ORIGINAL ARTICLE

DNA context and promoter activity affect gene expression in lentiviral vectors

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Abstract. Several varieties of lentiviral delivery systems have been used to generate stable cell lines and transgenic animals. Some enhancing elements have been identified to promote stable transgene expression. In this study, we describe that the promoter activity is affected by the lentiviral genomic context. We have examined the promoter activities using green fluorescence protein (GFP) as a reporter in different cell lines to demonstrate that the cytomegalovirus (CMV) promoter may not always be the best choice to overexpress transgenes in all cell types. Our data showed that the polypeptide chain elongation factor 1 alpha (EF1α) promoter is relatively active in all three model cell lines (293T, HOS, and Hela) while the CMV promoter is less effective in Hela cells. (www.actabiomedica.it)

Key words: DNA context, promoter activity, elongation factor 1 alpha

Introduction

Lentiviral vectors have been considered as an effective gene delivery system due to the following features: 1) ability to infect non-dividing cells, 2) high efficiency of gene delivery and broad range of infectivity while using the vescicular stomatitis virus G (VSVG) envelope protein, and 3) integration into host genome for long-term gene expression (1-6). At least three generations of lentiviral vectors have been evolved in the last decade (1, 7, 8). Different regulatory elements and promoters have been introduced into the lentiviral vectors to increase the overexpression efficacy. In this study, we have focused on the DNA context of the lentiviral vectors and the effect of cell types on promoter activities.

Materials and Methods

Construction of lentiviral vectors

The lentiviral vector pLenti6.2-GW/EGFP was purchased from Invitrogen (Carlsbad, CA, USA). The construction of lentiviral vector pLV-CMV-GFP has been described in (2, 3), plasmids pLV-EF1α-GFP and pLV-PGK-GFP were derived from pLV-CMV-GFP by replacing the CMV promoter. The DNA segment containing polypeptide chain elongation factor 1 alpha (EF1α) promoter was amplified from human liver genomic DNA using primers, EF1-F (ggccatcgatgcctccccgtcaccacccccccaa) and EF1-R (ggcctctagaggggtagttttcacgacacctgaaatgg) by PCR. The DNA segment containing phosphoglycerate kinase (PGK) promoter was amplified from 3T3 genomic DNA using primers PGK-F (ggccatcgataattctaccgggtaggggaggcg) and PGK-R (ggcctctctagacaggtcgaaaggcccggagatgagg). Both promoters were cloned into *Cla*I and *Xba*I sites of pLV-CMV-EGFP to replace the CMV promoter.

Lentiviral production

The transfection reagent lipofectamin 2000 and the lentiviral packaging mix were purchased from In-

vitrogen (Carlsbad, CA, USA). The cell culture medium and supplements were purchased from Hyclone (Logan, UT, USA). The transfection procedure was followed the manufacturer's protocol and the supernatants were collected at 48 hours post transfection. The lentiviral stocks were then treated with DNaseI (10 units/ml) at 37°C for 15 min, filtered through 0.45 mm filters, and stored at -80°C. The human cell lines 293T, HT1080, Hela, and HOS used in transduction experiments were purchased from ATCC (Manassas, VA, USA).

Determination of virus titer

The titers of different lentiviral stocks generated in this study were determined by FACS (functional GFP titer) and real-time PCR (DNA titer). To determine functional GFP titers, 100 μl 5-fold serial dilutions of lentiviruses were added to 5 x 10^4 293T cells per well in 6-well plates. The transduced cells were trypsinized, collected and resuspended for flow cytometry to determine the percentage of green fluorescent cells at 48 hours post transduction. To determine DNA titers, total DNA was isolated using Qiagen DNeasy kit at 24 hours after transduction. Quantitative PCR was carried out by using ABI Prism 7700 with Invitrogen LUX primers (LV-FL: cgccgtggaaaatctctagcagtggcg, and LV-RU: gctcctctggtttccctttcg). The amplification condition was described in (9).

