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Original Article

Differential bicistronic gene translation mediated by the internal ribosome entry site element of encephalomyocarditis virus

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ARTICLE INFO

Article history:
Received 11 November 2019
Accepted 16 June 2020
Available online 24 June 2020

Keywords:
Bicistronic vector
EMCV IRES
Gene therapy
Mammalian cells
Protein expression

ABSTRACT

Background: Internal ribosome entry sites (IRESs) allow the translation of a transcript independent of its cap structure. They are distributed in some viruses and cellular RNA. The element is applied in dual gene expression in a single vector. Although it appears the lower efficiency of IRES-mediated translation than that of cap-dependent translation, it is with the crucial needs to know the precise differences in translational efficacy between upstream cistrons (cap-dependent) and downstream cistrons (IRES-mediate, cap-independent) before applying the bicistronic vector in biomedical applications.

Methods: This study aimed to provide real examples and showed the precise differences for translational efficiency dependent upon target gene locations. We generated various bicistronic constructs with quantifiable reporter genes as upstream and downstream cistrons of the encephalomyocarditis virus (EMCV) IRES to precisely evaluate the efficacy of IRES-mediated translation in mammalian cells.

Results: There was no significant difference in protein production when the reporter gene was cloned as an upstream cistron. However, lower levels of protein production were obtained when the reporter gene was located downstream of the IRES. Moreover, in the presence of an upstream cistron, a markedly reduced level of protein production was observed.

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Peer review under responsibility of Chang Gung University.
https://doi.org/10.1016/j.bj.2020.06.006

Conclusion: Our findings demonstrate the version of the EMCV IRES that is provided in many commercial vectors is relatively less efficient than cap-dependent translation and provide valuable information regarding the utilization of IRES to facilitate the expression of more than one protein from a transcript.

At a glance of commentary

Scientific background on the subject

The internal ribosome entry site (IRES) element is widely used for cap-independent translation. Several vectors with IRES element are generated for dual proteins expression in one transcript. However, it is unclear the difference of precise efficiency between cap-dependent and IRES-mediated translation cap-independent translation.

What this study adds to the field

In the work, a quantifiable reporter system was constructed to evaluate the efficacy of EMCV IRES-mediated translation in mammalian cells. The results demonstrate the version of the EMCV IRES that is provided in many commercial vectors is relatively less efficient than cap-dependent translation and provide valuable information regarding the utilization of IRES to facilitate the expression of more than one protein from a transcript.

Gene therapy has been widely applied to treat tumors as well as inherited and infectious diseases [1–3]. The acquisition of an expression vector that efficiently produces the target protein is an essential requirement in the development of gene therapy [4]. Furthermore, the ability to express different genes from a single vector can greatly enhance the efficiency and versatility of gene therapy applications. One approach is to express different genes via separate expression cassettes in a single vector. However, this strategy may be problematic due to promoter attenuation [4]. Additionally, the size of the clone can sometimes become very large, causing increased handling difficulty. Another alternative approach is the utilization of bicistronic vectors, in which the genes are linked to each other by an internal ribosome entry site (IRES), allowing co-translational expression of both cistrons [5–7]. In fact, this bicistronic approach may offer a high degree of flexibility in the regulation of gene expression [8,9].

The IRES is a cis-acting RNA sequence that has been shown to mediate internal entry of the 40S ribosomal subunit into mRNA, which results in the initiation of mRNA translation [10,11]. This cap-independent translation mode was first identified in picornavirus [12,13]. The picornavirus genome is a positive-strand RNA molecule with a single, long open reading frame encoding a polyprotein. During virus infection, cap-dependent host cell translation is markedly inhibited, and viral protein expression continues via a cap-independent mechanism that is mediated by IRES elements [14,15]. It has been shown that IRES-mediated protein expression is not unique to picornavirus. Many IRES elements have also been identified in other viruses (for example, hepatitis C virus and retroviruses) [11,16–18] and in higher organisms such as mammals [19–23]. In fact, IRES elements have been successfully utilized in a wide variety of biotechnological applications, including heterologous protein expression, the production of transgenic animals, and gene therapy [24–26].

