# Advanced modular self-inactivating lentiviral expression vectors for multigene interventions in mammalian cells and *in vivo* transduction

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## ABSTRACT

**In recent years, lentiviral expression systems have gained an unmatched reputation among the gene therapy community for their ability to deliver therapeutic transgenes into a wide variety of difficult-to-transfect/transduce target tissues (brain, hematopoietic system, liver, lung, retina) without eliciting significant humoral immune responses. We have cloned a construction kit-like self-inactivating lentiviral expression vector family which is compatible to state-of-the-art packaging and pseudotyping technologies and contains, besides essential *cis*-acting lentiviral sequences, (i) unparalleled polylinkers with up to 29 unique sites for restriction endonucleases, many of which recognize 8 bp motifs, (ii) strong promoters derived from the human cytomegalovirus immediate-early promoter ($P_{hCMV}$) or the human elongation factor 1α ($P_{hEF1\alpha}$), (iii) $P_{hCMV-}$ or $P_{PGK-}$ (phosphoglycerate kinase promoter) driven G418 resistance markers or fluorescent protein-based expression tracers and (iv) tricistronic expression cassettes for coordinated expression of up to three transgenes. In addition, we have designed a size-optimized series of highly modular lentiviral expression vectors (pLentiModule) which contain, besides the extensive central polylinker, unique restriction sites flanking any of the 5′U3, R-U5-ψ+-SD, cPPT-RRE-SA and 3′LTR$_{\Delta U3}$ modules or placed within the 5′U3 (–78 bp) and 3′LTR$_{\Delta U3}$ (8666 bp). pLentiModule enables straightforward cassette-type module swapping between lentiviral expression vector family members and facilitates the design of Tat-independent (replacement of 5′LTR by heterologous promoter elements), regulated and self-excisable proviruses (insertion of responsive operators or LoxP in the 3′LTR$_{\Delta U3}$ element). We have validated our lentiviral expression vectors by transduction of a variety of insect, chicken, murine and human cell lines as well as adult rat cardiomyocytes, rat hippocampal slices and chicken embryos. The novel multi-purpose construction kit-like vector series described here is compatible with itself as well as many other (non-viral) mammalian expression vectors for straightforward exchange of key components (e.g. promoters, LTRs, resistance genes) and will assist the gene therapy and tissue engineering communities in developing lentiviral expression vectors tailored for optimal treatment of prominent human diseases.**

## INTRODUCTION

Gene therapy strategies rely on efficient transfer of therapeutic transgenes into desired target cells. A variety of viral and non-viral vectors and expression concepts have been designed and evaluated for their safety, high-level transduction, tropism and sustained expression in a variety of therapeutically relevant cells and tissues (1,2). Retroviral vectors derived from oncoretroviruses such as the murine leukemia virus (MLV) emerged as the most widely used gene therapy tools for transgene delivery currently in the clinics (3). The attractiveness of oncoretroviral transduction technologies resides in: (i) their ability to mediate stable integration in the target chromosomes likely promoting long-term expression of delivered transgenes; (ii) their large cloning capacity sufficient for most foreseeable clinical situations; (iii) their compatibility with pseudotyping strategies which extend the tropism of gene delivery; and (iv) the exclusive delivery of therapeutic transgenes in the absence of viral genes which precludes any potent humoral immune response eliminating transduced cells and enables recurring treatments (3,4).

In addition to these characteristics common to all retroviruses, lentiviruses such as the human immunodeficiency virus type 1 (HIV-1) can replicate in non-mitotic cells owing to their so-called pre-integration complex, a macromolecular

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structure comprising the viral genome, a few structural proteins and the enzymes responsible for reverse transcription and integration (5–7). Transduction of proliferation-incompetent cells is a decisive asset for molecular interventions in tissues considered key targets for future gene therapy including the brain, the heart, the hematopoietic system, the liver, the lungs and the retina (8–14). Like all retroviruses, the HIV-1 genome contains the *gag*, *pol* and *env* regions, which encode the core proteins, the virion-associated enzymes and the envelope (Env) glycoprotein (13,15). This coding region is flanked by the long terminal repeats (LTRs; 5′ and 3′LTR) and *cis*-acting sequences essential for integration, transcription and polyadenylation. In addition, HIV-1 contains two regulatory genes, *tat* and *rev*, required for viral replication (LTR transactivation and nuclear export of viral RNA) and four accessory genes, *vif*, *vpr*, *vpu* and *nef*, which are dispensable for viral growth but critical for *in vivo* replication and pathogenesis (15). Basal transcription of the HIV-1 provirus' 5′LTR initially results in small amounts of multiply spliced transcripts encoding Tat, Rev and Nef. Tat transactivates 5′LTR-mediated transcription until Rev reaches a threshold concentration and mediates cytoplasmic accumulation of unspliced and singly spliced viral transcripts followed by production of the late viral proteins (15).

