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ORIGINAL ARTICLE

A COMPARATIVE STUDY OF THE INTERNAL RIBOSOME ENTRY SITE-CONTAINING EXPRESSION SYSTEM

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Abstract We analyzed transient and stable eukaryotic gene expression using internal ribosome entry site (IRES) and green fluorescent protein (GFP)-S65T (IRES-GFP) expression systems. The IRES-containing vector enables simple construction of a plasmid and easy detection of transfected cells, as well as an estimation of the levels of the gene of interest. This system is useful for measuring changes in the intracellular calcium concentration using the fluorescent calcium indicator Fura-2 AM, although subcellular localization of the protein of interest is not detectable. Importantly, the IRES-GFP plasmid showed delayed expression and an approximate 40% reduction in fluorescence intensity, which suggests that usage of IRES-containing expression system might cause under-estimation of desired gene. Although this technique has some disadvantages, the IRES-containing vector is simple and straightforward for gene expression analysis, comparing with co-expression procedure of multiple plasmids.

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Key words: eukaryotic expression vector; bicistronic expression vector; green fluorescent protein; internal ribosome entry site.

Introduction

Several strategies can be employed to evaluate cells that express a gene of interest, including the generation of stable cell lines with two expression cassettes, antibodies (such as neomycin resistant gene), chimeric constructs containing marker genes, and co-transfection of marker genes[1]. The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* exhibits relatively strong fluorescence and is widely used as a marker of gene expression because of its simple detection[2]. Co-transfection with GFP expression plasmid is a rapid and efficient method to assess gene expression at the single-cell level, as in patch-clamp studies. However, in our experience, only one-third of cells with GFP fluorescence have enough expression of the interest gene with co-expression procedure[3]. Generation of a chimeric construct is time-consuming, and maintaining the open reading frames (ORFs) of the gene of interest and marker gene is often troublesome. A single vector harboring two discrete expression cassettes requires the construction of relatively complex and cumbersome vectors and is generally associated with unreliable expression of the gene of interest.

The internal ribosome entry site (IRES) is a sequence of approximately 500 bp that permits Cap-independent translation and, consequently, generation of two ORFs from one mRNA[4]. The use of an IRES in bicistronic expression vectors enables the production of both the protein of interest and a selective marker under the control of the same promoter.

Here we report the analysis of a bicistronic expression vector using a combination of IRES and GFP expression systems. A bicistronic vector is simple to construct and enables easy

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identification of cells expressing the gene of interest. This vector is useful not only for transient expression but for the generation of stable cell lines by G418 selection, enabled by a neomycin resistance gene in the parent vector.

Methods

Plasmid Construction

GFP-S65T (Clontech, Mountain View, CA, USA) was inserted into the Ncol site of the pGT1.8IresBgeo vector (kindly supplied by Dr. A. Smith, University of Edinburgh), and the Xbal fragment, which includes IRES and GFP-S65T gene, was inserted into the human cytomegalovirus (CMV) expression vector pcDNA3 (Invitrogen, Tokyo, Japan). IRES sequence was originated from poliovirus[4].

IRES/lacZ and IRES/Bgeo fragments were also excised from the pGT1.8IresBgeo vector and inserted into the EcoRI site of IRGFP/pcDNA3 (IRBgeo/IRGFP/pcDNA3). For the trp-like (transient receptor potential-like gene) cDNA expression construct, the Xbal fragment from IRGFP/pcDNA3 was inserted into the Xbal site of pcDNA3 harboring Drosohpila trp-like cDNA (kindly supplied by Dr. L.E. Kelly, University of Melbourne).

Cell Culture and Transfection

Cell culture and lipofection were performed as described previously[3]. Human embryonic kidney cells (HEK 293, ATCC CRL 1573) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Exponentially growing cells were plated into 35 mm dishes, and lipofection was performed using commercially prepared lipofectamine (Invitrogen, Carlsbad, CA, USA). Two micrograms of DNA were used for each experiment. The ImaGene Red™ C*12*RG lacZ Gene Expression Kit (Thermo Fisher, Yokohama, Japan) was used to visualize fluorescence in cells expressing the lacZ gene product. Briefly, the ImaGene Red C*12*RG substrate reagent was added to the original medium to a final concentration of 33 μM and incubated for 20–60 min, and the resulting fluorescent product was visualized using a rhodamine filter set.

Microscopy

Standard epifluorescence optics (Zeiss cube #10) were used to visualize GFP (excitation wavelength 470 nm, emission wavelength 520 nm).

