

Evidence for expression of promoterless GFP cassette: Is GFP an ideal reporter gene in biotechnology science?

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Abstract

Green fluorescent protein (GFP) has played an important role in biochemistry and cell biology as a reporter gene. It has been used to assess the potency of promoters for recombinant protein production. This investigation reveals evidences suggesting that the *gfp* GFP gene (*EGFP*) could be expressed without the promoter. In a study, a pLenti-F/GFP vector was constructed with the purpose to allow GFP expression in transduced cells but not in packaging cells; however, after transfecting the HEK293T cell line, *GFP* gene was expressed, compared to pLOX/CW*gfp*-transfected cells showed expression lag, lower levels and reduced percentage of GFP expression in the cells. This unexpected result could be due to auto transduction in packaging cell, possible retrotransposon activity in the cell line, possible contamination of pLenti-F/GFP with the pLOX/CW*gfp* and possible presence of a promoter within backbone of the vector. All the possibilities were ruled out. To exclude the possibility that a sequence within the region might act as a promoter, the fragment to be transfected was minimized to a region containing "from the start of the *GFP* gene to 5'LTR R". The *GFP* gene was again expressed. Therefore, our findings suggest the *EGFP* does not need promoter for expression. This should appeal to the researchers designing GFP based assays to evaluate the potency of promoters, since possible aberrant expression may have a potential to influence on the results of a planned experiment.

Keywords: Green fluorescent proteins, Viral packaging, Promoterless.

INTRODUCTION

Fluorescent proteins (FPs) are a homologous class of proteins containing a chromophore, which is composed of three amino acids, within their polypeptide which produce a visible wavelength upon emission. As a common research procedure, genes encoding engineered FPs are introduced into living cells to trace the location of the gene product and its dynamics using fluorescence microscopy (1).

For a successful experiment involving FPs, several criteria should be met: the FP should be expressed efficiently while causing no toxicity for the cell, the signal should also be strong enough well above the level of auto fluorescence, it should have adequate photo stability necessary for the duration of the experiment and finally not to be influenced by environmental effects.

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In case the FP is to be used as a fusion protein, it should not be oligomerized (2). Green FP (GFP) is one of the FPs first discovered by Shimomura *et al.* (3) as a companion protein to aequorin, a chemiluminescent protein produced in *Aequorea* jellyfish. During the last 20 years, GFP has promoted from a largely unknown protein to a commonly used tool in molecular biology, biochemistry, and pharmaceutical biotechnology. Its remarkable ability to create an efficiently emitting internal fluorophore is highly valuable (4). The *GFP* gene was first sequenced in 1992 (5). GFP is an 11-stranded β -barrel threaded by an α -helix which makes the axis of the cylinder. The chromophore is connected to the α -helix and is buried nearly completely in the center of the cylinder, called a β -can (6). Interestingly, GFP is resistant to heat, alkaline pH, organic and chaotropic salts, detergents, photo bleaching, and numerous proteases (7). Its mutants optimized for codon usage have been created for mammals (8), plants (9), yeast (10), and fungi (11). The stronger the promoters/enhancers, the higher are the emission intensity of the GFP (12). GFP can be expressed with sufficient efficiency in a cell-free *in vitro* translation system confirming the fact that the protein is capable of folding autonomously (13). Today, GFP has found its broad use in many organisms. Widely used as a real-time molecular and cellular analysis tool in bacteria, transgenic plants, fungi, and mammals. GFP is now considered to be a vital marker (14,15). As a general approach, the *GFP* gene is fused in frame with the gene of interest forming a chimera which upon expression can be detected using fluorescence microscopy. GFP is believed to rarely affect the protein activity or mobility, and is not generally toxic except in rare cases involving high GFP concentrations, for example, in retroviral packaging cells (16). GFP has been successfully used to indicate protease action, dimerization of transcription factors, calcium sensitivity, and quantitative monitoring of gene expression in organisms (1). However, so far, several pitfalls have been found with GFPs: the signal might be confounded by background fluorescence effect

especially if it is not densely localized or highly expressed, and the chromophore formation occurs slowly post-translationally and requires oxygen (5). Thus far, there has been no report of a cryptic promoter within the *GFP* gene. Cryptic promoters differ from regular promoters in that they do not have a well-defined promoter initiation site sequence like TATAA box. They are independently regulated and the initiation mechanisms are not well-characterized. The existence of a cryptic promoter has been proven for some genes (17-19). In previous studies, as we were involved in designing and constructing a viral transfer vector which was supposed to express GFP in transduced cell line but not in packaging HEK293T cells, after transfection of the cells, we unexpectedly noticed that the promoterless GFP cassette was expressed (20,21). Therefore, the present study was launched to investigate the reason behind the observation. Our data demonstrate that the *GFP* gene can be expressed without any promoter.

