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Full Length Research Paper

pLIVE-EGFP: A liver specific vector carrying the EGFP reporter for transgenic screening and expression

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pLIVE-EGFP is a liver-specific expression vector constructed by insertion of IRES-EGFP gene at the *Xho* I site of the pLIVE vector. The pLIVE-EGFP vector permits simultaneous expression of a gene of interest in addition to the EGFP reporter, specifically within liver cells, both *in vivo* and *in vitro*. When expressed in liver cells, the EGFP reporter produces a bright green fluorescence, which can easily be detected with fluorescence microscopy or flow cytometry. Compared with its parental vector, the pLIVE-EGFP is more powerful in providing a simple, direct method for monitoring gene transfection efficiency, gene expression and for screening positive clones.

Key words: Enhanced green fluorescent protein, gene expression, liver specific vector, screening.

INTRODUCTION

Many *in vitro* and *in vivo* transgenesis require reporter system for monitoring gene expression, intracellular protein trafficking or localization. Several types of reporter systems, such as secretory alkaline phosphatase (SEAP), luciferase, β-galactosidase, or fluorescent proteins (FPs), have been widely applied in these researches (Naylor, 1999; Branchini, et al., 2007; Teng and Wu, 2007; Choi et al., 2008). Of the reporter genes known to date, green fluorescent protein (GFP) is a naturally fluorescent protein that requires no cofactors or substrates for fluorescence, making it a tremendously useful direct visualization marker in both *in vivo* and *in vitro* studies (Zhang et al., 1996; Tang et al., 2008, 2009).

The pLIVE is a commercial vector preceded by the chimeric minimal mouse albumin (ALB) promoter and the mouse alpha fetoprotein (AFP) enhancer II, which enables its specific expression in mouse liver tissues and cells over a prolonged time. In many ways, the vector works well, but it still has limitations. As the vector is not designed to have any reporter system or selection system, the opportunity to monitor targeted gene expression or to select positive clones is very limited. Although some individual vectors such as pLIVE-*LacZ* and pLIVE-SEAP, which are derived from the pLIVE by insertion of the reporter gene (*LacZ* or SEAP) at its MCS, could be employed as a positive control in tagging expression of a gene of interest. But the drawback of this method is obvious, as this reporter system can only be applied with another parallel experimental system; it is not possible to tell if the target gene in the testing system is really expressed. Hence, if a determination of the target gene delivery efficiency or visual readout of gene expression is desired, other choices need to be considered.

In the present research, we described a modified pLIVE vector, pLIVE-EGFP, containing the EGFP reporter, which allows simultaneous expression of a gene of interest via the internal ribosome entry sites (IRES) element of the encephalomyocarditis virus (ECMV) at its upstream position. Compared with its parental vector, the pLIVE-EGFP provided an easy method to monitor gene delivery efficiency, to target gene expression and also to screen positive clones.

MATERIALS AND METHODS

Cells culture and transfection

Hepa1-6 cells were cultured in Dubecco's Modified Eagle Medium

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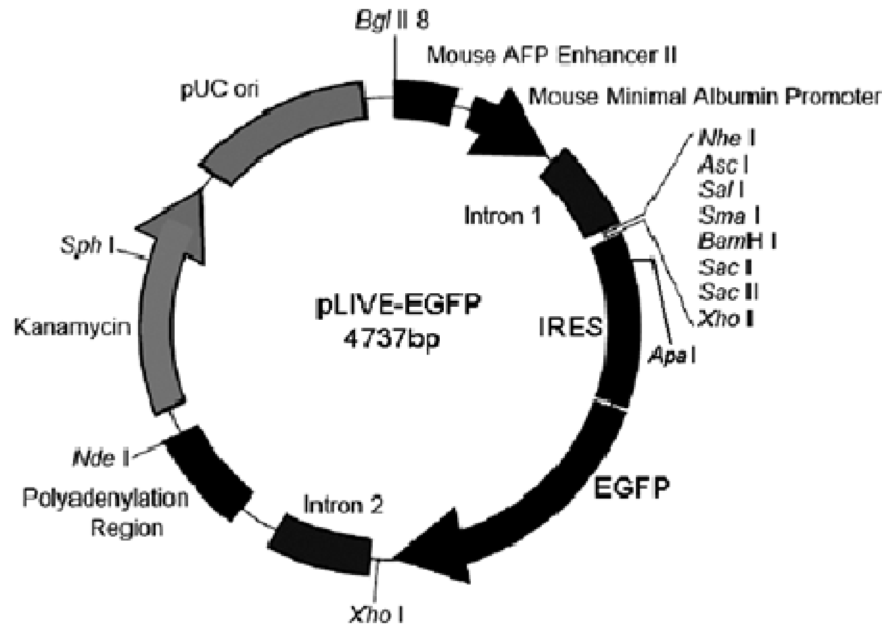