Measurement of the Intracellular Calcium Concentration

The intracellular calcium concentration was determined using Fura-2 AM as described previously[5]. Prior to the experiment, cells were loaded with 1 μM Fura-2 AM dissolved in dimethyl sulfoxide for 20–60 min. Each cell showed a homogeneous fluorescence signal, thus suggesting that Fura-2 AM is not compartmentalized within the major intracellular organelles. The calcium concentration was calculated according to the equation described by Grynkiewicz[6]. R_{min} and R_{max} (fluorescence ratio F*340*/F*360*) were determined *in vitro* (analysis of calibration solutions). Fluorescence emission was observed through a Schott KV 500 filter (500 nm cut-off wavelength). Data are expressed as the ratio of fluorescence intensity at 340 nm to that at 380 nm.

Results

In Figure 1, plasmid maps were shown. As a positive control, GFP-S65T was inserted into pcDNA3-multiple cloning site (GFP/pcDNA3, Fig.1A). For IRES-containing expression, IRES-GFP fragment was inserted into XbaI site of pcDNA3 (IRGFP/pcDNA3, Fig.1B). To confirm expression of the foreign gene in the IRGFP/pcDNA3 vector, IRES/Bgeo, a fusion gene of

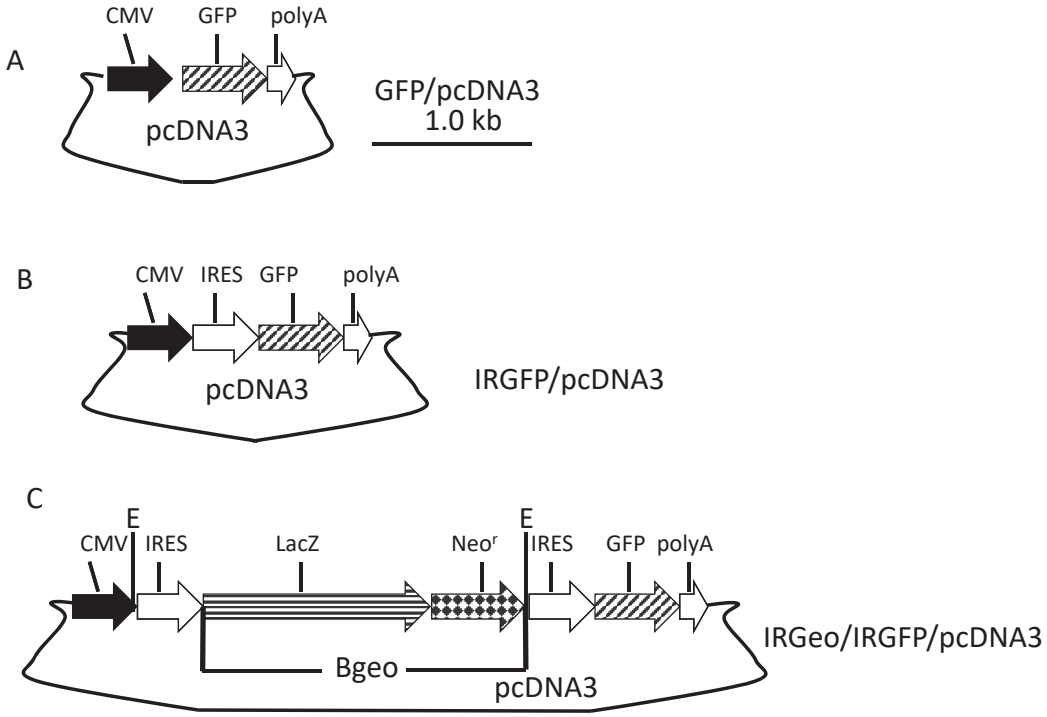

Figure 1 IRES-GFP/pcDNA3 plasmid construction. (A) GFP-expressing plasmid (GFP/pcDNA3, positive control). (B) The bicistronic vector harboring IRES-GFP, which contains one IRES sequence, to enable the expression of one or two genes (IRGFP/pcDNA3, bicistronic vector). (C) The IRES-Geo sequence was inserted into the IRES-GFP plasmid (IRGeo/IRGFP/pcDNA3) to generate a tricistronic vector.

lacZ and the neomycin resistance gene, was inserted into IRGFP/pcDNA3 (IRGeo/IRGFP/pcDNA3, Fig. 1C).