Capitalizing on a refined understanding of HIV-1 molecular biology following decades of intensive research on acquired immunodeficiencies, a group of researchers pioneered the transition of this well-evolved pathogen into a high leverage gene therapy tool (16–18). HIV-1-based vectors have been generated using a multiply attenuated packaging system, which is activated in its most advanced third generation configuration by transient transfection of four plasmids encoding: (i) *gag* (coding for the virion main structural proteins) and *pol* (responsible for lentivirus-specific enzymes); (ii) *rev* (a post-transcriptional regulator for *gag* and *pol* expression as well as nuclear RNA export); (iii) *vsv-g* (required for pseudotyping); and (iv) the desired transgene in a lentiviral expression configuration (lentiviral expression vector or lentivector) (19). Latest developments include packaging cell lines, which express all packaging components and require exclusive transfection of the lentiviral expression vector (20–23). The lentiviral expression vector, which can be combined with any generation of packaging system, is the only genetic material transferred to the target cell. It typically comprises the transgene cassette flanked by *cis*-acting elements required for encapsidation, reverse transcription and integration including the packaging signal (ψ+), the polypurine tracts (PPT), 5′ and 3′LTRs as well as *env*-derived sequences encompassing the Rev response element (RRE) (16–19,24). Latest generation HIV-1-based expression technologies include self-inactivating (SIN) vectors, which loose the transcriptional capacity of the LTR once integrated into target cells. SIN vectors show improved performance and are devoid of transcriptional interference and *in vivo* suppression associated with standard vectors and enable construction of more-stringent tissue-specific and/or regulatable expression configurations (25,26).

Unfortunately, current lentiviral expression vectors are the result of a progressive development rather than a bottom-up rational design and are therefore often lacking convenient multiple cloning sites (MCS) and a modular set-up to increase compatibility with existing expression technologies (gene regulation, recombination) (16,17,19). This situation has significantly delayed the dissemination of lentiviral expression technology beyond the pioneering laboratories to a broad scientific community. We describe here a size-optimized highly modular construction kit of SIN lentiviral expression vectors containing extended MCS, multicistronic expression cassettes as well as various promoter and resistance elements.

## MATERIALS AND METHODS

### Cell culture and fluorescence microscopy

Chinese hamster ovary cells (CHO-K1; ATCC CCL-61), baby hamster kidney cells BHK-21 (ATCC CCL-10), human fibrosarcoma cells HT-1080 (ATCC CCL-121), human cervical adenocarcinoma cells HeLa (ATCC CCL-2), human hepatocellular carcinoma cells HepG2 (ATCC HB-8065) and human chronic myelogenous leukemia cell line K-562 (ATCC-243) were cultivated in FMX-8 (Cell Culture Technologies GmbH, Switzerland), Dulbecco's modified Eagle medium (DMEM) (BHK-21, HT-1080, HeLa, HepG2; catalog no. 52100-039; Life Technologies AG, Basel, Switzerland) or Iscove's modified Dulbecco's medium (K-562, catalog no. 4220-022; Life Technologies AG) supplemented with 10% fetal calf serum (all other cell lines; PAA Vienna, Austria; Lot. no. A01129-242). The chicken bursal cell line DT40 was cultivated as described before (27). Adult rat cardiomyocytes (ARC) were prepared and cultivated as described previously (28). Hippocampal slices prepared from 6-day postnatal rats were cultured using the roller-tube technique (29).

For DRAQ5-mediated DNA-specific staining of transduced cells, $4 \times 10^3$ CHO-K1 cells were seeded into chamber slides (Lab-Tek, Nalge Nunc International, IL) and grown for 24 h before they were infected with $2 \times 10^6$ c.f.u./ml of desired lentiviruses (pMF365-, pBM40- and pBM45-derived). After 48 h, DRAQ5 (Biostatus Ltd, Leicestershire, UK) was added to the culture at a final concentration of 25 μM for 7 min. The medium was removed and cells washed twice with PBS (Dulbecco's phosphate-buffered saline, Sigma, catalog no. D5773) before they were fixed with 4% paraformaldehyde in PBS for 8 min and washed another five times with PBS. After removing the PBS, the cells were covered with a drop of Lisbeth's medium [Tris-buffered glycerol: 3:7 mixture of 0.1 M Tris–HCl (pH 9.5) and glycerol plus 50 mg/ml *n*-propyl-gallat] and sealed with a cover slip for confocal microscopy.

