# Human β-Globin Second Intron Highly Enhances Expression of Foreign Genes from Murine Cytomegalovirus Immediate-Early Promoter

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**Abstract**   To develop a highly efficient mammalian expression vector, a series of vectors were constructed based on the murine cytomegalovirus (MCMV) immediate-early (IE) promoter and human β-globin second intron. The resulting MCMV promoter was several-fold stronger than the HCMV promoter in various mammalian cell lines, such as the NIH3T3, Neuro-2a, 293T, and HT1080 cell lines, and was only slightly weaker than the HCMV promoter in HeLa and CHO cells. The inclusion of the human β-globin second intron behind the MCMV promoter or HCMV promoter markedly enhanced the promoter activity in various mammalian cell lines, and the resultant MCMV/Glo-I expression system was stronger than the HCMV promoter from 4.7- to 11.2-fold in every cell line tested. Also, the MCMV/Glo-I promoter induced a higher level of the VSV-G protein in a transiently transfected 293T cell line, which is useful for the production of recombinant retrovirus and lentivirus vectors.

**Key words:** MCMV IE promoter, human β-globin second intron, high-level gene expression

The expression of foreign genes in mammalian cells is essential for gene therapy, DNA vaccines, and cell biology research, as well as the production of medicinal products in mammalian cells. It is already known that the synthesis of foreign recombinant proteins from mammalian expression systems is mainly linked to the strength of the promoter in the expression vector, thus determining the optimal promoter

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as the primary requirement for the high-level expression of foreign genes in such systems [23–25]. Until now, the SV40 promoter/enhancer [12], HCMV promoter/enhancer [5], RSV LTR [14], or EF-1α promoter [17] have all been shown to be quite efficient promoters in a variety of cell lines, and are widely used in many commercial vectors. However, a recent publication reported that the murine cytomegalovirus immediate-early (MCMV IE) promoter was more active than the HCMV promoter in both murine and human cell lines [1], and more stably and consecutively expressed than the HCMV promoter in various cell lines from diverse species [20]. Also, the MCMV IE promoter was found to direct higher levels of transgene expression than the HCMV IE promoter *in vivo* [1]. As such, the MCMV IE promoter/enhancer would appear to be a good starting point for developing a versatile vector with a high expression capacity and extremely wide host range.

To maximize or optimize gene expression in mammalian cells, many elements of the expression module, including promoters, enhancers, and polyadenylation [poly(A)] signals have been isolated, characterized, and examined. In addition, recent studies have shown that the use of homologous and heterologous introns can affect the efficiency of gene expression in recombinant mammalian systems. It is generally accepted that introns significantly augment the expression of genes depending on the nature of the gene and cell line [6, 8, 10, 11, 18, 22], and many commercial vectors (for instance, pSI and pCI from Promega, U.S.A., or pSG5 from Stratagene, U.S.A.) adopt heterologous introns to drive a higher level of expression. The effect of introns in the augmentation of eukaryotic gene expression has been shown to be mainly in the posttranscriptional stage [15]. Heterologous

introns are only effective when they are located between the promoter and the gene of interest, while introns in the 3' untranslated region hardly enhance gene expression, even sometimes inhibiting it [13, 16]. Therefore, based on these reports, the combination of a strong promoter/enhancer and the above-mentioned elements needs to be examined to achieve a high level of gene expression.

Accordingly, this study constructed a mammalian expression vector containing a combination of the MCMV IE promoter and the human β-globin second intron. The resultant MCMV/Glo-I expression vector achieved a high level of expression in various human and rodent cell lines. As such, this new expression vector will be useful in both gene expression experiments and *in vivo* gene therapy trials, including DNA vaccine trials.