Under control of the CMV promoter (GFP/pcDNA3), GFP expression (Fig. 1A) in HEK293 cells was readily detected within 3 days after transfection (Fig. 2A). GFP fluorescence was resistant to photobleaching. Because HEK293 cells grew well after transfection, GFP did not appear to affect cell growth. No morphological changes were observed after transfection.

Figure 2A shows the time course of GFP expression in GFP-trasfected cells (transient expression). The GFP gene inserted into pcDNA3 (GFP/pcDNA3, Fig. 1A) reached its peak expression between days 3 and 4, while the GFP gene inserted into the IRGeo/IRGFP/pcDNA3 vector (Fig. 1C) reached its peak expression between days 6 and 7. These results indicate a 2–3-day delay in expression by the IRGFP system. The fluorescence intensity of GFP expressed from IRGeo/IRGFP/pcDNA3 was approximately 2–3-fold weaker on days 4–7 (i.e., GFP gene expression was somewhat decreased due to the insertion of IRES for transient expression) (Fig.1C, 2B).

To evaluate the GFP-induced fluorescence, we performed an excitation wavelength scan (emission 520 nm). Figure 3A shows the excitation wavelength scans for cells expressing GFP (broken line) or IRES-GFP (dotted line) and cells without GFP (solid line). No difference was observed between transfected cells and non-transfected cells at wavelengths less than 400 nm, while background fluorescence was detected at excitation wavelengths above 430 nm. Nevertheless, as emission fluorescence was fairly low with 340 nm or 380 nm, it became possible to use calcium indicator Fura-2 AM, whose excitation wavelength is 340 or 380 nm,

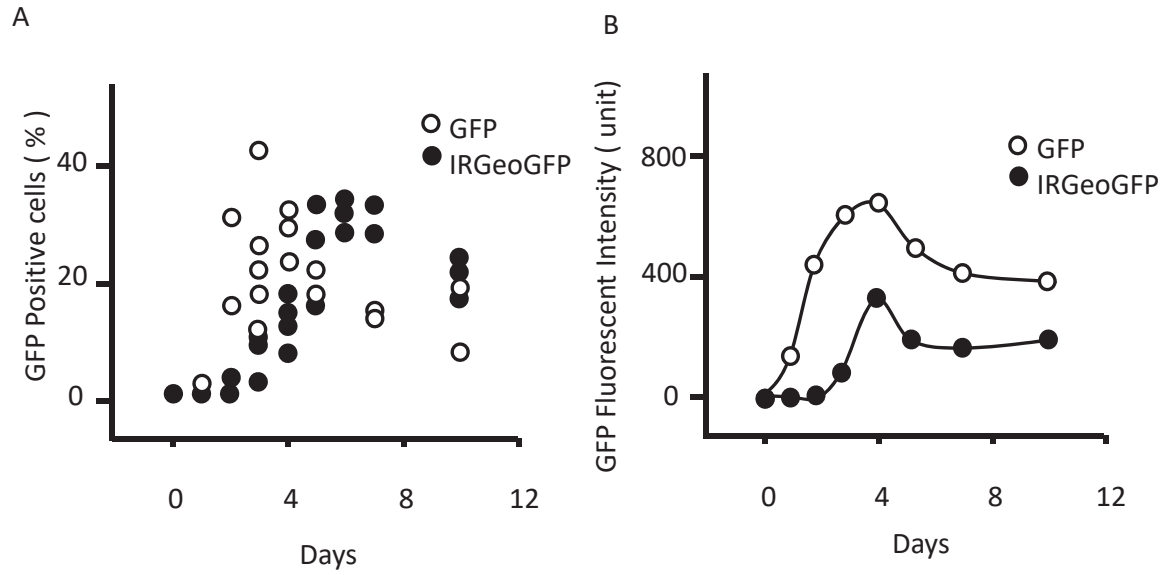

Figure 2 (A) Expression time course of GFP-positive cells harboring the GFP/pcDNA3 (GFP, open circle) and IRGeo/IRGFP/pcDNA3 vectors (IRGeoGFP, closed circle). The percentage of GFP-positive cells was recorded. (B) Time course of GFP fluorescence using different constructs (GFP and IRGeoGFP). Fluorescence was detected using a GFP filter set (excitation 470 nm, emission 520 nm).