MATERIALS AND METHODS

The HEK293T cell line

The HEK293T cell line was provided by Pasteur Institute of Iran, Tehran, I.R. Iran. The cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Missouri state, USA) containing 100 U/mL of penicillin and 100 mg/mL streptomycin, and supplemented with 10% fetal calf serum (Sigma-Aldrich, Missouri state, USA).

Vector constructs

pLenti-final/GFP (pLenti-F/GFP)

We designed a new lenti transfer vector. The back bone of this vector was pLenti4-GW/H1/TO-laminshRNA (Invitrogen, California, USA). The vector was modified for the purpose of expressing GFP in transduced cells with no expression in packaging cells. In this vector, the CMV promoter (PCMV) was positioned between R and U5 in 5'LTR on the opposite strand. The position of the *GFP* gene was between 5' and 3' LTR and next to the 3' LTR on the opposite strand

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and with the same direction as the promoter (Fig. 1A).

pLenti-promoterless/GFP (pLenti-pl/GFP)

During the construction of the pLenti-final/GFP, the promoter fragment was inserted in the final stage. Therefore, a stage before the completion of construction, the vector had no promoter (Fig. 1B).

p-Prokaryotic optimized/GFP (p-pro opti/GFP)

The GFP gene sequence was optimized according to *E. coli* expression system codon preferences, and placed under the control of a lacUV5 promoter on pUC57 vector (GeneCust Luxembourg) (Fig. 1C).

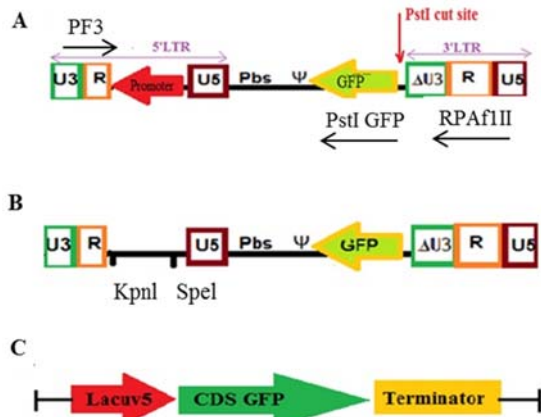


Fig. 1. Vector constructs. (A) pLenti-F/GFP, (B) pLenti-pl/GFP, and (C) p-pro opti/GFP. GFP, Green fluorescent protein.

Production of lentivirus in the HEK293T cell line

The HEK293T cell line was transfected with polyfect (Qiagen, Germany), following manufacturer's instructions. In the first step, the HEK293T cell line was co-transfected with the PMD2G (envelope plasmid), psPAX2 plasmid (packaging plasmid), and pLenti-F/GFP (transfer plasmid). In parallel, the HEK293T cell line was co-transfected with the PMD2G, psPAX2, and pLOX/CWgfp plasmids as the positive control. After overnight transfection, the cells were analyzed for the expression of GFP using fluorescence microscopy (Nikon Inverted Microscope, Japan) and flow cytometry

(FACS, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and the medium was replaced with fresh DMEM + 10% FBS + penicillin/streptomycin. The cells were incubated at 37 °C, 5% CO₂. After 24, 48, and 72 hrs, culture media were harvested and pooled and then fresh media added. The pooled media now contains the lentiviral particles.

Vector digestion and self-ligation to rule out contamination with pLOX/CWgfp

The maps of pLOX/CWgfp, pLenti-F/GFP, and pLenti-promoterless/GFP (pLenti-pl/GFP) were evaluated. Two restriction enzymes *EcoRI* and *PstI* were selected producing different digestion patterns in the vectors. The pLenti-F/GFP, pLenti-pl/GFP, and pLOX/CWgfp were digested separately with *EcoRI* and *PstI*.

The products of pLenti-F/GFP and pLenti-pl/GFP digestion with *EcoRI* were run on 1% agarose gel. The fragments of both plasmids were extracted from gel by DNA extraction kit according to the company's instructions (QIAGEN, Germany). The extracted products were self-ligated by T4 DNA ligase (fermentas, Waltham, Massachusetts, USA) and transformed into *E.coli* strain TOP10F competent with CaCl₂, and then cultured in LB agar medium containing ampicillin. After overnight culture, some colonies were picked up and cultured in LB broth medium containing ampicillin. After 16 h, plasmids were extracted (Bioneer, Korea) and used to transfect the HEK293T cell line.