## MATERIALS AND METHODS

### Plasmid Construction

The plasmid pHYK was constructed by the self-ligation of pCR™3 (Invitrogen, Carlsbad, CA, U.S.A.) linearized by *Eco*RI, and the resulting pHYK was nearly identical to pcDNA3.1 (Invitrogen, U.S.A.), which is in widespread use. The plasmid pMYK was then constructed by inserting the *Sca*I/*Hin*dIII fragment of the MCMV promoter from pUCMV18 [19] into *Sca*I/*Hin*dIII-digested pHYK. pMYKluc and pHYKluc were constructed by inserting a 2.7 kb *Hin*dIII/*Bam*HI luciferase gene isolated from pGL2/basic (Promega, Madison, WI, U.S.A.) into *Hin*dIII/*Bam*HI-digested pMYK and pHYK, respectively. To facilitate subsequent subcloning, the *Hin*dIII/*Sal*-digested luciferase gene of pGL2/basic was inserted into *Hin*dIII/*Sal*I-digested pBluescript/KS(+) (Stratagene, U.S.A.) to make pKS/luc(HS). pEFluc was constructed by inserting the *Bam*HI/*Hpa*I luciferase fragment from pKS/luc(HS) into *Bam*HI/*Eco*RV-digested pEF [21]. The plasmids pHYK/Glo-I and pMYK/Glo-I were constructed by inserting an 875 bp *Eco*RI fragment from pGEM-T/β-globin, kindly donated by Dr. P. L. Hahn (Ewha Woman's University, Seoul, Korea), into *Eco*RI-linearized pHYK and pMYK, respectively. The plasmids pHYK/Glo-I/luc and pMYK/Glo-I/luc were constructed by inserting the *Sma*I/*Hpa*I luciferase gene fragment from pKS/luc(HS) into *Eco*RV-digested pHYK/Glo-I and pMYK/Glo-I, respectively. The plasmids pMG/VSV-G and pME/VSV-G were constructed by inserting the *Not*I/*Xba*I VSV-G gene fragment into *Not*I/*Xba*I-digested pMYK/Glo-I and pMYK/EF-I, respectively.

### Cell Culture, Transfection, and Luciferase Assay

The HeLa (ATCC, CCL-2), 293T (ATCC, CRL-1573), HT1080 (ATCC, CCL-121), NIH3T3 (ATCC, CRL-1658), and CHO (ATCC, CCL-6) cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%

FBS, 100 U/ml penicillin G sulfate, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B (Life Technologies, Rockville, MD, U.S.A.). The U937 cells were grown in RPMI media supplemented with 10% FBS, 100 U/ml penicillin G sulfate, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B. All cultures were maintained at 37°C in an incubator with 5% $CO_2$. The adherent and suspended cells were transfected by electroporation using an EquiBio electroporator (The Wheelwrights Boughton Monchelsea, KENT, U.K.). In the case of the adherent cells, $1.5 \times 10^6$ cells were resuspended in 400 μl of RPMI with 10% FBS, then 3 μg of reporter plasmids and 2 μg of pRL/TK (Promega) plasmids were added to normalize transfection efficiency, along with an electric pulse of 260 V and 1,080 μF. Thereafter, the cells were transferred into 6-well plates filled with 3 ml of DMEM including 10% FBS, washed twice with phosphate buffer saline (PBS) after 6 h of incubation, the 3 ml of DMEM replenished, and the cells incubated for a further 48 h. The U937 cell lines were also transfected under the same electroporation conditions, but after the electric shock, the cells were transferred to 3 ml of RPMI media and incubated for 48 h without any media change. Thereafter, the cells were washed twice with PBS, harvested, and subjected to a dual luciferase assay according to the manufacturer's instruction (Promega). The transfection efficiencies were normalized by the relative luciferase activity of an independently transfected *Renilla* luciferase gene. All transfections were performed in triplicate, and the results were the means of at least two independent experiments.

### Retroviral Infection and X-Gal Assay

293T cells were plated onto 6-well plates the day before transduction at a density of $1 \times 10^5$ cells per well. A viral supernatant containing 8 μg of polybrene (H-9268, Sigma) per ml was added to each well, and the cells incubated for 4 h at 37°C. The viral supernatant was then removed, and the cells incubated in the culture medium for 48 h. After removing the culture media, the infected cells were fixed with 1 ml of a fixing solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min at room temperature, washed three times with phosphate-buffered saline (PBS), and incubated with an X-gal staining solution (0.1 mg/ml X-gal, 5 mM $K_3Fe (CN)_6$, 5 mM $K_4Fe(CN)_6 3H_2O$, 1 mM $MgCl_2$, all ingredients prepared in PBS) at 37°C overnight.