On the other hand, the advantage of utilizing a viral IRES element is the ability to sustain protein synthesis in a broad range of cell types. The tissue tropism displayed by certain types of cellular IRES elements could be useful for targeting specific organs [7,27]. Among the various IRES elements, the IRES element of encephalomyocarditis virus (EMCV) is the most commonly used for constructing bicistronic expression vectors [5]. However, compared with the cap-dependent translation of the upstream cistron, EMCV IRES-mediated translation of downstream cistrons appears less efficient [17,28]. To date, little is known about the precise differences in translational efficacy between upstream cistrons (cap-dependent) and downstream cistrons (IRES-mediate, cap-independent), which may be because the bicistronic vector pRF, which was used in firefly and Renilla luciferase-based bicistronic assays, was found to generate spliced transcripts [29,30]. Therefore, the aim of the current study was to utilize another quantifiable reporter to evaluate the efficacy of EMCV IRES-mediated translation in mammalian cells. In the current work, we generated a series of bicistronic reporter constructs composed of enhanced green fluorescent protein (EGFP), Renilla luciferase (Rluc), firefly luciferase (Fluc), interleukin-2 (IL-2), or interleukin-4 (IL-4) gene coding sequences as the upstream or the downstream cistron in a commonly used pIRES vector. The expression level of reporter proteins such as Rluc, Fluc, IL-2, and IL-4 can be accurately measured by dual-luciferase assay or enzyme-linked immunosorbent assay (ELISA). Our results indicated that the IRES elements resulted in lower levels of gene translation. Furthermore, we also demonstrated a significant reduction in the level of protein translation mediated by IRES in constructs in which the cap mediating another reporter expression.

Materials and methods

Generation of bicistronic reporter constructs

p*IRES* (Clontech, Mountain View, CA, USA), a parental eukaryotic expression vector used for hybrid construction, served as a control plasmid in this study. To generate the mono- or bicistronic reporter constructs pEGFP*IRES*φ, pφ*IRES*EGFP, pRluc*IRES*φ, pφ*IRES*Rluc, pRluc*IRES*Fluc, pFluc*IRES*Rluc, pIL2*IRES*φ, pφ*IRES*IL2, pIL2*IRES*IL4, and pIL4*IRES*IL2, the EGFP, Rluc, Fluc, IL-2, and IL-4 reporter gene coding regions were cloned into the p*IRES* vector using the appropriate restriction enzyme sites. In the nomination of constructs, the symbol, φ, indicates the multiple cloning sites without gene inserted. The pIRES used in this study is a "crippled" IRES in order to reduce IRES expression and increase expression of the upstream cap-dependent open reading frame [31,32]. Plasmid DNA was purified from a transformed *Escherichia coli* (strain DH5α) using endotoxin-free Qiagen Plasmid Mega Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The plasmid DNA was then stored at −80 °C as dry pellets before use. The plasmid DNA was reconstituted in sterile water at a concentration of 1 mg/mL prior to use in the transfection experiment [33]. One μg plasmid DNA was used for transfection.

Cell culture

The 3T3 mouse embryonic fibroblast cell line was maintained in RPMI medium containing penicillin, streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and cultured under 5% CO_2-95% air at 37 °C.

Transient transfection

One day before transfection, 3T3 cells (1−3 x 105 cells/well) were seeded in a 6-well plate in 3 mL of growth medium. For each transfection sample, appropriate quantities (1 μg, in most experiments) of DNA and Lipofectamine™ 2000 (Invitrogen) were diluted in 100 μL of Opti-MEM (Invitrogen) and incubated for 5 min at room temperature. Next, both materials were mixed gently (200 μL total volume) and incubated at room temperature for 30 min. Then, the DNA-Lipofectamine mixture was added to the cells. After 800 μL of Opti-MEM was added to each well, the cells were then incubated at 37 °C under 5% CO_2-95% air for 6 h. An additional 3 mL of RPMI-1640-10% (v/v) FBS was added to each well, and the transfected cells were cultured at 37 °C in a CO_2 incubator for a further 48 h to allow transgene expression.

Fluorescence microscopy analysis

EGFP fluorescence images of transfected cells were obtained using an inverted fluorescence microscope (Nikon, Tokyo, Japan).

Real-time PCR analysis

Transgene expression (EGFP) was also analyzed by real-time PCR. At 48 h after transfection, the cells were harvested by trypsinization, washed, and resuspended in PBS. RNA was isolated using the TRIzol method (Invitrogen) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed using random primers in a final volume of 75 μL (Reverse Transcription System; Promega, Madison, WI). Real-time PCR was then performed, and the sequences of the primers used were 5′- CGACGGCAACTACAAGA and 3′-TCTATATCATGGCCGACAAG. The mRNAs encoding β-actin or GAPDH were served as internal controls, and their sequences of the primers used were listed as followings:β-Actin 5′-GAA ATC GTG CGT GAC ATT AAG, 3′-CTA GAA GCT TTT GCG TGG ACG ATG GAG GGG CC; GAPDH: 5′- AAG GTC GGT GTG AAC GGA TT, 3′-TGG TGG TGC AGG ATG CAT TG.