Vector linearization for separation of the CDS of green fluorescent protein from plasmid backbone

The pLenti-F/GFP and pLenti-pl/GFP plasmids were linearized at the start of GFP gene using *PstI* (Fig. 1A). The linearized plasmids were then treated by calf intestinal alkaline phosphatase (CIAP), following the company's instruction (Invitrogen, California, USA), in order to prevent self-ligation. The CIAP treated products were analyzed by agarose gel (1%)

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electrophoresis followed by gel extraction (gel extraction kit, QIAGEN, Hilden, Germany).

Amplification of the regions from 5'LTR R to 3'LTR R and from the *GFP* gene to 5'LTR R was conducted. A fragment spanning 5'LTR R to 3'LTR R containing the *GFP* gene was amplified from each of the pLenti-F/GFP and pLenti-pl/GFP vectors. Forward and reverse primers (forward 3 and reverse A/II) are shown in Table 1. Another fragment encompassing from *GFP* to 5'LTR was amplified from both vectors using forward 3 and reverse *Pst*I-GFP primers (Table 1 and Fig. 1A). Agarose gel (1%) electrophoresis was carried out to analyze these polymerase chain reaction (PCR) products and gel extraction was performed.

Transfection of the HEK293T cells by different fragments and constructs

The HEK293T cells were transfected with the pLenti-F/GFP, pLenti-pl/GFP, linearized pLenti-F/GFP, linearized pLenti-pl/GFP, newly extracted pLenti-F/GFP (plasmid without any contamination with pLOX/CWgfp), newly extracted pLenti-pl/GFP (plasmid without any contamination with pLOX/CWgfp), 5'LTR R to 3'LTR R fragment, the *gfp* gene to 5'LTR R fragment, p-prokaryotic optimized/GFP (p-pro opti/GFP), and pLOX/CWgfp in separate experiments. After overnight transfection, the cells were analyzed for the expression of GFP using fluorescence microscopy and flow cytometry.

RESULTS

Production of lentivirus in the HEK293T cell line

In the process of lentivirus production, in the HEK293T cell line transfected with the three plasmids including psPAX2, pLenti-F/GFP, and PMD2G, the *GFP* gene was found to be expressed although with delay (after 20 h) and reduced intensity compared to the HEK293T cells transfected with pLOX/CWgfp, psPAX2, and PMD2G (test cells) which became green after 8 h (Fig. 2). Furthermore, the percentage of the cells expressing GFP was 55% for the former vs. 90% for the latter (Fig. 3).

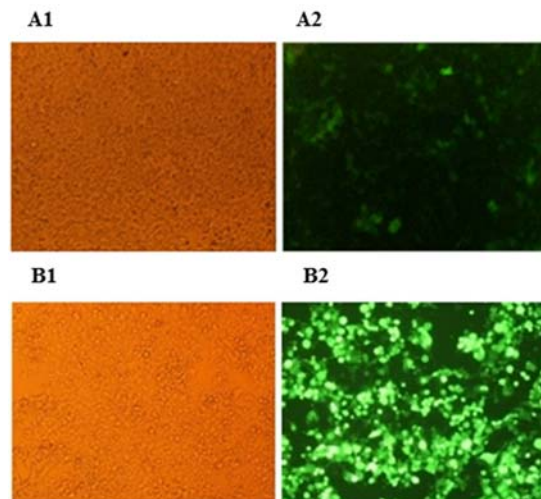


Fig. 2. Green fluorescent protein expression in the HEK293T cells under fluorescence microscopy. (A1 and A2) The HEK293T cells were co-transfected with PMD2G, psPAX2 and pLenti-F/GFP; (B1 and B2) the HEK293T cells were co-transfected with PMD2G, psPAX2 and pLOX/CWgfp. GFP, Green fluorescent protein.

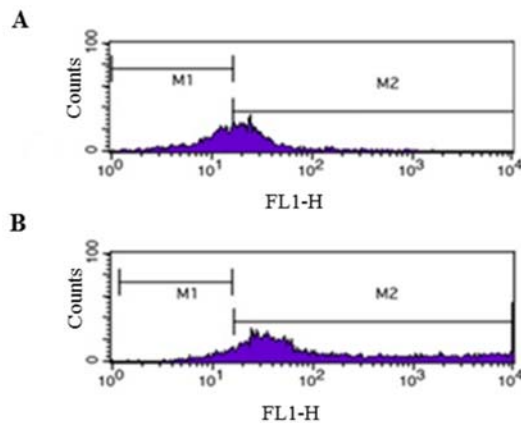


Fig. 3. The flow cytometry of GFP expression in HEK293T cells. (A) The HEK293T cells were co-transfected with PMD2G, psPAX2 and pLenti-F/GFP; (B) The HEK293T cells were co-transfected with PMD2G, psPAX2 and pLOX/CWgfp (control). The horizontal axis shows the fluorescence intensity of GFP. GFP, Green fluorescent protein.

