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Brief Report

Lentivirus Vectors Encoding Both Central Polypurine Tract and Posttranscriptional Regulatory Element Provide Enhanced Transduction and Transgene Expression

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ABSTRACT

Incorporation of a central polypurine tract (cPPT) and a posttranscriptional regulatory element (PRE) into lentivirus vectors provides increased transduction efficiency and transgene expression. We compared the effects of these elements individually and together on transduction efficiency and gene expression, using lentivirus vectors pseudotyped with vesicular stomatitis virus G protein (VSV-G) and encoding enhanced green fluorescent protein (GFP) and rat erythropoietin (EPO). The transduction efficiency was greater than 2-fold higher in the vector containing the PRE element, 3-fold higher in vector encoding the cPPT element, and 5-fold increased in the GFP virus containing both cPPT and PRE elements relative to the parent virus. In comparison with parent vector the mean fluorescence intensity (MFI) of GFP expression was 7-fold higher in cells transduced with virus containing PRE, 6-fold increased in cells transduced with virus containing cPPT, and 42-fold increased in GFP-virus containing both cPPT and PRE elements. EPO-virus containing a PRE element showed a nearly 5-fold increase in EPO secretion over the parent vector, and the vector encoding both PRE and cPPT showed a 65-fold increase. Thus, lentivirus vectors incorporating both PRE and cPPT showed expression levels significantly increased over the sum of the components alone, suggesting a synergistic effect.

INTRODUCTION

LENTIVIRAL VECTORS have the advantage over murine leukemia viral vectors of enabling provirus integration into nondividing cells (Corbeau *et al.*, 1996; Naldini *et al.*, 1996b; Blomer *et al.*, 1997; Kafri *et al.*, 1997; Zufferey *et al.*, 1998; Miyoshi *et al.*, 1999). These vectors were constructed by incorporating elements from human immunodeficiency virus type 1 (HIV-1) that interact with the nuclear import system and mediate transport via the nucleopore into the cell nucleus (Corbeau *et al.*, 1996; Naldini *et al.*, 1996b; Kafri *et al.*, 1997). It is significant that, when using this vector system, *in vivo* transduction of terminally differentiated brain tissue (Naldini *et al.*, 1996a,b; Blomer *et al.*, 1997; Kordower *et al.*, 1999), liver and muscle (Kafri *et al.*, 1997; Park *et al.*, 2000), and human bone marrow stem cells (Miyoshi *et al.*, 1999) has been achieved.

Because HIV-1 primarily infects $CD4^+$ cells, heterologous viral glycoproteins have been employed to improve tropism of HIV-based replication-defective retroviral vectors. In most reports lentiviral vectors have been pseudotyped with envelope glycoproteins from amphotropic murine leukemia virus (MuLV) or vesicular stomatitis virus G protein (VSV-G) (Yee *et al.*, 1994; Corbeau *et al.*, 1996; Naldini *et al.*, 1996a,b; Parolin *et al.*, 1996; Kafri *et al.*, 1997; Luo *et al.*, 1998). Two major benefits conferred by VSV-G pseudotyping are a broad tropism and a more robust virus that can be easily concentrated by centrifugation.

Current lentiviral vectors are of three main types: the first-generation HIV long terminal repeat (LTR) *tat* system (Parolin *et al.*, 1996; Reiser *et al.*, 1996); the next generation, which does not use the HIV *tat* promoter for transgene expression; and a third generation with improved safety that encodes a self-

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inactivating (SIN) construct in the 3′ LTR to attenuate any production of wild-type lentivirus. The latter virus is made *tat* independent by substituting the U3 region of the 5′ LTR with the Rous sarcoma virus (RSV) promoter (Naldini *et al.*, 1996b; Dull *et al.*, 1998; Zufferey *et al.*, 1998) (Fig. 1). However, the principle of vector design is essentially the same in these vectors and the alterations were made to improve vector safety. The incorporation of a central polypurine tract (cPPT) (Charneau and Clavel, 1991; Follenzi *et al.*, 2000; Zennou *et al.*, 2000) and a posttranscriptional regulatory element (PRE) (Patzel and Sczakiel, 1997; Zufferey *et al.*, 1999) into lentivirus vectors has shown increased transduction efficiency and transgene expression. The cPPT elements from the *pol* region of HIV-1 have been shown to act by increasing nuclear transport of the virus preintegration complex and hence increasing transduction efficiency (Charneau and Clavel, 1991; Follenzi *et al.*, 2000; Sirven *et al.*, 2000; Zennou *et al.*, 2000). It has been shown that a 99-nucleotide plus-strand overlap, a central DNA flap, acts as a *cis* determinant of HIV-1 genome nuclear import, and is a crucial determinant of gene transduction of human hematopoietic stem cells (Sirven *et al.*, 2000). Studies have shown that PRE elements stabilize virus vector mRNA and lead to increased transgene expression (Patzel and Sczakiel, 1997; Zufferey *et al.*, 1999). Both of these elements act in an orientation- and location-specific manner.