Results

DNA context of lentiviral vectors affects gene expression

All of the lentiviral vectors used in this study contain GFP gene as a reporter (Fig. 1). The major differences among pLenti6.2-EGFP and pLV-GFP vectors are no central polypurine tract (cPPT) sequence and Woodchuck hepatitis posttranscriptional regulatory element (WPRE) in the pLenti6.2-EGFP vector. To determine whether the level of EGFP expression is only dependent on the strength of promoters, 293T cells were transfected with pLenti6.2-EGFP and three pLV-GFP vectors. The GFP expression

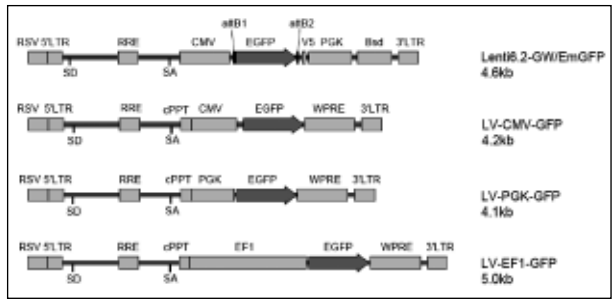

Figure 1. Schematic structure of the lentiviral vectors. The abbreviations are described as follow: RRE, Rev-response element; SD, splice donor; SA, splice acceptor; cPPT, central polypurine tract; WPRE, Woodchuck hepatitis posttranscriptional regulatory element; Bsd, blasticidin; attB1 and attB2, Gateway site-specific recombination sequences

from pLV-CMV-GFP, pLV-EF1α-GFP, and pLV-PGK-GFP vectors are varied (Fig. 2B-D). The data showed that 293T cells harboring CMV-GFP, EF1α-GFP, and PGK-GFP vectors displayed high, medium, and low intensity of green fluorescence, respectively (Fig. 2B-D). It suggests that the CMV, EF1α, and PGK promoters are listed in the order of promoter activity from the highest to the lowest in the 293T cells. By comparing the intensity of green fluorescence expressed from CMV promoters on pLenti6.2-EGFP and pLV-CMV-GFP vectors, the level of GFP expressed from pLV-CMV-GFP is much higher than GFP expressed from pLenti6.2-EGFP (Fig. 2A and D). The lentiviral stocks were further analyzed by

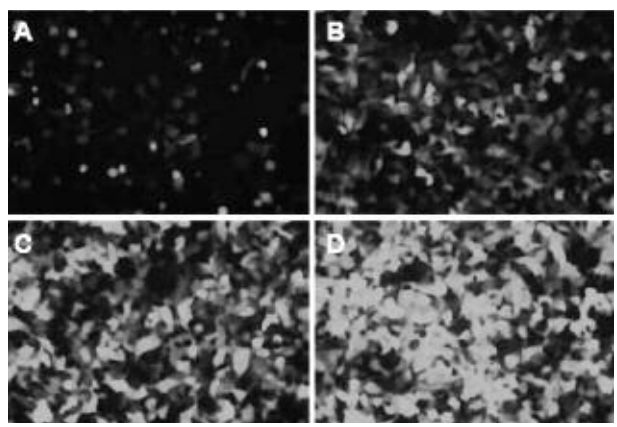

Figure 2. Transfecting 293T cells with lentiviral vectors. The 293T cells were transfected with (A) pLenti6.2-GW/EGFP, (B) pLV-PGK-GFP, (C) pLV-EF1α-GFP, and (D) pLV-CMV-GFP

Table 1. Titering pLV-CMV-GFP and pLenti6.2-GW/EGFP by FACS and realtime PCR

Lentivirus	DNA copy no./ml	GFP positive cells/ml	Mean Fluorescence Signal
LV-CMV-GFP	$2.3 \pm 0.1 \times 10^8$ (100%)	$6.3 \pm 0.2 \times 10^6$ (100%)	1395 ± 52 (100%)
Lenti6.2-GW/EGFP	$1.3 \pm 0.0 \times 10^8$ (57%)	$5.6 \pm 0.4 \times 10^5$ (8.9%)	64 ± 12 (4.6%)

quantitative PCR to determine the DNA titer and the same DNA titers of pLenti6.2-GW/EGFP and pLV-CMV-GFP lentiviral stocks were used to transduce 293T and HT1080 cells (Table 1). The pLV-CMV-GFP vector in 293T and HT1080 cells expressed higher levels of GFP than the GFP expressed from pLenti6.2-GW/EGFP vector while both cell lines were transduced with same DNA titers of viruses (Fig. 3B vs. 3A, 3D vs. 3C). The results confirm the lentiviral vector sequences affect the level of transgene expression.