Measurement of Rluc and Fluc production by dual-luciferase assays

On the indicated day of culture, the growth medium was removed from the cultured cells, and a sufficient volume of 1x PBS was applied to wash the surface of the culture vessel. Passive lysis buffer was dispensed into each culture well (0.5 mL/well), and the culture plates were placed on a platform with gentle rocking at room temperature for 15 min. The lysate was transferred into an Eppendorf tube to determine the firefly and Renilla luciferase activities with the dual luciferase assay system (Promega). The cell lysate was transferred into a luminometer tube containing LAR II and mixed by pipetting two or three times. The tubes were placed in the luminometer (GloMax 20/20 single-tube luminometer; Promega, the linear dynamic range more than 8 logs), and reading was initiated. Then, the Stop & Glo® reagent was added into the same luminometer tube, pipetted two or three times to mix, and a second reading was obtained.

Measurement of IL-2 and IL-4 production by ELISA

The method has been described in detail elsewhere [34,35]. Briefly, on the indicated day of culture, cell-free supernatants were harvested and immediately assayed for IL-2 using an ELISA detection system. The microtiter plates (Maxisorb, Nunc, Denmark) were first coated with an anti-IL-2 (or IL-4) capture mAb (Pharmingen, San Diego, CA, USA) at a concentration of 2 μg/mL in bicarbonate buffer (0.79 g Na_2CO_3 and 1.46 g $NaHCO_3$ in 500 mL distilled water, pH 9.6) and incubated overnight at 4 °C. After washing with washing buffer (PBS with 0.1% [v/v] Tween 20), the plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Next, standards (at initial concentrations of 5 ng/mL in 1% [w/v] BSA in PBS), were doubly diluted in a plate (Maxisorb, Nunc, Denmark), and the samples (i.e., cell culture supernatants) were added to these dilutions. The mixtures were then incubated at room temperature for 2 h. After this step, and before all subsequent steps, the washing steps were performed with a minimum of four changes of washing buffer. Bound IL-2 (or IL-4) was detected by incubation with a biotinylated monoclonal antibody (Pharmingen) (0.5 μg/mL in 1% (w/v) BSA in PBS) for 1 h at room temperature followed by incubation with a 1:200 dilution of avidin peroxidase (R&D Systems Inc., Minneapolis, MN, USA) for 30 min at room temperature. The reaction was developed by the addition of an enzyme substrate solution