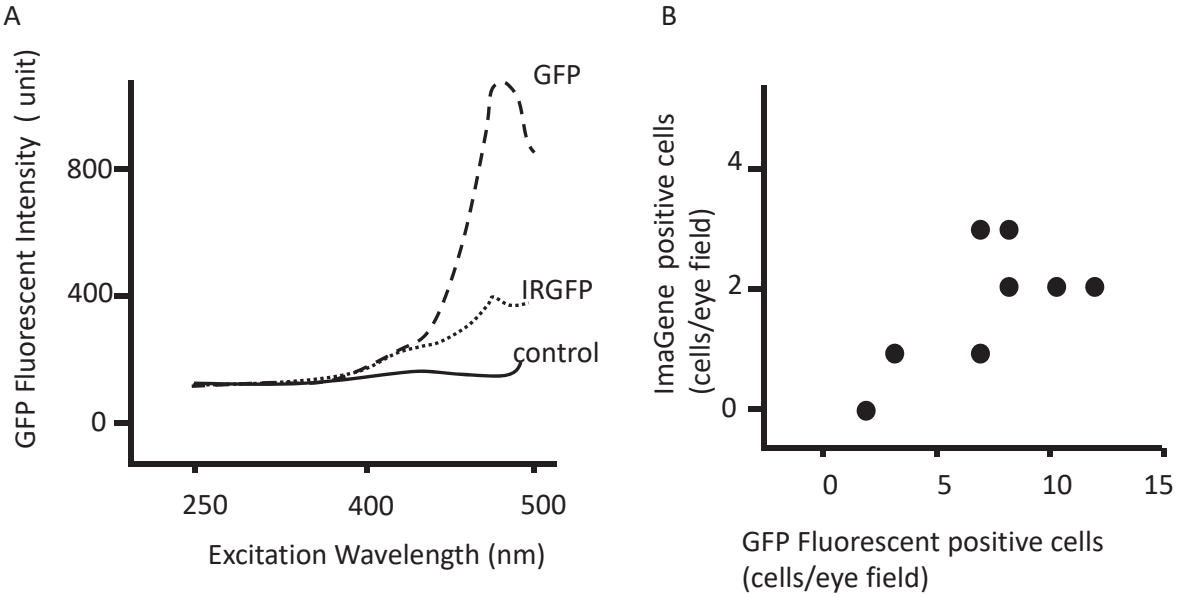

Figure 3 (A) Fluorescence excitation spectra obtained from HEK293T cells: control (solid line), GFP/pcDNA3 (broken line), and IRES-GEP (IRGFP; dotted line). Excitation spectra were recorded at a fixed emission wavelength of 520 nm. (B) Relationship between GFP- and lacZ-positive cells harboring the IRES-Bgeo/IRES-GFP/pcDNA3 construct. The number of positive cells per eye field (x200) is plotted.

with the IRES-GFP expression construct.

The numbers of GFP- and ImaGene Red™ C*12*RG lacZ-positive HEK293 cells transfected with IRES-Bgeo/IRES-GFP/pcDNA3 were plotted (Fig. 3B). As expected, GFP expressed from the bifunctional vector was resistant to photobleaching; however, rapid photobleaching was observed for lacZ gene expression, making