### Western Blotting

(0.5 μg) pMYK, pHE/VSV-G, and pMG/VSV-G plasmid DNA were cotransfected with 0.5 μg of pMYK/eGFP, a GFP expression plasmid vector, into 293T ($5 \times 10^5$ cell/6 well) cells, using a lipofectamine reagent, as described in the manufacturer's manual. The transfection efficiency was normalized by the amount of GFP expression. After 48 h, the cells were harvested and lysed in a 1× SDS gel-
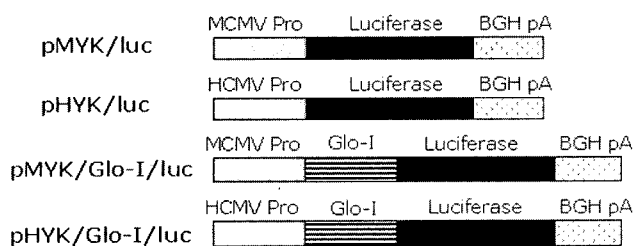
**Fig. 1.** Schematic representation of luciferase gene expression plasmid vectors containing various promoters.
MCMV Pro, murine cytomegalovirus immediate-early promoter; HCMV Pro, human cytomegalovirus immediate-early promoter; BGH pA, bovine growth hormone polyadenlation signal; Glo-I, human beta-globin second intron. See Materials and Methods for details.

loading buffer containing 50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. The lysate was then boiled for 5 min, separated on a 12% SDS-PAGE gel, and transferred onto a PVDF (Bio-Rad Laboratories, Hercules, CA, U.S.A.) membrane transfer buffer containing 48 mM Tris base, 39 mM glycine, and 20% methanol. Next, the membranes were incubated with a blocking buffer consisting of 5% skim milk in a TBST buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05%

Tween-20) for 1 h and then with an anti-VSVG monoclonal antibody (Sigma-Aldrich Co.) diluted 1:5,000 in the same buffer for 1 h, washed 3 times with TBST, and incubated with HRP-conjugated anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, U.S.A.) diluted in 1:5,000 for 1 h. After washing the membrane three times with TBST, the signal was visualized with ECL detection reagents (Amersham Bioscience, U.K.), and the band intensity measured using Biorad Quantity 1-D Analysis software (version 4.51).

