearly all of the 154 genes of AcMNPV, including the ETL gene, appeared to be expressed in both permissive (SF9) and nonpermissive (BmN, a silkworm-derived cell line) cells, although the peak expression levels of these genes were delayed approximately 12 h in nonpermissive BmN cells. Interestingly, the product of the ETL gene is necessary, either directly or indirectly, for the expression of many other AcMNPV genes^[14]. So, it is important to determine how many ETL gene-dependent baculovirus intrinsic genes are expressed in mammalian cells before the application of recombinant baculoviruses for gene therapy.

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