Expression of fluorescent proteins was visualized using a Leica DM-RB fluorescence microscope (Heerbrugg, Switzerland) or a confocal microscope set-up (Zeiss Axioplan fluorescence microscope, Biorad MRC-600 confocal scanner, Silicon Graphics workstation) equipped with appropriate filters. The fluorescence of the enhanced green fluorescent protein (EGFP) and the enhanced yellow fluorescent protein (EYFP) were visualized using the XF114 filter (Omega Optical Inc., Brattleboro, VT), the enhanced cyan fluorescent protein (ECFP) was detected using the XF105 filter (Omega Optical Inc.) and the red fluorescent protein was monitored using the N2.1 filter by Leica Inc (Heerbrugg, Switzerland).

**Table 1.** Plasmids used and constructed in this study

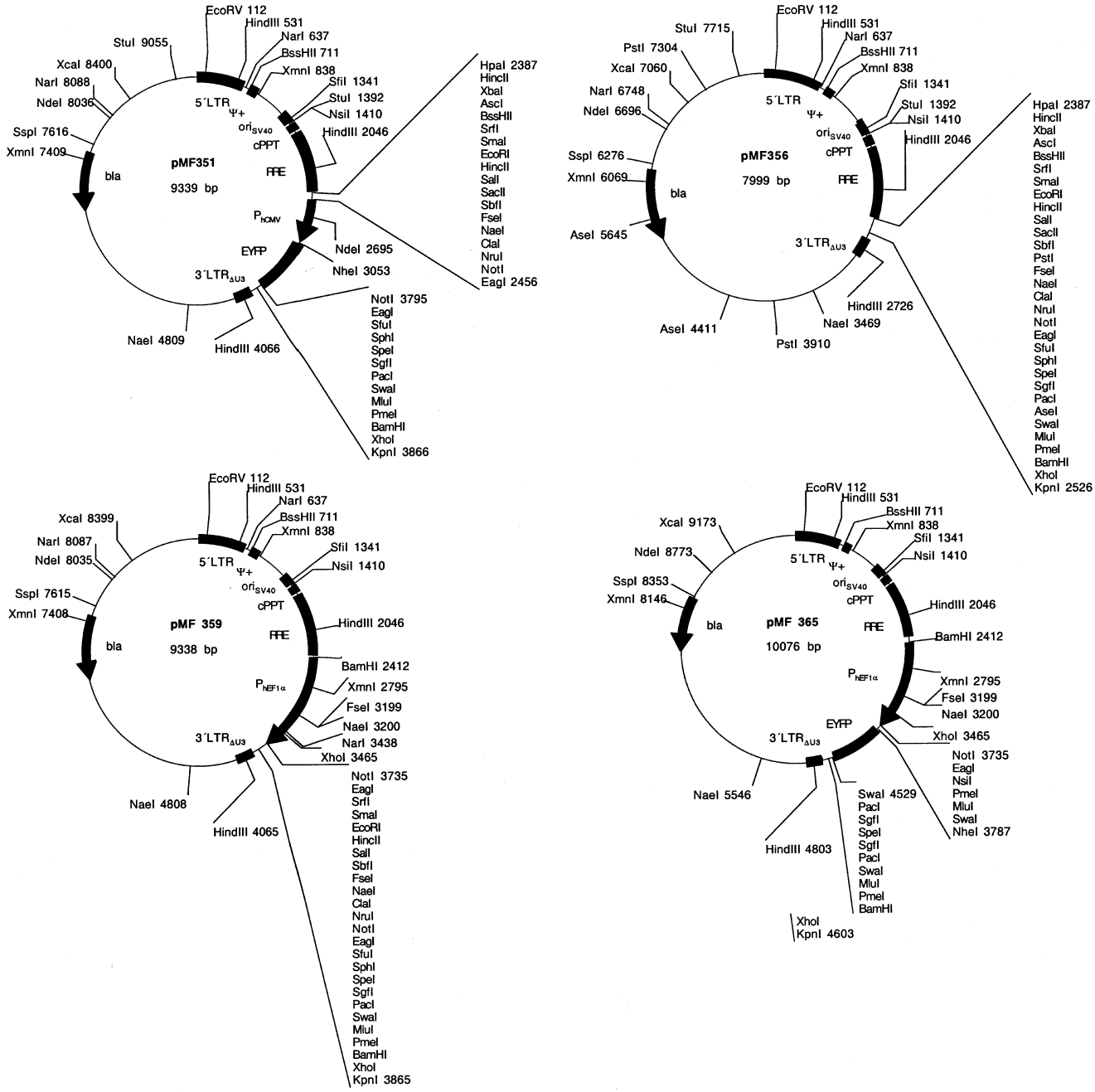
| Plasmid | Description | Reference or source |
|---|---|---|
| pcDNA3.1/V5-His TOPO | Mammalian expression vector | Invitrogen, Carlsbad, CA |
| pcDNA3 | Mammalian expression vector vector | Invitrogen, Carlsbad, CA |
| pcDNA3-$VEGF_{121}$ | pcDNA3 encoding human $VEGF_{121}$ | Weber, unpublished |
| pCF10 | Dual-regulated expression vector | Fux and Fussenegger, submitted for publication |
| pEF4/MycHisB | Expression vector containing the human elongation factor 1α promoter ($P_{EF1\alpha}$ ) | Invitrogen, Carlsbad, CA |
| pEYFP-C1 | Expression vector encoding the enhanced yellow fluorescent protein under control of $P_{hCMV}$ | Clontech, Palo Alto, CA |
| PMSCVneo | Retroviral expression vector derived from the murine embryonic stem cell virus | Grez *et al*., 1990 |
| PLPCX | Retroviral expression vector derived from the Moloney MLV (MoMuLV) and Moloney murine sarcoma virus (MoMuSV) | Miller and Rosman, 1989 |
| pNL-EGFP | Lentiviral expression vector (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-3′LTR) | Mochizuki *et al*., 1998 |
| pNL-EGFPΔU3 | pNL-EGFP containing an *Eco*RV/*Pvu*II deletion in the U3 region of the 3′LTR eliminating any enhancers (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-3′$LTR_{\Delta U3}$) | Reiser *et al*., unpublished |
| pSS173 | Mammalian expression vector encoding the human placental SEAP | Schlatter *et al*., 2002 |
| pWW85 | Triple-fluorescent expression vector encoding ECFP, RFP and EYFP | Weber *et al*., 2002 |