Transfection of HEK293T cell line with pLenti-F/GFP plasmid and pLenti-pl/GFP

To assess the GFP expression pattern, HEK293T cells were transfected with *pLenti-F/GFP* and *pLenti-pl/GFP*, separately. After 20 h, the cells became green, with the similar pattern of expression described above under fluorescence microscopy.

Table 1. The primer sequences used in this study.

Primers	Primer sequences
F3 (Forward and revers primers)	5' ATA CTT AAG CCT CAA TAA AGC TTG CCT TGA GTG CTT CAG GTA CC 3'
RA/II (Forward and reverse primers)	5' TGA GGC TTA AGC AGT GGG TTC C 3'
RPstI GFP (Forward and reverse primers)	5' ATT CTG CAG GTC GCC ACC ATG GTG AGC 3'

Vector digestion and self-ligation to rule out contamination with pLOX/CWgfp

pLenti-F/GFP and pLenti-pl/GFP have no restriction site for *EcoRI* but one for *PstI*, pLOX/CWgfp has four restriction sites for *EcoRI* and three for *PstI*. Following digestion of pLenti-F/GFP and pLenti-pl/GFP by *EcoRI* and ruling out the possible existence of pLOX/CWgfp within them, the HEK293T cell line was transfected with the resultant purified plasmid which yielded the same mentioned expression pattern.

Transfection of HEK293T cell line with p-pro opti/GFP

After 24 h of transfection of HEK293T cells with p-pro opti/GFP, GFP was not expressed.

Vector linearization for separation the CDS of GFP from plasmid backbone

After separation the CDS of *GFP* from plasmid backbone, HEK293T cells were transfected with *GFP* CDS. After 20 h, the cells became green, with the similar pattern of expression described above under fluorescence microscopy.

DISCUSSION

Fluorescent labeling is an important tool in biological studies nowadays. While a number of chemical dyes are available to label biological specimens, they have to be added exogenously and are generally incompatible with living systems. However, genetically encoded fluorophore such as members of the GFP family provide a solution for the purpose (22).

We demonstrated here evidence suggesting that the *GFP* gene (*EGFP*) can be expressed in promoterless cassettes. During an investigation, we designed and constructed a retroviral transfer vector for the purpose

of expressing GFP in transduced cells and with no expression in packaging cells (20). In the production of lentivirus, we expected the *GFP* not to be expressed in the packaging cells due to the absence of any promoter in the upstream of *GFP*. However, after 20 h, in the HEK293T cell line transfected with the three plasmids including psPAX2, pLenti-F/GFP, and PMD2G, the *GFP* gene was found to be expressed although with delay and reduced intensity compared to the HEK293T cells transfected with pLOX/CWgfp, psPAX2, and PMD2G which became green after 8 h. Furthermore, the percentage of the cells expressing GFP was 55% for the former vs. 90% for the latter. To ensure the validity of the results, all the experiments were repeated and the same findings were obtained. Given the odd observation, the process of viral packaging was ceased and our investigations were focused on understanding the underlying reason.

To explain the result, we hypothesized either of several reasons could be the case:

a) Auto transduction of the cells with the viruses produced during the process of packaging. In this view, the viral RNA could be reverse-transcribed, imported into the nucleus and stably integrated into the host genome. Since the promoter will now be positioned upstream of the *GFP* gene, it can be expressed (23,24). This might also explain the observed delayed expression of the gene after 20 h. To rule out the hypothesis, the cells were transfected with the pLenti-F/GFP plasmid alone to ensure the virus not to be produced. We found that *GFP* was expressed again, with the same pattern of delayed, decreased intensity and the same percentage of the expressing cells.

b) Retrotransposase re-activation. In cell lines especially those derived from cancer cells, possible epigenetic modifications that inactivate retrotransposases can be removed

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leading to retrotransposase activity. If retrotransposons are active in the cell line, the promoter can be placed upstream of the *GFP* gene and results in its expression. To investigate the notion, the HEK293T cell line was transfected with the pLenti-pl/*GFP* vector, the promoterless version of our vector. Again, under fluorescence microscopy the cells became green, with the similar pattern of expression described above. Therefore, we conclude retrotransposition plays no role in the expression of the *GFP*.