The promoter activities are altered by different types of cells

In the experiments of 293T cells transfected with pLV-GFP vectors, the CMV promoter showed the highest activity in GFP expression in 293T cells. However, when the 293T cells were transduced with same copy number of pLV-CMV-GFP, pLV-EF1α-GFP, and pLV-PGK-GFP viruses and the level of GFP expression was determined by flow cytometry; the data showed that the overall GFP expression from CMV promoter is 4.2 and 6.3 folds higher than GFP

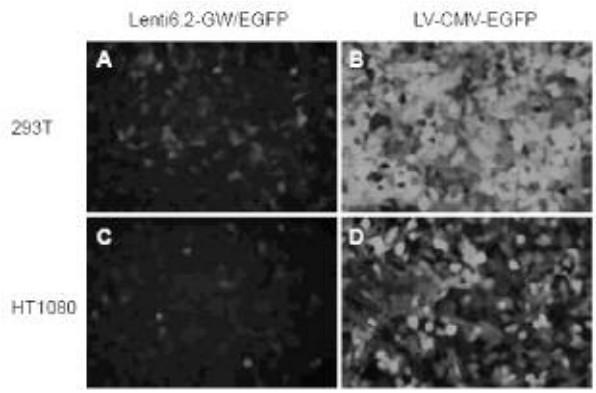

Figure 3. Examination of lentiviral GFP expression in 293T and HT1080 cells. The 293T and HT1080 cells was transduced with the same titer of pLV-CMV-GFP and pLenti6.2-GW/EGFP lentiviruses for 48 hours

Table 2. FACS analysis of pLV-GFP transduction in 293T cells

Lentivirus	GFP positive cells/ml	Mean Fluorescence Signal
LV-CMV-GFP	3.0 ± 0.1 x 106 (100%)	476 ± 14 (100%)
LV-EF1α-GFP	3.0 ± 0.2 x 106 (100%)	74 ± 0.4 (16%)
LV-PGK-GFP	2.8 ± 0.3 x 106 (93%)	104 ± 4.8 (22%)

expressing from PGK and EF1α promoters, respectively (Table 2, Fig. 4D-F). To investigate whether the promoter activities are influenced by different types of cells; The HOS and Hela cells were transduced with same DNA titers of viruses. In the HOS cells, the CMV and EF1α showed a similar level of activity (Fig. 4G-I). On the contrary, the EF1α is the most active promoter in the Hela cells (Fig. 4 J-L).

Discussion

The lentiviral transduction is one of the most effective methods to overexpress transgenes. However, the influence of the lentiviral DNA context on overexpression still has to be considered. In this study, we have shown that the GFP expression from the CMV promoter encoded in two lentiviral vectors, pLenti6.2-GW/EGFP and pLV-CMV-GFP, reveals 20-fold differences (Table 1 and Fig. 2). The differential expression of GFP reporter may be due to pLenti6.2-GW/EGFP vector was lacking some cis-acting elements such as central polypurine tract (cPPT) sequence and Woodchuck hepatitis posttranscriptional regulatory element (WPRE). It has been reported that the cPPT sequence and WPRE in the lentiviral vectors contribute to a higher transgene expression and virus titer (8, 10). Cloning cPPT sequence and WPRE into pLenti6.2-GW/EGFP has increased GFP expression up to 10-fold (data not shown). In addition to transfecting 293T cells, transducing 293T and HT1080 cells with pLenti6.2-GW/EGFP and