Figure 1. The map of the pLIVE-EGFP vector. The liver-specific pLIVE-EGFP vector was constructed by inserting IRES-EGFP gene at the *Xho* I restriction site of pLIVE plasmid. This enables simultaneous expression of the targeted gene inserted at MCS and EGFP reporter.

(DMEM) supplemented with 10% fetal calf serum. Cells were maintained at 37ºC in 5% CO_2 until the cells were 80% confluent. For transfection, cells were incubated with 3 µg of DNA and 9.6 µl of jetPEI^{TM} reagent (Polyplus Transfection, France) in 2 ml of serum-free medium per well in 6-well plates for 4 h at 37 ºC. The cells were then subcultured at a 1:2 dilution 48 h for further analyses.

Construction of the pLIVE-EGFP vector

The IRES-EGFP gene was prepared by PCR (primers: 5´-GCACTCTAGATATGGTGAGCAAGGGCG-3´ and 5´-GCTATCTAGATTACTTGTACAGCTCGTCCA-3´, *Xho* I is underlined) from the plasmid pIRES2-EGFP (Clontech, USA). The PCR-amplified product was digested with *Xho* I and then cloned into pLIVE vector (Mirus, USA); the resultant plasmid with the correct IRES-EGFP insertion was designated pLIVE-EGFP.

Application of pLIVE-EGFP vector in transgenic screening and expression

To characterize the usefulness of the pLIVE-EGFP vector, we designed a DNA fragment that inserted pLIVE-EGFP at *Nhe* I and *BamH* I restriction sites. In this test, the *Escherichia coli* alkaline phosphatase (AP) gene was used and was amplified from pDAP2 (Kerschbaumer et al., 1996) by PCR with the following primers: 5´-AAAGAATTCATGGTAGACAACAAATTCAACAAAAGAAC-3´ and 5´-AAACTGCAGTTCGGCGCCTGAGCATC-3´ (*Nhe* I and *BamH* I sites are underlined). The 1.5 kb PCR products were digested with *Nhe* I and *BamH* I and were cloned into pLIVE-EGFP. The recombinant vector pLIVE-EGFP-AP was transfected into mouse Hepa1-6 cells. For fluorescence analyses, the transfected Hepa1-6 cells were grown on glass coverslips under the conditions described above for 12 h. Then the glass coverslips were washed three times with TBS buffer (20 mM Tris-HCl, 50 mM NaCl; pH 7.5) and viewed under a fluorescence microscope (Leica, Germany). Subsequently, the slides were tested for AP expression, using a colorimetric assay, Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (BCIP/NBT, Ameresco) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The directional IRES-EGFP insertion was confirmed by double restriction enzyme assay (*Bgl* II + *Apa* I) which produced 1.1 kb and 3.7 kb fragments, otherwise produced 2.1 and 2.6 kb fragment. The plasmid with the correct IRES-EGFP insertion was named pLIVE-EGFP (Figure 1).

To test the pLIVE-EGFP vector, AP gene was inserted pLIVE-EGFP at *Nhe* I and *BamH* I restriction sites. Hepa1-6 cells transfected with pLIVE-EGFP-AP vector produced bright green fluorescence, whereas those transfected with pLIVE vector did not (Figures 2A and B). As shown in Figures 2C and D, the pLIVE-EGFP-AP transfected cells stained dark blue, which allowed them to be easily distinguished from cells transfected with pLIVE, which only gave rise either to a light blue staining or no color reaction. This result confirmed that the novel vector designed here was able to simultaneously express both the EGFP reporter and a gene of interest, such as AP.