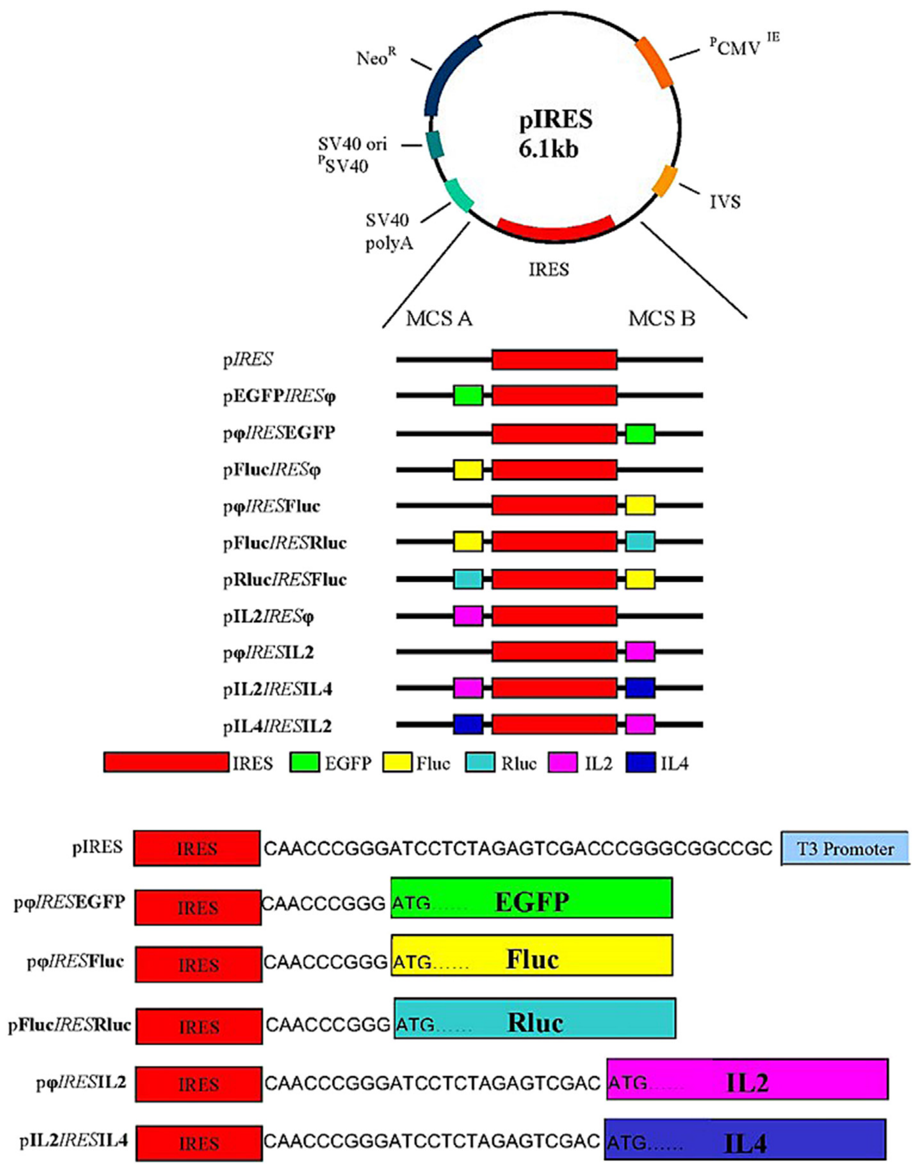

Fig. 1 Schematic diagrams of the mono- and bi-cistronic reporter vectors pEGFPIRESφ, pφIRESEGFP, pRlucIRESφ, pφIRESRluc, pRlucIRESFluc, pFlucIRESRluc, pIL2IRESφ, pφIRESIL2, pIL2IRESIL4, and pIL4IRESIL2. The plasmid pIRES, in which the multiple cloning sites (MCS) and IRES sequence are downstream of the immediate early promoter of cytomegalovirus (PCMVIE), was used as the vector backbone. The target genes encoding enhanced green fluorescent protein (EGFP) and Renilla luciferase (Rluc) were separated by the IRES element of encephalomyocarditis virus (EMCV) and expressed under the control of the CMV promoter. The intervening sequence (IVS) between PCMVIE and the MCS is an intron that is efficiently spliced out following transcription. SV40 polyadenylation signals downstream of the MCS direct the proper processing of the 3′ end of the mRNA of the gene of interest. Bacteriophage T7 and T3 promoters are located upstream and downstream of MCS A and B, respectively. pIRES contains the neomycin resistance gene (NeoR) to permit the selection of transformed cells.

consisting of 10 μg/mL 3,3,′5,5′-tetramethyl-benzidine (TMB) (Biosource, Carlsbad, CA, USA) in phosphate/citrate buffer (0.92 g $Na_2HPO_4 \cdot 2H_2O$ and 0.51 g citric acid in 100 mL dH_2O, pH 5.0) containing 0.3–30% (v/v) hydrogen peroxide (H_2O_2) (Pharmingen, San Diego, CA) for 5–20 min. The reaction was terminated after 10–20 min by the addition of 2 M sulfuric acid (H_2SO_4) (Sigma, St. Louis, MO, USA). The absorbance was measured at 450 nm using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). The levels of cytokines were estimated by regression analysis from standard curves constructed using recombinant cytokines; the measurements were performed in duplicate. Less than 10% difference between duplicates was noted.

Statistics and presentation of results

Statistically significant differences between groups were determined using an unpaired Student's *t*-test. In all cases, the confidence level was set at 5%; thus, a *p*-value of less than 0.05 was considered to represent a significant difference.