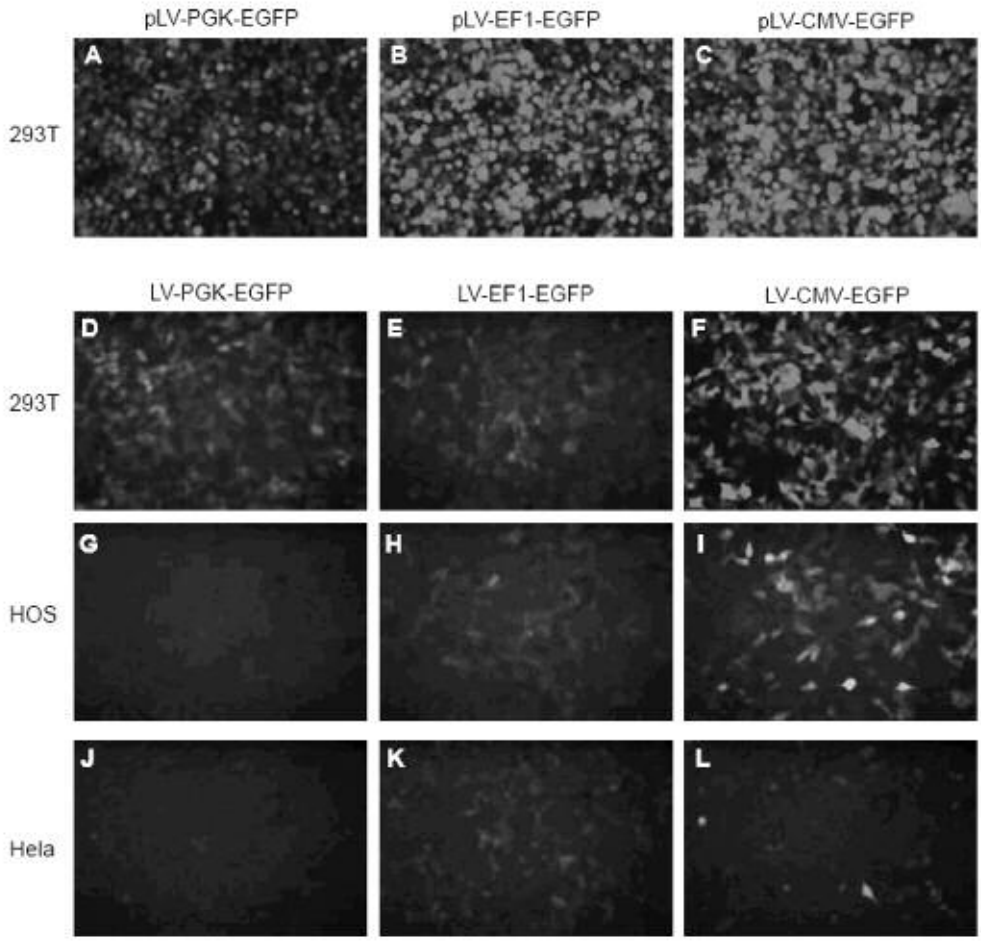

Figure 4. Comparing promoter activities in different cell lines. (A-C). The 293T cells were transfected with pLV-GFP vectors. (D-L). The 293T, HOS, and Hela cells were transduced with same titer of lentivirus to compare the level of gene expression

pLV-CMV-GFP showed the consistent results (Fig. 3). The results indicate that the differential expression of GFP between pLenti6.2-GW/EGFP and pLV-CMV-GFP is due to the lentiviral genomic sequences but not the plasmid topology.

The multiplicity of infection (MOI) may be underestimated if using functional GFP titer. In table 1 the numbers of GFP-positive cells were 10-fold difference between pLV-CMV-GFP and pLenti6.2-GW/EGFP transductions while the DNA titers showed that the DNA copy numbers only differed in 1 fold. The data suggest if the context of lentiviral genome affects the production of GFP, then determining the copy number of lentivirus using GFP titer may not be accurate. Moreover, it has been reported that the estimation of lentiviral cDNA copy number in the transduced cell lines is more reliable than using the level of GFP expression to titer lentiviruses (11).

The CMV promoter may not always be the best choice for transgene overexpression in all cell types. By comparing to other promoters, while CMV promoter gave more than 6-fold of GFP expression than human house keeping gene EF1α promoter in 293T cells, the GFP production by EF1α promoter revealed the highest intensity in Hela cells.

Acknowledgements

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