In comparison with the generation of murine leukemia viral vectors, current methods for producing lentivirus vectors are relatively inefficient and labor intensive. The production of sufficient amounts of virus for *in vivo* delivery in preclinical studies employing large outbred animals is time and reagent consuming. Therefore, we investigated the incorporation of cPPT and PRE elements into lentivirus vectors to achieve increases in transduction efficiency and gene expression that would permit reduced levels of both virus production and administration in preclinical animal studies. We compared the effects of the cPPT and PRE elements individually and together on transduction efficiency and gene expression, using a third-generation lentiviral vector pRRL encoding enhanced green fluorescent protein (GFP) and erythropoietin (EPO), a secreted growth factor.

MATERIALS AND METHODS

Construction and packaging of vectors

All manipulations were performed on the vector pRRL-CMV-GFP-SIN, a generous gift of R. Zufferey and D. Trono (University of Torino, Turin, Italy). We inserted a multiple cloning site (X) in place of the GFP gene. The polylinker contained sites (5′ to 3′) *Bam*HI, *Xma*I, *Pst*I, *Eco*RI, *Asc*I, *Mlu*I, *Sac*II, *Spe*I, *Xho*I, *Pac*I, *Nhe*I, *Nsi*I, and *Kpn*I. The X polylinker consisted of two separate oligonucleotides that were annealed together and ligated into the *Eco*RI–*Kpn*I sites in the base vec-