| pWW265 | $VEGF_{121}$ was excised from pcDNA-$VEGF_{121}$ by *Hin*dIII/*Spe*I and ligated into the corresponding sites (*Hin*dIII/*Spe*I) of pCF10 ($P_{hCMV}$-$VEGF_{121}$-pA) | Weber *et al*., unpublished |
| pTRIDENT1 | Tricistronic mammalian expression vector harboring a tetracycline-responsive, $P_{hCMV*-1}$-driven expression unit ($P_{hCMV*-1}$-MCSI-*IRES*I-MCSII-*IRES*II-MCSIII-pA) (pMF125) | Fussenegger *et al*., 1998 |
| pTRIDENT3 | Tricistronic mammalian expression vector harboring a tetracycline-responsive, $P_{hCMV*-1}$-driven expression unit ($P_{hCMV*-1}$-MCSI-*IRES*-MCSII-*CITE**-MCSIII-pA) (pMF122) | Fussenegger *et al*., 1998 |
| pMF201 | Dual-regulated expression vector encoding EYFP and ECFP | Fussenegger *et al*., 2000 |
| pMF242 | pcDNA3.1/V5-His TOPO encoding mouse erythropoietin (mEPO) | Fussenegger *et al*., 2000 |
| pMF320 | $P_{EF1\alpha}$ was amplified from pEF4/MycHisB by OMF164/OMF165 and cloned in antisense orientation into pcDNA.3.1/V5-His TOPO | This work |
| pMF351 | $P_{hCMV}$-EYFP was amplified from pEYFP-C1 with OMF179/OMF180 and cloned as *Hpa*I/*Kpn*I fragment into the corresponding sites (*Hpa*I/*Kpn*I) of pNL-EGFPΔU3 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCSI-$P_{hCMV}$-EYFP-MCSII-3′$LTR_{\Delta U3}$) | This work |
| pMF352 | U3 was amplified from pNL-EGFPΔU3 using OMF183/OMF184 and cloned into pcDNA3.1/V5-His TOPO. The U3 fragment contains a *Sbf*I site | This work |
| pMF353 | R-U5-ψ+-SD was amplified from pNL-EGFPΔU3 using OMF185/OMF186 and cloned into pcDNA3.1/V5-His TOPO | This work |
| pMF355 | PPT-3′$LTR_{\Delta U3}$ was amplified from pNL-EGFPΔU3 using OMF189/OMF190 and cloned into pcDNA3.1/V5-His TOPO | This work |
| pMF356 | The $P_{hCMV}$-EYFP cassette of pMF351 was excised by *Not*I and the backbone religated (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-3′$LTR_{\Delta U3}$) | This work |
| pMF357 | U3 was excised from pMF352 by *Xho*I/*Srf*I and cloned into the corresponding sites (*Xho*I/*Srf*I) of pTRIDENT1 | This work |
| pMF358 | R-U5-ψ+-SD was excised from pMF353 by S*rf*I/*Swa*I and cloned into the corresponding sites (*Srf*I/*Swa*I) of pMF357 | This work |
| pMF358x | R-U5-ψ+-SD was excised from pMF376 by S*rf*I/*Swa*I and cloned into the corresponding sites (*Srf*I/*Swa*I) of pMF357 | This work |