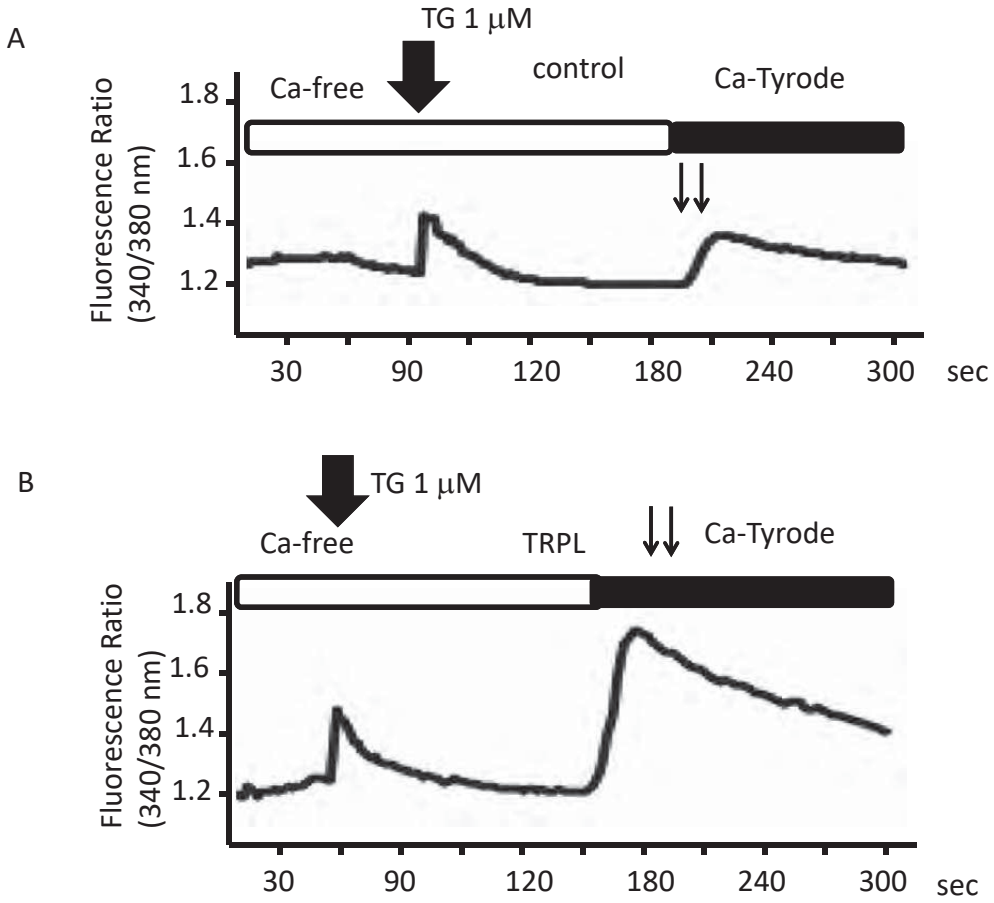

Figure 4 Measurement of changes in the intracellular calcium concentration in HEK293 cells. (A) Endogenous SOCE was observed using the IRES-GFP construct (double arrows). (B) Large SOCE was observed using the trp-l/IRES-GFP construct (double arrows). TG (thapsigargin, 1 μM), Ca-free (calcium-free Tyrode), and Ca-Tyrode (normal Tyrode) (n = 7～10).

it impossible to acquire images. A correlation between GFP fluorescence and lacZ staining was observed (Fig. 3B). Most of the lacZ-positive cells exhibited GFP fluorescence, but only approximately one-fourth of the GFP-positive cells exhibited fluorescence using the ImaGene Red™ C*12*RG lacZ method (Fig. 3B). Taken together, these results suggest that ImaGene Red™ C*12*RG lacZ staining is less sensitive than GFP fluorescence, and that a correlation exists between GFP and lacZ expression levels.

Figure 4 shows the intracellular calcium concentration in HEK293 cells transfected with the IRGFP plasmid with or without the trp-like channel. After replacing normal Tyrode's solution with calcium-free Tyrode's solution, thapsigargin (1 μM) increased the intracellular calcium concentration (thick arrow). After a subsequent decrease in the calcium concentration, calcium-containing Tyrode's solution was added. The intracellular calcium concentration increased as a result of the so-called "store-operated calcium entry" (SOCE; double arrows, Fig. 4A). After transfection of the IRES-GFP plasmid without the trp-like channel, a small increase in SOCE was observed (double arrows). On the other hand, after transfection of the IRES-GFP plasmid containing the trp-like channel, a large increase in SOCE was observed (Fig. 4B), indicating successful expression of