**RESULTS**

**Comparison of MCMV, HCMV, and EF-1α Promoter Activities**

The immediate-early promoter activity of MCMV and HCMV was compared in six or seven different cell lines encompassing human and rodent cell lines (Fig. 2A). Generally, the MCMV promoter exhibited higher promoter strength than the HCMV promoter, except for two cases (HeLa and CHO), where the MCMV promoter showed a slightly lower promoter activity. The MCMV promoter was 6.71-fold more active than the HCMV promoter in NIH3T3 (mouse fibroblast cell line), 7.87-fold more active
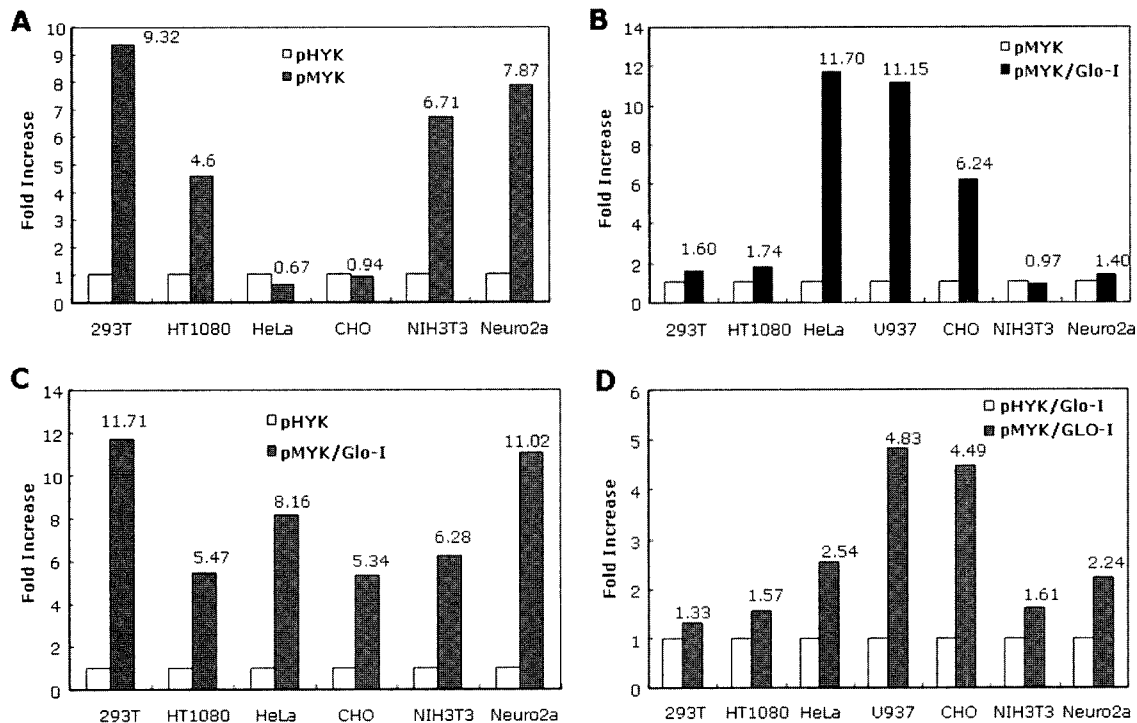


**Fig. 2.** Comparison of promoter activity by dual luciferase assay in various cell lines.
A. Comparison of immediate-early promoter activity of MCMV and HCMV in six different cell lines. B. Promoter activity resulting from insertion of human β-globin second intron downstream of the MCMV promoter. C. Comparison of the promoter strength of MCMV/Glo-I and HCMV. D. Promoter activity resulting from insertion of human β-globin second intron downstream of the MCMV and HCMV promoter. The lucifease assay was performed according to the Promega standard protocol. The activity values were normalized by the relative luciferase activity of an independently transfected *Renilla* luciferase gene. The values are the means of at least two independent experiments.

in Neuro-2a (mouse neuronal cell line), 9.32-fold more active in 293 (human embryonic kidney cell line), and 4.6-fold more active in HT1080 (human fibroblast cell line). The MCMV promoter was also compared with the elongation factor one-alpha promoter (EF-1$\alpha$), known as a strong promoter in human cell lines, particularly the HeLa cell line. The results showed that the EF-1$\alpha$ promoter was 2-fold stronger than the MCMV promoter (data not shown).

### Effect of Inserting Human $\beta$-Globin Second Intron Downstream of the MCMV Promoter

The effect of inserting the human $\beta$-globin second intron downstream of the MCMV promoter was examined (Fig. 2B). The results showed that the human $\beta$-globin intron dramatically enhanced the MCMV promoter strength in most cell lines (from 1.4-fold to 11.7-fold), except for the NIH3T3 cell line, in which the intron effect was negligible (0.97-fold). The most significant increase was observed in the HeLa (11.7-fold) and U937 (11.2-fold,

human monocytoid cell line) cell lines, and a moderate increase (6.24-fold) was seen in the CHO cell line.

### Comparison of the MCMV/Glo-I Promoter with the HCMV and HCMV/Glo-I Promoters

When the promoter strength of MCMV/Glo-I was compared with that of the HCMV promoter, which is currently considered as a strong promoter, the results showed that the MCMV/Glo-I promoter was consistently more active (from 5.34-fold to 11.71-fold) than the HCMV promoter in all cell lines tested (Fig. 2C).