| pMF359 | $P_{EF1\alpha}$ was excised from pMF320 by *Eco*RV/*Xma*I and cloned into the corresponding sites (*Hpa*I/*Xma*I) of pMF356 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-MCS-3′$LTR_{\Delta U3}$) | This work |
| pMF360 | cPPT-RRE-SA was excised from pMF375 by *Swa*I/*Mlu*I and cloned into the corresponding sites (*Swa*I/*Mlu*I) of pMF358 | This work |
| pMF360x | cPPT-RRE-SA was excised from pMF375 by *Swa*I/*Mlu*I and cloned into the corresponding sites (*Swa*I/*Mlu*I) of pMF358x | This work |
| pMF363 | $P_{hCMV}$ was excised from pLPCX using *Stu*I/*Eco*RI and cloned into the compatible sites (*Hpa*I/*Eco*RI of pMF356 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hCMV}$-MCS-3′$LTR_{\Delta U3}$) | This work |
| pMF365 | EYFP was excised from pMF201 by *Eco*RV/*Spe*I and ligated to the compatible sites (*Sma*I/*Spe*I) of pMF359 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-EYFP-3′$LTR_{\Delta U3}$) | This work |
| pMF371 | ECFP was excised from pMF201 by *Eco*RI/*Not*I and ligated to the corresponding sites (*Eco*RI/*Not*I) of pMF363 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hCMV}$-ECFP-3′$LTR_{\Delta U3}$) | This work |
| pMF375 | cPPT-RRE-SA was amplified from pNL-EGFPΔU3 using OMF191/OMF192 and cloned into pcDNA3.1/V5-His TOPO | This work |
| pMF376 | R-U5-ψ+-SD was amplified from pNL-EGFPΔU3 using OMF185/OMF193 and cloned into pcDNA3.1/V5-His TOPO | This work |
| pMF378 | $Ori_{SV40}$ was amplified from pNL-EGFPΔU3 using OMF195/OMF196 and cloned into pcDNA3.1/V5-His TOPO | This work |
| pMF388 | The 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-3′$LTR_{\Delta U3}$ was amplified from pMF356 by OMF199/OMF200 and cloned as *Aat*II/*Nhe*I fragment to the compatible sites (*Aat*II/*Xba*I) of the pTRIDENT1 backbone (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-3′$LTR_{\Delta U3}$) | This work |
| pMF394 | $P_{hCMV}$-EYFP was excised from pMF351 using *Hpa*I/*Kpn*I and cloned into the corresponding sites (*Hpa*I/*Kpn*I) of pMF388 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hCMV}$-EYFP-3′$LTR_{\Delta U3}$) | This work |
| pMF402 | PPT-3′$LTR_{\Delta U3}$ was excised from pMF355 by *Mlu*I/*Xba*I and cloned into the corresponding sites (*Mlu*I/*Xba*I) of pMF360 | This work |