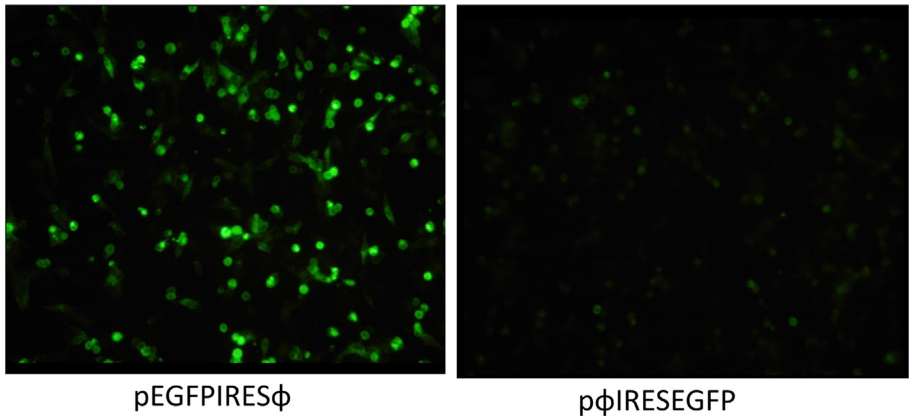

Fig. 2 Comparison of EGFP expression from different constructs in mammalian cells. 3T3 cells were transfected with the indicated bicistronic vectors, including pEGFP*IRES*φ and pφ*IRES*EGFP, by the liposome method. The EGFP expression in the cells was determined by fluorescence microscopy at 48 h post-transfection. The magnification is 100X.

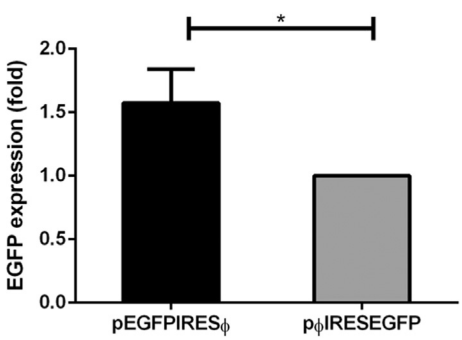

Fig. 3 Real-time PCR evaluation of EGFP mRNA expression in cells transfected with the different IRES constructs. The RNA of 3T3 cells transfected with pEGFP*IRES*φ and pφ*IRES*EGFP was isolated and reverse transcribed. Real-time PCR was performed to evaluate EGFP expression. The relative difference in EGFP cDNA levels was calculated and shown between the cells transfected with pEGFP*IRES*φ and pφ*IRES*EGFP.

Results

The attenuated EMCV IRES in commercial vectors has been frequently used to co-express dual proteins from a single transcript. However, the translational efficiency of cistron downstream of the EMCV IRES element in mammalian cells has not been clearly determined. Here, we show that gene positioning downstream of the commonly used EMCV IRES could result in a major difference in protein production. To precisely evaluate the efficacy of IRES-mediated translation in mammalian cells, we generated several mono- and bicistronic reporter constructs [Fig. 1] in which single genes (EGFP, Rluc, or IL-2 mRNA only) or dual genes (Rluc and Fluc as well as IL-2 and IL-4) were placed under transcriptional control of the cytomegalovirus (CMV) promoter. In general, the translation of the upstream cistron or the downstream cistron was controlled by either cap-dependent or IRES-mediated machinery, respectively.

Initially, to determine whether EMCV IRES-mediated translation from an attenuated EMCV IRES is less efficient than cap-dependent translation, we designed two reporter constructs in which the *EGFP* gene was either cloned upstream (pEGFP*IRES*φ) or downstream (pφ*IRES*EGFP) of the IRES. These two constructs were transfected into 3T3 mouse embryonic fibroblast cells. The cells were harvested at 48 h post-transfection, and EGFP expression was analyzed. [Fig. 2] shows that many cells displayed strong EGFP fluorescence after transfection with pEGFP*IRES*φ, whereas only a small amount of weakly fluorescent cells could be identified in the pφ*IRES*EGFP-transfected group. RT-PCR analysis was carried out to determine the efficiency difference between transfection and transcription. As shown in [Fig. 3], only small differences were observed in the EGFP mRNA levels. Similar results were obtained with two additional mammalian cell lines, CT26 murine colon cancer cells and P815 murine mastocytoma cells (data not shown), indicating that the difference was not due to tissue tropism. These results suggest that IRES-mediated EGFP translation (pφ*IRES*EGFP) is less efficient than cap-dependent translation (pEGFP*IRES*φ).