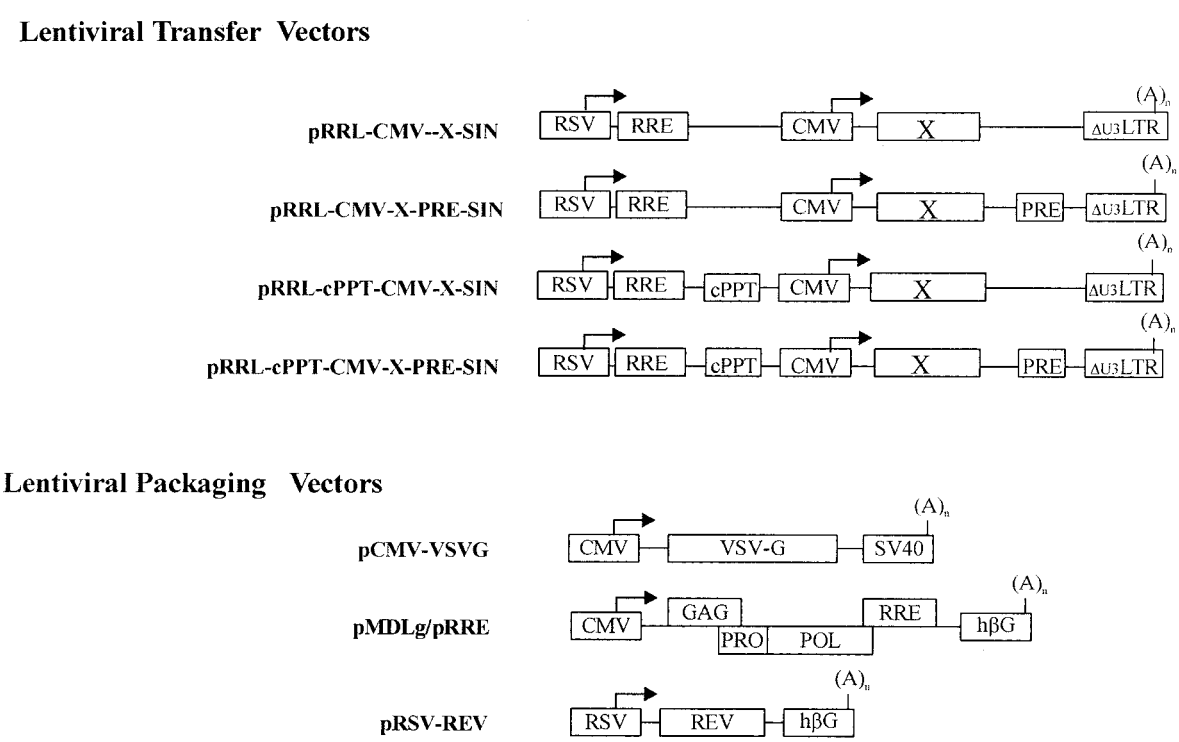

FIG. 1. Diagram of pRRL-based lentiviral transfer vectors and packaging vectors. Abbreviations: RSV, Rous sarcoma virus and HIV chimeric long terminal repeat; RRE, HIV Rev response element; CMV, human cytomegalovirus promoter; cPPT, HIV-1 central polypurine tract; PRE, human hepatitis virus posttranscriptional regulatory element; X, multiple cloning site; VSV-G, vesicular stomatitis virus G glycoprotein; GAG, HIV viral Gag-coding region; POL, HIV viral Pol-coding region; PRO, HIV Pro-coding region; Rev, HIV rev-coding region; SV40, simian virus 40 polyadenylation sequence; hβG, human β-globin polyadenylation sequence; Δ, deletion in the HIV-1 LTR U3 sequence.

tor to give pRRL-CMV-X-SIN. In brief, the cPPT insert was amplified from the *pol* region of the packaging virus pCMV-delta8.91 obtained from D. Trono (Naldini *et al.*, 1996b; Zufferey *et al.*, 1998). Primers were designed to incorporate a 5′ *Hpa*II site and a 3′ *Cla*I site (cPPT 5′, TCGCGACCGGTTTTAAAAGAAAAGGGGGG; cPPT3′, AAGCTTATCGATAAAATTTTGAATTTTTGTAATTTG). The polymerase chain reaction (PCR) product was digested with *Hpa*II and *Cla*I and ligated into the *Cla*I site directly 5′ to the cytomegalovirus (CMV) promoter in pRRL-CMV-X-SIN to yield pRRL-cPPT-CMV-X-SIN and checked for orientation by restriction enzyme digestion and sequencing. A plasmid containing the PRE element from human hepatitis B virus (Patzel and Sczakiel, 1997) was used to generate the PRE insert by PCR, using primers to incorporate a 5′*Nhe* I site and a 3′ *Kpn*I site (PRE5′, GCAGCGGCTAGCCTAGTGCCATTTGTTCAGT; PRE3′, GCACCCGGTACCCGCTCTAGTGATACATGG). The PRE element (1161 bp) was ligated into the *Nhe*I–*Kpn*I site in the X linker of either pRRL-CMV-X-SIN or pRRL-cPPT-CMV-X-SIN, to give, pRRL-CMV-X-PRE-SIN or pRRL-cPPT-CMV-X-PRE-SIN respectively. The cDNAs encoding rat erythropoietin (rEPO) (Wen *et al.*, 1993; Lejnieks *et al.*, 1998) and enhanced green fluorescent protein (eGFP) (Seppen *et al.*, 2000) were cloned into the multiple cloning site X to obtain the required expression vectors (Fig. 1).

Packaging of these viruses was performed by transient transfection of 293T cells (Naldini *et al.*, 1996b; Zufferey *et al.*, 1998). The day prior to transfection confluent 10-cm plates of 293T cells were split 1:5. pRRL-based lentiviral vectors were generated by calcium phosphate cotransfection of the transfer vector, the HIV *gag/pol* packaging construct, a *rev* expression plasmid, and the VSV-G expression plasmid (Soneoka *et al.*, 1995) into 293T cells as we have previously described (Barry *et al.*, 2000; Seppen *et al.*, 2000). Briefly, for each 10-cm-diameter dish 10 μg of transfer vector, 6.6 μg of pMDL-g/p-RRE packaging plasmid, 5 μg of pRSV-REV, and 3.5 μg of pCMV VSV-G envelope were mixed. The DNA was resuspended in 450 μl of 0.1 × TE (1× TE is 10 m*M* Tris [pH 8.0], 1 m*M* EDTA); 50 μl of 2.5 *M* $CaCl_2$ was added and the mixture was incubated at room temperature for 10 min. The DNA–$CaCl_2$ solution was added dropwise to 500 μl of 280 m*M* NaCl, 100 m*M* HEPES (pH 7.12) and 1.5 m*M* Na_2HPO_4 under vigorous bubbling, and once slightly turbid the solution was immediately added to the cells. All transfections proceeded for 16 hr, with medium replacement after 16 hr and virus collection 48 hr later. Viral supernatants were filtered through 0.2-μm pore size filters and stored at −80°C.