The liver-specific expression vector pLIVE has no a reporter system or selection marker, which makes it difficult to read out the real status of the target gene expression or to select a positive clone. In order to resolve this problem, we presented a modified pLIVE-EGFP vector by

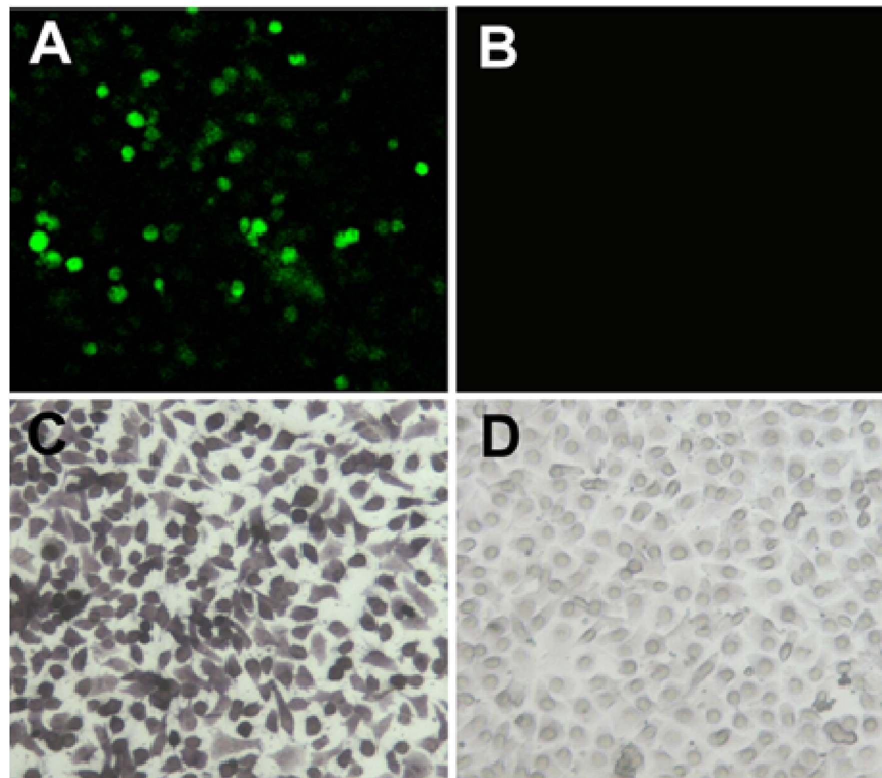

Figure 2. Coexpression of the reporter gene and a gene of interest. (A) Monitoring the EGFP by fluorescence microscopy. Cells with efficient pLIVE-EGFP-AP transfection show bright green fluorescence. (B) Cells transfected with pLIVE show no fluorescence. (C) Monitoring AP expression through colorimetric assay. Hepa1-6 cells transfected with pLIVE-EGFP-AP express high levels of Alkaline Phosphatase (AP) activity and are stained dark blue. (D) Hepa1-6 cells transfected with pLIVE express only low level of AP and show only light enzyme activity staining (light blue background).

inserting the IRES-EGFP gene at the *Xho* I restriction site of pLIVE. The presence of IRES enables simultaneous regulation of both the gene of interest and a reporter by a single promoter (Martinez-Salas, 1999). Therefore, virtually all transfected cells expressing the reporter would also express the gene of interest, thus providing a method for determining the status of expression of the target gene (Wan and Flavell, 2005). Under the control of IRES element, the EGFP gene was able to be co-expressed with a target gene inserted at the MCS upstream of IRES; EGFP within cells provided an easy method to monitor gene delivery efficiency, to target gene expression and also to positively screen clones. Moreover, the existence of the AFP enhancer and the ALB promoter ensured that the expression of the novel vector was constrained to only liver tissues and cells. Furthermore, the pLIVE-EGFP vector reserved most of the multiple cloning sites of pLIVE, so that target gene cloning and expression is as free as with its parental vector. The pre- sence of the EGFP reporter provides a direct, convenient way to detect gene delivery in living cells or to select positive clones, especially when this type of information is essential for extensive analysis.

Conclusion

We have developed a modified liver-specific pLIVE-EGFP expression vector that contains the EGFP reporter system. The vector we have devised provides a direct, convenient and effective way to monitor gene delivery efficiency, gene expression level, gene product localization and positive clone selection in liver cells and tissues.

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