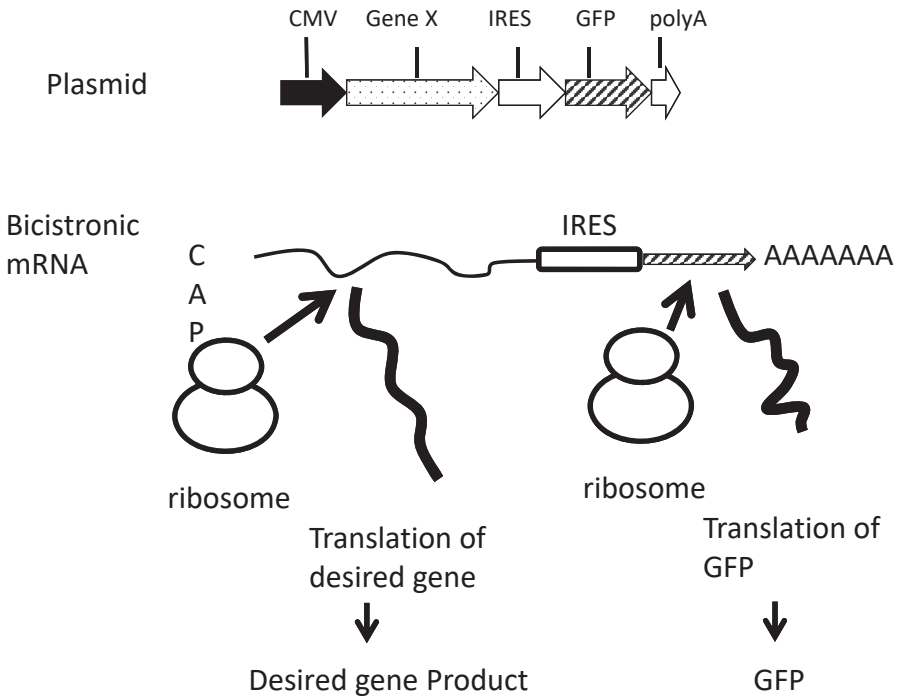

Figure 5 Schematic diagram of the IRES-GFP/pcDNA3 system. The IRES permits translation of the protein of interest (Protein X) and GFP from the same mRNA driven by the CMV promoter.

the trp-like gene using this IRES-GFP plasmid.

Discussion

A bicistronic expression vector makes it possible to identify cells that have been transfected successfully, thus expressing the gene of interest.

Figure 5 shows an overview of the IRES-GFP/pcDNA3 system. A bicistronic mRNA containing the gene of interest and GFP is transcribed. Ribosomes bind to the Cap complex and the IRES. The gene of interest and GFP are then translated together.

GFP-S65T fluorescence is very stable, 4–6-fold brighter than that produced by wild-type GFP, and not capable of being photobleached; thus, this expression vector is a useful tool to evaluate gene function at the single-cell level. In our experience, wild-type GFP is not always bright enough to detect the gene of interest (data not shown). GFP-S65T requires longer excitation and emission wavelengths (490 and 510 nm, respectively) and shows lower emission signals at an excitation wavelength between 340 and 380 nm relative to wild-type GFP[7]. This red-shift mutation makes it possible to measure changes in the intracellular calcium concentration using the IRES-GFP/pcDNA3 expression vector and the calcium indicator Fura-2 AM.

With pcDNA3, only a portion of the surviving clones typically express the gene of interest after G418 selection, because the promoters of the gene of interest and the selective marker gene are independent (e.g., CMV and SV40). In the IRES-GFP expression vector system, both the gene of interest and GFP are under the control of the same promoter (e.g., CMV promoter). The expression of the gene of interest is determined by measuring GFP fluorescence. As a result, it is also possible to obtain stable cell lines using this expression system (M.M., personal communication).

Translation of the gene of interest and GFP is performed by different ribosomes; therefore, IRES-GFP/pcDNA3 is not associated with issues such as subcellular localization or processing of the recombinant gene product. One possible disadvantage of this system is that it causes decreased expression or delayed expression compared with a simple CMV-driven construct, in which two different genes share a single promoter. This may lead to difficulties to analyze biological function. However, by detecting GFP fluorescent intensity, the high-expressing cells can be distinguished from other cells.

At present, it is difficult to examine interest genes using a eukaryotic expression system, due to the low efficiency of transient expression. A number of screening methods are available to analyze high cell volumes[8]. Some laboratories have reported the use of antisense experiments with pcDNA3[9]. Ideally, it is possible to screen the expression of short hairpin RNAs targeting unknown genes with this vector.

Enhanced GFP was engineered by Cormack et al.[10], and this GFP mutant is much brighter than GFP-S65T, which was used in this study. In contrast, the conditions used for the IRES system have been optimized[11]. These GFP and IRES mutants allow for simple gene expression studies in eukaryotic cells and transgenic animals.

The intensity of GFP fluorescence is correlated with the intensity of lacZ staining. GFP fluorescence is measured to estimate the relative expression level of the gene of interest. Thus, this IRES-GFP plasmid will also be useful for overexpression of multiple genes. Due to the low efficiency of transfection, it is difficult to analyze several genes simultaneously in eukaryotic cells. Therefore, a single IRES-GFP plasmid construct harboring multiple genes with an IRES for each gene could be produced. Another possibility is co-transfection, in which one gene is expressed from the IRES-GFP plasmid and the other from the pcDNA3 plasmid. A further possibility is to establish a stable cell line and transfect it with another plasmid with or without IRES-GFP.

In conclusion, the IRES-GFP/pcDNA3 expression vector is simple to construct and enables detection of cells expressing the gene of interest. This expression system is especially suitable for gene function analyses at the single-cell level, such as determining changes in the intracellular calcium concentration using Fura-2 AM.

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