Virus titer

Virus titer for the eGFP lentivirus was determined by infection of HeLa cells in the presence of DEAE-dextran (10 μg/ml) followed 3 days later by fluorescence-activated cell sorting (FACS) analysis (Barry *et al.*, 2000; Seppen *et al.*, 2000). Briefly, 5×10^4 HeLa cells were plated in 6-cm dishes and virus from pRRL-CMV-eGFP-SIN vector was serially titrated on duplicate plates in Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum (FCS)–2 m*M* glutamine–1 m*M* sodium pyruvate–penicillin (100 U/ml)–streptomycin (100 μg/ml) in the presence of DEAE dextran (10 μg/ml). After 16 hr the medium was replaced and the plates were incubated for a further 48 hr. Plates were then trypsinized and after washing three times in phosphate-buffered saline (PBS)–5% FCS and fixing in 4% paraformaldehyde, samples were analyzed by flow cytometry with a Beckton Dickinson (Franklin Lakes, NJ) FACS+ instrument.

Analysis of transduction by eGFP-encoding virus was performed with untransduced cells to set the negative control gates for fluorescein isothiocyanate (FITC) (FL1). Data were analyzed with CellQuest software (Beckton Dickinson). Viral $p24^{gag}$ protein was determined with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Coulter, Miami, FL) and compared with titers of eGFP virus obtained by FACS analysis. This assay showed a functional titer of 2.5×10^6 infectious units/ml for eGFP virus and this was equivalent to 1100 ng of p24 protein per milliliter. Lentiviruses encoding rEPO were assayed for $p24^{gag}$ content and expressed as infectious units/ml by comparison with eGFP virus titer determined by flow cytometry. This method is based on the assumption that the frequency of functional viral particle production is essentially the same for all preparations (Reiser *et al.*, 1996; Mochizuki *et al.*, 1998; Zufferey *et al.*, 1998) and we routinely observed about 2×10^3 transducing units/ng of $p24^{gag}$ protein.

TABLE 1. GENE EXPRESSION FROM LENTIVIRUS VECTORS CONTAINING cPPT AND PRE ELEMENTS[a]

	Transduction efficiency		*MFI of GFP*		*Secreted EPO*	
Lentivirus	*Value*	*Fold increase*	*Value*	*Fold increase*	*Value*	*Fold increase*
pRRL-CMV-X-SIN	17 ± 4	1	99 ± 24	1	134 ± 4	1
Vector + PRE	35 ± 2	2	733 ± 34	7	607 ± 64	5
Vector + cPPT	53 ± 2	3	627 ± 29	6	381 ± 55	3
Vector + cPPT + PRE	91 ± 3	5	4118 ± 180	42	8690 ± 81	65

[a]Transduction efficiency is expressed as percent GFP-positive cells, and mean fluorescence intensity (MFI) measures gene expression. EPO secretion is expressed as mU/ml per 10^6 cells per 24 hr. Parent vector is pRRL-CMV-eGFP-SIN or pRRL-CMV-rEPO-SIN. Vector + PRE is pRRL-CMV-eGFP-PRE-SIN or pRRL-CMV-rEPO-PRE-SIN. Vector + cPPT is pRRL-cPPT-CMV-eGFP-SIN or pRRL-cPPT-CMV-rEPO-SIN. Vector + cPPT + PRE is pRRL-cPPT-CMV-eGFP-PRE-SIN or pRRL-cPPT-CMV-rEPO-PRE-SIN. The data represent means ± SD of duplicate determinations from four separate virus batches.

Screening for replication-competent virus

Virus preparations were screened for replication-competent virus by using HeLa MAGI cells. These cells have a genomic β-galactosidase (β-Gal) reporter gene under transcriptional regulation of the HIV-1 LTR, such that wild-type HIV Tat activity activates the β-Gal reporter gene only in cells infected by virus encoding wild-type *tat* gene product (Emerman and Bissel, 1979). In brief, 5×10^4 HeLa MAGI cells were plated in 6-cm dishes 1 day prior to assay. On day 2 the medium was replaced, and 1 ml, 100 μl, or 10 μl of supernatant from test samples was added to the plate in the presence of DEAE-dextran (10 μg/ml). After 16 hr of incubation the medium was replaced, and the cells were cultured for a further 24 hr. Cells were then fixed and stained for β-galactosidase, using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate (Sigma, St. Louis, MO). Cells were scored positive if there was visible blue staining in the cytoplasm after 4–16 hr at 37°C as compared with the staining of untransduced controls over the same time period (Ramesh *et al.*, 1996). In addition, as an indicator of replication-competent virus the supernatant of serially passaged transduced cells was screened for $p24^{gag}$ protein, using a specific ELISA (Barry *et al.*, 2000).