To precisely quantify the translational efficacy mediated by the IRES element, we first performed systematic comparisons between 3T3 cells transfected with several other mono- and bi-cistronic reporter constructs, including pFluc*IRES*φ, pφ*IRES*Fluc, pFluc*IRES*Rluc, and pFluc*IRES*Fluc, as the production of Fluc or Rluc could be quantitatively measured by dual luciferase assay analysis. At 48 h post-transfection with the indicated constructs, the cells were harvested for dual luciferase assays. Significantly lower levels of Fluc were obtained when the *Fluc* gene was cloned downstream of the IRES (pφ*IRES*Rluc) versus upstream of the IRES (pFluc*IRES*φ) [Fig. 4A]. The mean difference in Fluc production between pFluc*IRES*φand pφ*IRES*Fluc was 4.2-fold ($n = 3$, [Fig. 4B]). In contrast, the mean difference in Fluc production between pφ*IRES*Fluc and pRluc*IRES*Fluc was approximately 0.5 fold ($n = 3$, [Fig. 4C]), suggesting that IRES-mediated translation from an attenuated EMCV IRES is less efficient than cap-dependent translation.

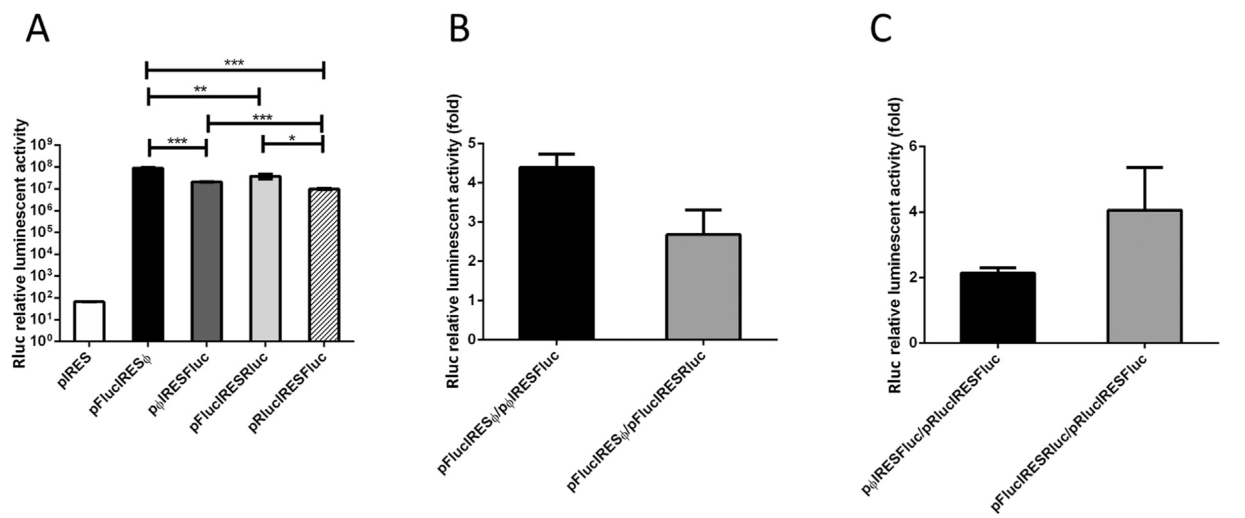

Fig. 4 Comparison of Rluc protein expression from different constructs in mammalian cells. 3T3 cells were transfected with the indicated mono- and bi-cistronic constructs by the liposome method. (**A**) Fluorescence activity generated in p*IRES*, pRluc*IRES*φ, pφ*IRES*Rluc, pRluc*IRES*Fluc, and pFluc*IRES*Rluc, (**B**) Ratios of Rluc expression by pRluc*IRES*φ, pφ*IRES*Rluc, and pRluc*IRES*Fluc. (**C**) Ratios of Rluc expression by pφIRESRluc and pFluc*IRES*Rluc The cells from cultures were harvested at 48 h post-transfection, and Rluc was measured by dual luciferase assay. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Next, the IL-2 and IL-4 genes were utilized for efficacy quantification. Several other mono- and bicistronic reporter constructs were developed, including pIL2*IRES*φ, pφ*IRES*IL2, pIL2*IRES*IL4, and pIL4*IRES*IL2. Systematic comparisons were conducted as described above. At 48 h post-transfection with the indicated constructs, the cell culture supernatants were harvested, and the production of the IL-2 or IL-4 cytokines was quantitatively measured by ELISA [Fig. 5]. Significantly lower levels of IL2 were obtained when the *IL2* gene was cloned downstream of the IRES (pφ*IRES*IL2) versus upstream of the IRES (pIL2*IRES*φ) [Fig. 5A]. The mean difference in IL2 production between pφ*IRES*IL2 and pIL2*IRES*φ was 36.7-fold (n = 5, [Fig. 5B]). The mean difference in IL2 production between pIL2*IRES*φ and pIL2*IRES*IL4 was approximately 1 (n = 4, [Fig. 5A]). Taken together, the difference of IRES-mediated translation by cytokines as reporter genes was 36.7-fold, while the difference by luciferases was 14.2-fold, revealing that the sensitivity of cytokines could be 2.5-fold greater than that of luciferase. It suggests that IRES-mediated translation from an attenuated EMCV IRES is less efficient than cap-dependent translation.