The human $\beta$-globin second intron was also inserted between the HCMV promoter and BGH pA signals, and the expression activity compared with that of the MCMV/Glo-I vector. Although the inclusion of the human $\beta$-globin intron in the HCMV promoter system significantly enhanced the HCMV promoter activity, the MCMV/Glo-I promoter system remained stronger than the HCMV/Glo-I vector (1.33-fold to 4.83-fold) (Fig. 2D), illustrating that the combination of the MCMV promoter and human $\beta$-
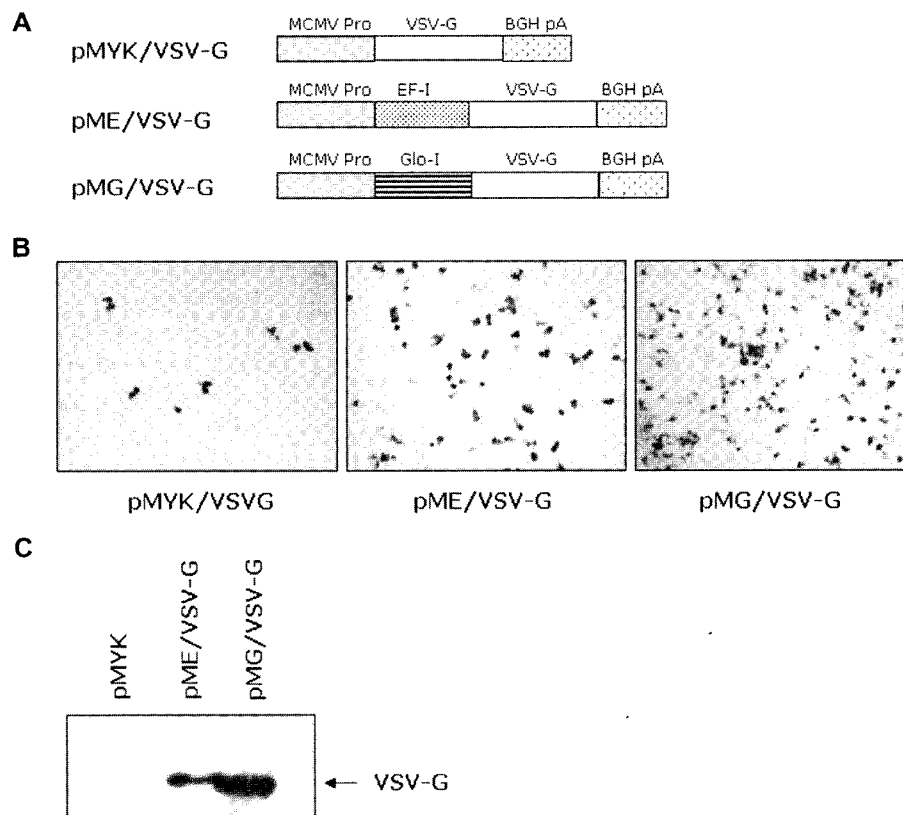


**Fig. 3.** Structures of VSV-G expression plasmid vectors and comparison of transduction efficiency.
(A) Structures of VSV-G expression recombinant plasmid vectors. (B) Infection assay of MCMV/VSV-G, MCMV/EF-I/VSV-G, and MCMV/GlO-I/VSV-G promoter expression systems. The transfer vector was pMFG/lacZ/Puro, which is a $\beta$-galactosidase gene expression retroviral vector, while the packaging cell GP293 was a *gag-pol* expression system. The stained cells were observed using a light microscope (×100). (C) Western blot analysis of SDS-PAGE (12%) of cell lysates from 293T cells transiently cotransfected with pMYK, pME/VSV-G, and pMG/VSV-G, respectively, using pMFG/eGFP. Plasmid pMYK, a backbone vector without VSV-G; pME/VSV-G, a VSV-G expression vector containing the MCMV/EF-1$\alpha$ promoter, the first intron; pMG/VSV-G, a VSV-G expression vector containing the MCMV/human $\beta$-globin second intron promoter system.

globin second intron was quite productive in both human and rodent cell lines.