c) Random contamination of our plasmids with the pLOX/CWgfp vector. To rule out this possibility, all the vectors, pLenti-F/*GFP*, pLenti-pl/*GFP*, and pLOX/CWgfp, were digested separately with two restriction enzymes, *EcoRI* and *PstI*, which generated plasmid-specific digestion patterns as expected. Following digestion of pLenti-F/*GFP* and pLenti-pl/*GFP* by *EcoRI* and ruling out the possible existence of pLOX/CWgfp within them, the HEK293T cell line was transfected with the resultant purified plasmid which yielded the same mentioned expression pattern. From this point on, all the tests were performed with either the newly extracted pLenti-F/*GFP* or pLenti-pl/*GFP* plasmids.

d) Possibility of a promoter sequence within the transfer vector backbone. For ruling out the likelihood, the pLenti-F/*GFP* and pLenti-pl/*GFP* were digested with *PstI* resulting in vector linearization at the adjacent of the *GFP* gene to separate the *GFP* gene from plasmid backbone at its upstream (Fig. 1A). After transfection of the HEK293T cell line with the linearized vector, the same GFP expression pattern was achieved.

Having ruled out all the logical possibilities mentioned above, we concluded that the *GFP* gene could be expressed without promoter. To confirm the idea, a shortened fragment of the vector spanning 5'LTR R to 3'LTR R fragment was amplified by PCR and transfected into the HEK293T cell line. Even under the circumstances, the *GFP* gene was found to be expressed. We repeated the experiment by producing a fragment from start codon (ATG) of the *GFP* gene to 5'LTR R fragment by PCR which led to the same finding.

In a parallel experiment, the HEK293T cells were transfected with p-pro opti/*GFP*. Unlike our expectations, *GFP* was not expressed. This could largely be due to the fact that the *GFP* gene on this vector had been optimized for prokaryotic codon usage. Although its sequence was different from the native one, its open reading frame was the same. The disruption in the native *GFP* sequence can turn off *GFP* expression. Therefore, there must be a eukaryotic cryptic promoter sequence in CDS of *GFP* that is interrupted.

Cryptic promoters have homology to the promoter sequences. They might be distributed randomly throughout the genome and are capable of driving transcription (25).

However, no significant attention had been paid to cryptic promoters from reporter genes until 2008 when Vopalensky *et al.* reported a cryptic promoter activity in the firefly luciferase gene (19). Recently, several researchers have revised their published results after finding cryptic promoters or cryptic splicing sites in the genomic fragments that were claimed to bear an internal ribosome entry sites (26-29).

The results of our experiment strongly suggest the presence of a cryptic promoter within the *GFP* gene and advocate the notion that this reporter gene might not be ideal for some experiments.

In a study in order to prove the cryptic promoter activity of a firefly luciferase (*FLuc*) gene, the investigators removed the CMV IE promoter, positioned upstream of *FLuc* and *GFP* genes, from the pFG vector creating a promoterless variant pFG(-P) in which *FLuc* was followed by *GFP*. Based on the finding that a significant amount of GFP had been produced in cells transfected with promoterless pFG(-P) vector, they suggested that it was a strong evidence for the cryptic promoter activity within the *FLuc* gene (19). In the light of our new finding, their conclusion might not be completely valid. Interestingly, results of the studies launched to investigate whether gene editing tools had been acting based on *GFP*, as the reporter gene, might not be valid, either. For instance, in a research, a reporter plasmid

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was designed in which the PCMV had been positioned upstream of a monomeric RFP (mRFP), as a reporter gene, a programmable nuclease target site and the enhanced *GFP* gene. mRFP was constitutively expressed from the PCMV, whereas the functional GFP was not expressed because of its out-of-frame position under normal conditions. Thus, cells transfected with this reporter plasmid expressed mRFP but not GFP. However, in case of programmable nuclease activity, a double-strand break was made in the target sequence, which upon its repair by non-homologous end-joining mechanism, could occasionally lead to frameshift mutations that position GFP in frame (30). Such experiments relying on the expression of GFP may give rise to false positive results.

Although the data obtained in this study are valid enough, further investigation is required to determine the transcription initiation site of the cryptic promoter using such techniques as 3'RACE. To find the exact sequence of the cryptic promoter, transfection of the truncated *GFP* gene cassettes with poly A signal could address the issue.

CONCLUSION

Finding a cryptic promoter within the *GFP* gene should appeal to the researchers working in the field and encourage them, when possible, to investigate the possible presence of cryptic promoters in plasmid backbones as well as other reporter genes. Failing to take the fact into consideration could potentially lead to confounded results of the planned experiments due to aberrant expression induced by these cryptic promoter elements. In summary, we could demonstrate that *GFP* does not need promoter for expression and this fact can negatively affect its role as a reporter gene to evaluate the power of promoters.

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