**Table 1.** *Continued*

| Plasmid | Description | Reference or source |
|---|---|---|
| pBM1 | The ECFP-IRESI-RFP cassette was excised from pWW85 by *Eco*RI/*Asc*I and cloned into the corresponding sites of pLentiTRIDENT2 (*Eco*RI/*Asc*I) 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-IRESI-RFP-IRESII-MCSIII-3′LTR$_{\Delta U3}$ | This work |
| pBM6 | The P$_{PGK}$-neo cassette was amplified from pMSCVneo using OBM1/OBM2 and cloned in sense orientation into pcDNA3.1/V5-His TOPO | This work |
| pMB7 | P$_{EF1\alpha}$ was excised from pMF320 using *Eco*RV/*Xma*I and cloned into the compatible sites (*Hpa*I/*Xma*I) of pMF388 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-MCS-3′LTR$_{\Delta U3}$) | This work |
| pBM8 | The P$_{PGK}$-neo cassette was excised from pBM6 by *Pac*I/*Mlu*I and cloned into the corresponding sites (*Pac*I/*Mlu*I) of pMF356 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$) | This work |
| pBM9 | The P$_{PGK}$-neo cassette was excised from pBM6 by *Pac*I/*Mlu*I and inserted into the corresponding sites (*Pac*I/*Mlu*I) of pMF359 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{EF1\alpha}$-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$) | This work |
| pBM13 | The P$_{PGK}$-neo cassette was excised from pBM6 using *Pac*I/*Mlu*I and cloned into the corresponding sites (*Pac*I/*Mlu*I) of pMF388 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$) | This work |
| pBM14 | The P$_{PGK}$-neo cassette was excised from pBM6 using *Pac*I/*Mlu*I and cloned into the corresponding sites (*Pac*I/*Mlu*I) of pBM7 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{EF1\alpha}$-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$) | This work |
| pBM40 | EYFP was excised from pLentiModule4 by *Nhe*I/*Mlu*I and cloned into the compatible sites (*Spe*I/*Mlu*I) of pBM7 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-EYFP-3′LTR$_{\Delta U3}$) | This work |
| pBM42 | mEPO was excised from pMF242 by *Eco*RI/*Eco*RV and cloned into the compatible sites (*Eco*RI/*Pme*I) of pMF359 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-EPO-3′LTR$_{\Delta U3}$) | This work |
| pBM43 | VEGF$_{121}$ was excised from pWW265 by *Sal*I/*Mlu*I and ligated into the corresponding sites (*Sal*I/*Mlu*I) of pMF359 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-VEGF$_{121}$-3′LTR$_{\Delta U3}$) | This work |
| pBM44 | SEAP was excised from pSS173 by *Eco*RI/*Eco*RV and ligated into the compatible sites (*Eco*RI/*Pme*I) of pMF359 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-SEAP-3′LTR$_{\Delta U3}$) | This work |
| pLentiTRIDENT1 | The MCSI-IRESI-MCSII-IRESII-MCSIII cassette was excised from pTRIDENT1 by *Eco*RI/*Bgl*II and cloned into the compatible sites (*Eco*RI/*Bam*HI) of pMF363 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-MCSI-IRESI-MCSII-IRESII-MCSIII-3′LTR$_{\Delta U3}$) (pMF370) | This work |
| pLentiTRIDENT2 | The MCSI-IRES-MCSII-CITE*-MCSII cassette was excised from pTRIDENT3 by *Eco*RI/*Bgl*II and cloned into the compatible sites (*Eco*RI/*Bam*HI) of pMF363 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-MCSI-IRES-MCSII-CITE*-MCSIII-3′LTR$_{\Delta U3}$) (pMF369) | This work |
| pLentiTFT1 | The triple-fluorescent expression cassette ECFP-IRESI-RFP-IRESII-EYFP was excised from pWW85 using *Eco*RI/*Mlu*I and cloned into the corresponding sites (*Eco*RI/*Mlu*I) of pLentiTRIDENT1 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-IRESI-RFP-IRESII-EYFP-3′LTR$_{\Delta U3}$) (pMB2) | This work |
| pLentiTFT2 | EYFP was excised from pWW85 by *Spe*I/*Mlu*I and cloned to the corresponding sites (*Spe*I/*Mlu*I) of pBM1 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-IRES-RFP-CITE*-EYFP-3′LTR$_{\Delta U3}$) (pMB3) | This work |
| pLentiModule1 | PPT-3′LTR$_{\Delta U3}$ was excised from pMF355 by *Mlu*I/*Xba*I and cloned into the corresponding sites (*Mlu*I/*Xba*I) of pMF360x (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-MCS-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I) (pBM4) | This work |
| pLentiModule2 | The P$_{hCMV}$-EYFP cassette was excised from pMF351 by *Eco*RI/*Mlu*I and cloned into the corresponding sites (*Eco*RI/*Mlu*I) of pLentiModule1 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hCMV}$-EYFP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I) (pBM5) | This work |
| pLentiModule3 | ori$_{SV40}$ was excised from pMF378 by *Xba*I/*Nhe*I and cloned into the *Xba*I sites of pLentiModule1 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-MCS-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I-ori$_{SV40}$) (pBM15) | This work |
| pLentiModule4 | ori$_{SV40}$ was excised from pMF378 by *Xba*I/*Nhe*I and cloned into the *Xba*I sites of pLentiModule2 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hCMV}$-EYFP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I-ori$_{SV40}$) (pBM12) | This work |
| pLentiModule5 | The P$_{hEF1\alpha}$-EYFP cassette was excised from pMF365 by *Bam*HI and ligated in sense orientation into the corresponding site (*Bam*HI) of pLentiModule1 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hEF1\alpha}$-EYFP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I) (pBM45) | This work |
| pLentiModule6 | The P$_{hEF1\alpha}$-VEGF$_{121}$ cassette was excised from pBM43 by *Bam*HI and ligated in sense oritentation into the corresponding site (*Bam*HI) of pLentiModule1 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hEF1\alpha}$-VEGF$_{121}$-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I) (pBM46) | This work |
| pLentiModule7 | The P$_{hEF1\alpha}$-EPO cassette was excised from pBM42 by *Bam*HI and ligated in sense orientation into the corresponding site (*Bam*HI) of pLentiModule1 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hEF1\alpha}$-EPO-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I) (pBM47) | This work |
| pLentiModule8 | The P$_{hEF1\alpha}$-SEAP cassette was excised from pBM44 by *Bam*HI and ligated in sense orientation into the corresponding site (*Bam*HI) of pLentiModule1 (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hEF1\alpha}$-SEAP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I) (pBM48) | This work |