## Effect of VSV-G Expression from the MCMV/Glo-I Promoter System on Recombinant Retrovirus Production

To examine the effect of VSV-G expression from the MCMV/Glo-I promoter system on recombinant retrovirus production, VSV-G expression plasmid vectors were constructed (Fig. 3A). The VSV-G protein is a vesicular stomatotitis virus envelope glycoprotein that interacts with the phospholipid component of the cell membrane to mediate viral entry through membrane fusion [20]. The viruses were produced by transient cotransfection, as described in Materials and Methods. Briefly, 0.5 μg of pMYK/VSV-G (VSV-G expression under the MCMV promoter), pME/VSV-G (VSV-G expression under the MYK/EF-I promoter), or pMG/VSV-G (VSV-G expression under the MYK/Glo-I promoter) plasmid DNA was cotransfected with 0.5 μg of the transfer vector pMFG/LacZ/puro, a β-galactosidase gene expression retroviral vector, into GP293 packaging cells by a lipofection method using a lipofectamine reagent (Gibco BRL). After 48 h, the culture medium containing the recombinant retrovirus particles was harvested and used to transduce the 293 cells. Figure 3B shows that the VSV-G expression from the pMG/VSV-G expression vector produced the highest titer of recombinant retrovirus particles (~$10^7$ TU/ml) when compared with pME/VSV-G (<$10^4$~$10^5$ TU/ml) and pMYK/VSV-G (<$10^2$ TU/ml). Finally, a Western blot analysis was performed to examine the expression level of the VSV-G protein from the VSV-G expression vectors. As shown in Fig. 3C, the MCMV/Glo-I promoter system synthesized a higher level of VSV-G expression (3.13-fold increase) than the MCMV/EF-I promoter system, as expected. The transfection efficiency was normalized by measuring the GFP expression (data not shown). The results demonstrated that the newly developed vector was efficient in producing useful recombinant proteins and in the luciferase assay.

## DISCUSSION

Previously, Kim and Risser [19] showed that the MCMV promoter activity could be increased 22-fold in the HeLa and NIH3T3 cell lines by deleting 890-bp far upstream negative regulatory sequences, and the inhibitory effect of the upstream negative regulatory sequences also affected heterologous promoters. As such, the shorter MCMV IE promoter was selected as the backbone in the current study. However, there has also been a contradictory report that the presence of upstream negative regulatory sequences does not have a consistent effect on promoter activity [1].

As with previous results [1, 24], the present data clearly show that the MCMV promoter was stronger than or at least as strong as the HCMV promoter in a variety of human and rodent cell lines. In general, the MCMV promoter was moderately stronger than the HCMV promoter in murine cell lines, such as NIH3T3 (6.71-fold) and Neuro2a (7.87-fold), yet exhibited similar activities to the HCMV promoter in the CHO cell line (0.94-fold). However, the MCMV promoter was surprisingly much stronger than the HCMV promoter in the 293 (9.32-fold) and HT1080 (4.6-fold) cell lines that were established from human cells.

In various systems, including mammalian tissue culture cells, transgenic mice, insects, and plants, the optimal expression of many endogenous genes requires the presence of one or more introns. The individual steps in gene expression reported to be affected by introns include transcription, polyadenylation, mRNA export, translational efficiency, and mRNA decay. It is also known that synergistic interactions between the splicing and polyadenylation machineries contribute to more efficient 3' end processing of intron-containing transcripts. In some cases, different introns positioned identically within a single gene elicit entirely opposite effects on protein expression. In others, the same intron placed into different exons yields opposite results. However, the intronic characteristics that contribute to these differences remain largely undefined.