Lentiviral infection and analysis of EPO production

For cell transduction a viral equivalency of 2 ng of $p24^{gag}$ protein was used to infect 5×10^3 HeLa cells in the presence of DEAE-dextran (10 μg/ml), giving a multiplicity of infection (MOI) of about 1. After infection for 16 hr the medium was changed, and cells were cultured for another 48 hr before harvesting cells encoding eGFP for FACS analysis and harvesting supernant from cells transduced to express rEPO to measure cytokine secretion. Conditioned supernatants of cells transduced with EPO-lentivirus were analyzed by human EPO ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer instructions.

RESULTS AND DISCUSSION

As a means to increase transduction efficiency and transgene expression of lentivirus vectors we incorporated into a parent vector (pRRL-CMV-X-SIN) a central polypurine tract (cPPT) and a posttranscriptional regulatory complex (PRE). We tested the effect on transduction and gene expression of virus vectors encoding both cPPT and PRE elements. To achieve this cD-

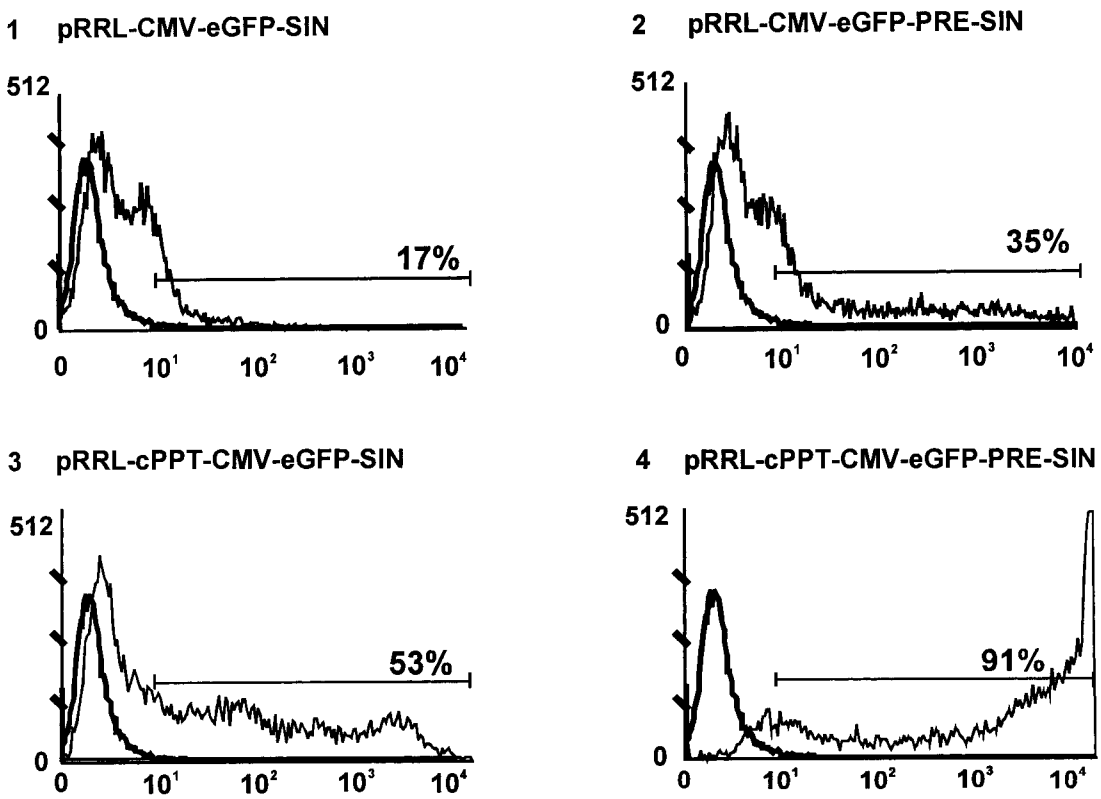

FIG. 2. Flow cytometric analysis of HeLa cells transduced with modified lentiviral vectors. Representative analysis of cells transduced with lentivirus expressing eGFP driven from the CMV promoter. The solid bar denotes the gate imposed on these data to calculate percent eGFP-positive cells. Staining of untransduced HeLa cells was used as a negative control (and are shown in all plots). HeLa cells transduced with the parent vector (RRL-CMV-eGFP-SIN) are shown in plot 1. HeLa cells transduced with RRL-CMV-eGFP-PRE-SIN demonstrated an increase in the range of intensities of eGFP expression (plot 2). Transduction by RRL-cPPT-CMV-eGFP-SIN produced a larger number of positive cells over a wider range of intensities than the parent vector (plot 3). When both elements were incorporated into the same virus, RRL-cPPT-CMV-eGFP-PRE-SIN, a significant increase in both the frequency and intensity of GFP marking was seen compared with all other panels (plot 4). Numbers on each plot represent percent eGFP-positive cells for the gate set on the FL1 histograms.

NAs encoding rat EPO and enhanced GFP were cloned into the multiple cloning site of pRRL-CMV-X-SIN to obtain eight expression vectors (Fig. 1). The resulting eight vectors were packaged and pseudotyped with VSV-G envelope protein and the $p24^{gag}$ protein content was determined for each virus stock. Transduction of 5×10^4 HeLa cells in duplicate from four separate replicates of each virus was performed at the same MOI of approximately 1. We compared the transduction efficiencies and expression levels of the parental vectors pRRL-CMV-eGFP-SIN and pRRL-CMV-rEPO-SIN with those modified to incorporate cPPT and/or PRE elements (Fig. 1 and Table 1).