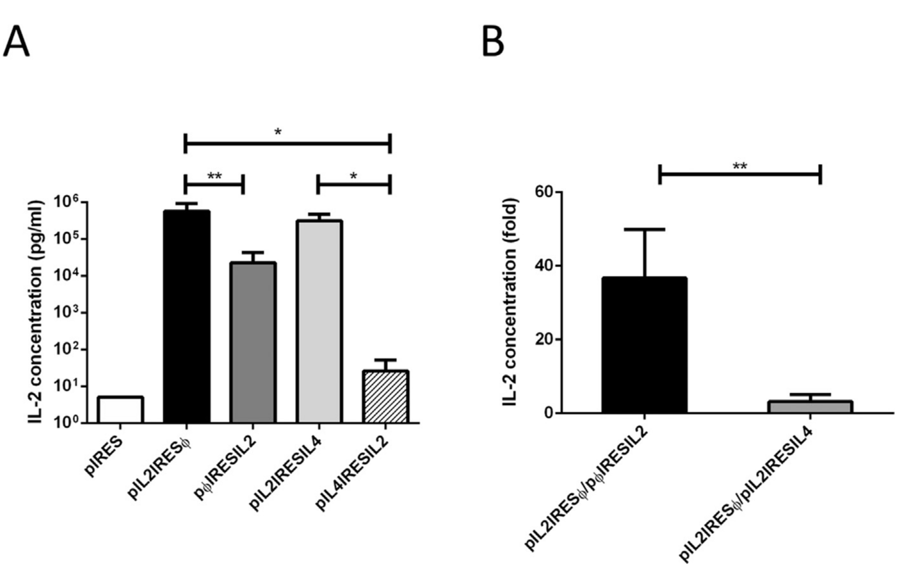

Fig. 5 Comparison of IL-2 protein expression from different constructs in mammalian cells. 3T3 cells were transfected with the indicated mono- and bi-cistronic constructs by the liposome method. (**A**) IL2 generated in pIRES, pIL2IRESφ, pφIRESIL2, pIL2IRESIL4, and pIL4IRESIL2. (**B**) Ratios of IL-2 expression by pIL2*IRES*φ, pφ*IRES*IL2, and pIL2*IRES*IL4. The cell culture media were harvested at 48 h post-transfection, and IL-2 was measured in the supernatants by ELISA. (*: $p < 0.05$, **: $p < 0.01$).

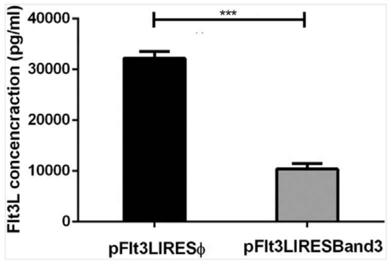

Fig. 6 Differences of Flt3L protein expression from different constructs in mammalian cells. 3T3 cells were transfected with the indicated mono- and bi-cistronic constructs, including pFlt3LIRESφ and pFlt3LIRESBand3, by the liposome method. The cell culture media were harvested at 48 h post-transfection, and Flt3L was measured in the supernatants by ELISA. (***: $p < 0.001$).

It was also noted that as compared with the pφ*IRES*IL2 construct, a markedly reduced level of IL-2 production was observed when the *IL-2* gene was cloned downstream of the IRES and the upstream cistron was the *IL-4* gene (pIL4*IRES*IL2). The mean difference was approximately 3,002-fold (n = 4). Moreover, when the level of IL-2 produced from pIL4*IRES*IL2 was compared to the level produced from pIL2*IRES*IL4, the mean difference was tremendously increased to more than 30,000-fold (n = 4), indicating that IRES-mediated protein translation may still be affected by cap-dependent translation.