The inclusion of the human β-globin second intron significantly enhanced gene expression in many cell lines, as expected. The effects of the intron were conspicuous in the HeLa (11.7-fold), U937 (11.2-fold), and CHO (6.24-fold) cell lines, yet only moderate to negligible in the HT1080 (1.74-fold), 293T (1.60-fold), Neuro2a (1.40-fold), and NIH3T3 (0.97-fold) cell lines. It was interesting to find that the cell lines (NIH3T3, Neuro-2a, 293T, and HT1080), in which the MCMV promoter activity was significantly higher than the HCMV promoter activity, were only slightly affected by the inclusion of the human β-globin intron behind the MCMV promoter, while the cell lines (HeLa and CHO), in which the MCMV promoter activity was similar to the HCMV promoter activity, were dramatically influenced by the insertion of the human β-globin intron. As such, it can be speculated that the MCMV promoter activity or translational capacity in the highly expressing cell lines, such as NIH3T3, Neuro-2a, 293T, and HT1080, was already so saturated that the inclusion of the human β-globin intron was unable to increase the expression level any further.

The effect of a heterologous intron on enhancing gene expression is different, along with the genes used as a reporter. For example, a comparison of the effect including a heterologous intron behind the HCMV promoter on chloramphenicol acetyltransferase (CAT) and luciferase gene expression showed a 3-fold increase in the luciferase expression, yet a 21-fold increase in the CAT gene expression [7], representing a 7-fold difference in the effect of the

intron between the CAT and luciferase genes. When applying the situation to the current experimental system, the fold increase in the luciferase assay, which ranged from 5.3- to 11.7-fold, represented a 37.1-fold to 81.9-fold increase in the CAT expression system, which is a dramatically elevated expression level when compared with other commercial expression vector systems.

In many applications, the vector size is also important in terms of effectiveness and safety. Thus, it is preferable to minimize the vector size as much as possible, as this can increase the copy number in *E. coli*, and also minimize possible adverse effects from foreign genetic materials [21]. With this in mind, a shorter version of the pMCMV/Glo-I vector was constructed by deleting the 1,626 bp fragment containing the neomycin resistance gene cassette, thereby making the vector useful for a transient *in vivo* direct injection gene therapy protocol. Another version of the pMCMV/Glo-I vector was also created by replacing the gene conferring resistance to the β-lactamase gene with the kanamycin resistance gene as a prokaryotic selection marker. This work was performed to follow CBER guidelines [9], which recommend the use of aminoglycoside antibiotics, such as kanamycin or neomycin, instead of β-lactam series antibiotics, including ampicillin.

The size of the pMYK vector consisting of the 671 bp MCMV IE promoter is 4489 bp. As such, when deleting the 1,626 bp *Bsu*36I fragment encompassing SV40 promoter/origin, the kanamycin/neomycin resistance gene, and TK poly A signal, the vector size was significantly reduced to 2,866 bp, and the vector named pMYKD (data not shown). Also, the ampicillin resistance gene of pMYKD was replaced with the kanamycin resistance gene to construct pMYKD/Kan. Then, the 875 bp human β-globin second intron was inserted between the MCMV IE promoter and the BGH polyadenylation signal of pMYK, pMYKD, and pMYKD/Kan, resulting in pMYK/Glo-I, pMYKD/Glo-I, and pMYKD/Kan/Glo-I, respectively (data not shown). These small and safe vectors could be used for DNA-based immunization and other gene therapy applications.

A previous research group created transgenic mice using the MCMV promoter cassette, and observed expression patterns from various tissues and cell types [2]. The results showed that although the MCMV promoter was active in a variety of tissues, it was not a pan-specific promoter, and the spectrum of organs positive for expression was narrower than that from HCMV IE promoter transgenic mice. The widely used HCMV IE promoter is also not a pan-specific promoter, and most of the expressing tissues correlate with tissues naturally infected by the HCMV in the human host [3, 4]. However, as the inclusion of heterologous introns greatly enhances gene expression in transgenic mice, this raises the issue of whether the current pMCMV/Glo-I vector may drive a wider expression than the MCMV or HCMV promoter-driven expression

in transgenic animals. This issue is currently under investigation.

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