The transduction efficiency, as determined by percent GFP-positive cells, was increased in all the modified vectors and was 2-fold higher in the vector containing the PRE element, and 3-fold higher in vector encoding the cPPT element (Table 1). However, in comparison with the parent virus there was a significant 5-fold increase in transduction efficiency in the GFP virus containing both cPPT and PRE elements (Table 1). The mean fluorescence intensity (MFI) of GFP expression was about 7-fold higher in cells transduced with pRRL-CMV-eGFP-PRE-SIN, as determined by a shift to the right of events in the gated region (Fig. 2), indicating that the posttranscriptional regulatory element increased transgene expression levels (Table 1). Cells transduced with pRRL-cPPT-CMV-eGFP-SIN showed a 6-fold increase in MFI in comparison with parent vector. Most notably, the GFP-encoding virus containing both cPPT and PRE elements showed a 42-fold increase in MFI in comparison with the parent virus, suggesting that a combination of these elements provided enhanced gene expression.

Representative data of flow cytometric analyses of HeLa cells transduced by the four eGFP viruses are shown in Fig. 2. Fluorescence in the FL1 (FITC) channel was plotted against frequency of events. Untransduced HeLa cells, shown in each plot, were used as a negative control and defined the 0% $eGFP^+$ gate. The same gate was set for all analyses. Cells transduced with the parent vector pRRL-CMV-eGFP-SIN showed 17% eGFP-positive cells (Fig. 2, plot 1). HeLa cells transduced with pRRL-CMV-eGFP-PRE-SIN (Fig. 2, plot 2, 35% $eGFP^+$) demonstrated an increase in the range of intensities of eGFP expression. Plot 3 in Fig. 2 shows cells transduced by pRRL-cPPT-CMV-eGFP-SIN (53% $eGFP^+$), which gave a larger number of cells transduced over a wider range of intensities than the parent vector. When both elements were incorporated into the same virus, pRRL-cPPT-CMV-eGFP-PRE-SIN, a significant increase in both the frequency (91% $eGFP^+$) and intensity of GFP marking was seen compared with the other transductions (Fig. 2, plot 4).

The levels of erythropoietin secreted from cells transduced with rat EPO-encoding viruses were in general agreement with the mean fluorescence intensity measurements obtained from GFP virus (Table 1). pRRL-CMV-rEPO-PRE-SIN, the virus vector containing a PRE element, showed a nearly 5-fold increase in EPO secretion over the parent vector, and pRRL-cPPT-CMV-rEPO-PRE-SIN, the vector encoding both PRE and cPPT, showed a 65-fold increase. The GFP-vector encoding both PRE and cPPT also showed expression levels that were much larger than their additive effects, even allowing for increased transduction efficiencies.

Taken together, these data indicate that lentivirus vectors encoding cPPT and PRE elements provide increases in both transduction efficiency and gene expression, and suggest that these elements act synergistically in these lentivirus vectors. Although lentivirus vectors may be produced at high titer, the current lack of an efficient stable packaging cell system necessitates batch production of virus that is consuming of both time and reagents. Vectors including both elements may benefit therapeutic gene expression and reduce the quantity of virus needed for animal tests.

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