It may be arguable that the translational efficacy of low molecular weight proteins was investigated in current study. Thus, we also examined the translational efficacy of various mono- and bi-cistronic reporter constructs encoding the ~103-kDa Band 3 protein (the erythrocyte anion channel protein) [36] and/or the ~30-kDa Fms-like tyrosine kinase 3 ligand protein (Flt3L). The constructs of Flt3L located upstream of the IRES were also tested in 3T3 cells, and the Flt3L expression is higher in the vector without gene located downstream of the IRES. The results were highly coincident [Fig. 6].

Discussion

Zhou and colleagues [37] utilized various fluorescent reporter proteins as the downstream cistron and the target gene as the upstream cistron to screen for activities of various IRES elements in mammalian cells. However, this system was limited by the fact that the translation efficiency mediated by individual IRES elements was relatively low, leading to inaccurate evaluation. To circumvent this problem, these authors utilized a positive feedback vector expressing a bicistronic mRNA with a reporter protein as the first cistron [37]. Additionally, there were studies utilized bicistronic retroviral vectors, in which IRES elements were used to facilitate co-expression of the dominant drug-selectable marker together with the therapeutic gene in a single bicistronic mRNA [38]. Their results also revealed a substantial reduction (1–1.5 log) of gene expression with the co-expression construct in the retroviral infected cells. Presumably, such reduction was due to the low level of IRES-mediated target gene translation.

Our current study was mainly designed to provide real examples and show the precise differences (such as 30 or 300-fold lower) for translational efficiency dependent upon target gene locations in the commonly available bicistronic expression vector containing EMCV IRES. Besides the dual-luciferase reporters, we had also utilized the low molecular weight proteins IL-2 and IL-4, which can be easily qualified precisely by immunoassays. Indeed, using this system, if the upstream cistron was absent, we found lower levels of protein production were obtained when the reporter gene was cloned into the downstream locus of EMCV IRES (~36.7-fold for IL-2 reporter). Surprisingly, a markedly reduced level of protein production was observed when the reporter gene was cloned downstream locus of EMCV IRES while the upstream cistron was present (~3002-fold for IL-2 reporter) suggesting EMCV IRES-mediated translation is relatively less efficient. Such extremely huge differences might directly affect the outcome when we apply the bicistronic vectors for gene therapy or other biomedical applications.

It may be argued why bother to use the above IL-2 and IL-4 but not only the commonly available duo-luciferase reporter system. In fact, more than two groups have published the lower translational efficiency of IRES-mediated translation as we mentioned earlier. However, people also noted and might question that the Fluc reading appeared to be usually higher than that of Rluc in the experiments, which was also found in our results. Indeed, a group had already addressed the question [39]. They consider that the firefly luciferase might have its own defect due to the fact that it is localized in small vesicular structures called peroxisomes *in vivo* such as in yeast, mammalian, and plant cells as in the firefly lantern [40]. Due to the presence of a peroxisomal translocation, signal is located at the C-terminal domain of the molecule and such peroxisomal localization appear to form as an additional membrane barrier.

In summary, here we provide the evidence that the gene expression of the commonly available vectors containing the EMCV IRES is relatively less efficient than cap-dependent translation, and 1.5-fold–3000-fold differences may occur if the locations of target genes vary. The data demonstrated from the study would be with great value regarding the utilization of IRES to facilitate the expression of more than one protein from a transcript, and appear to be beneficial for the applications of the bicistronic expression vectors.

Conclusion

Internal ribosome entry site (IRES) elements have been widely used to allow coexpressing more than one protein from a single transcript which offers a high degree of flexibility in gene therapy applications. However, compared with cap-dependent translation of the upstream cistron, IRES-mediated translation of the downstream cistron appears less efficient. In the current study, we generated various bicistronic reporter constructs which allow quantitatively evaluating the efficacy of IRES-mediated translation in mammalian cells. The study provide real examples and valuable information for the utilization of IRES to facilitate the efficiency and versatility of gene therapy applications.

Conflicts of interest

The authors have declared no conflicts of interest.

Acknowledgements

This study was financially supported by the Chang Gung Memorial Hospital Grants [CMRPD1H0621, BMRP440] and Ministry of Science and Technology [MOST 105-2221-E-131-035]. We gratefully acknowledge the support from Instrument Center of Chang Gung University, and the technical and editing assistance of Ms. Yi-Jing Lin, Tzu-Ying Liu and Lilian Chung.

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