## *In vivo* transduction of chicken embryos

Experiments on chicken embryos were conducted following the shell-free cultivation protocols by Djonov and co-workers (30). After 3 days of incubation at 37°C, Brown Leghorn eggs were opened, and their contents were carefully poured into plastic Petri dishes, 80 mm in diameter. The chicken embryos were incubated at 37°C in a humidified atmosphere. Recombinant lentiviruses were applied locally on the top of the growing chorioallontoic membrane (CAM) or injected intravenously at embryonic day 9. On embryonic day 11, the CAMs were examined by *in vivo* fluorescence microscopy following intravenous (i.v.) injection of 0.1 ml 2.5 fluoresceine isothiocyanate dextran (2 000 000; Sigma, St Louis,




MO) (31). The developing blood vessels were monitored using a Polyvar-Reichert fluorescent microscope at a magnification of 25×. The microscope was equipped with a custom-built heating table to maintain the temperature of the specimens at 37°C. Blood circulation and microvascular patterns were monitored for ~15 min for every CAM using an LE CCD Optronics video camera (Visitron system, Puchheim, Germany) and a digital video recorder (Sony, DHR-1000 VC).

### Construction of lentiviral expression vectors

*Lentiviral expression vectors (Table 1) containing extended multiple cloning sites*. The basic multi-purpose lentiviral expression vector pMF356 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-3′$LTR_{\Delta U3}$) is derived from pNL-EGFPΔU3, a pNL series lentiviral vector containing an *Eco*RV/*Pvu*II deletion in the U3 region (corresponding to 8666–9066 in Genbank accession no. AF033819) of the 3′LTR [3′$LTR_{\Delta U3}$ (25,32)].

pMF356 was constructed by a two-step procedure as follows. (i) The $P_{hCMV}$-EYFP cassette ($P_{hCMV}$, promoter of the human cytomegalovirus; EYFP) was amplified from pEYFP-C1 (Clontech, Palo Alto, CA) using oligonucleotides OMF179: GATCGTTAACTCTAGAGGCGCGCCCGGGCGAATTCGTCGACCCGCGGCCTGCAGGCCGGCCATCGATCGCGAGCGGCCGCtagttattaatagtaatc [annealing

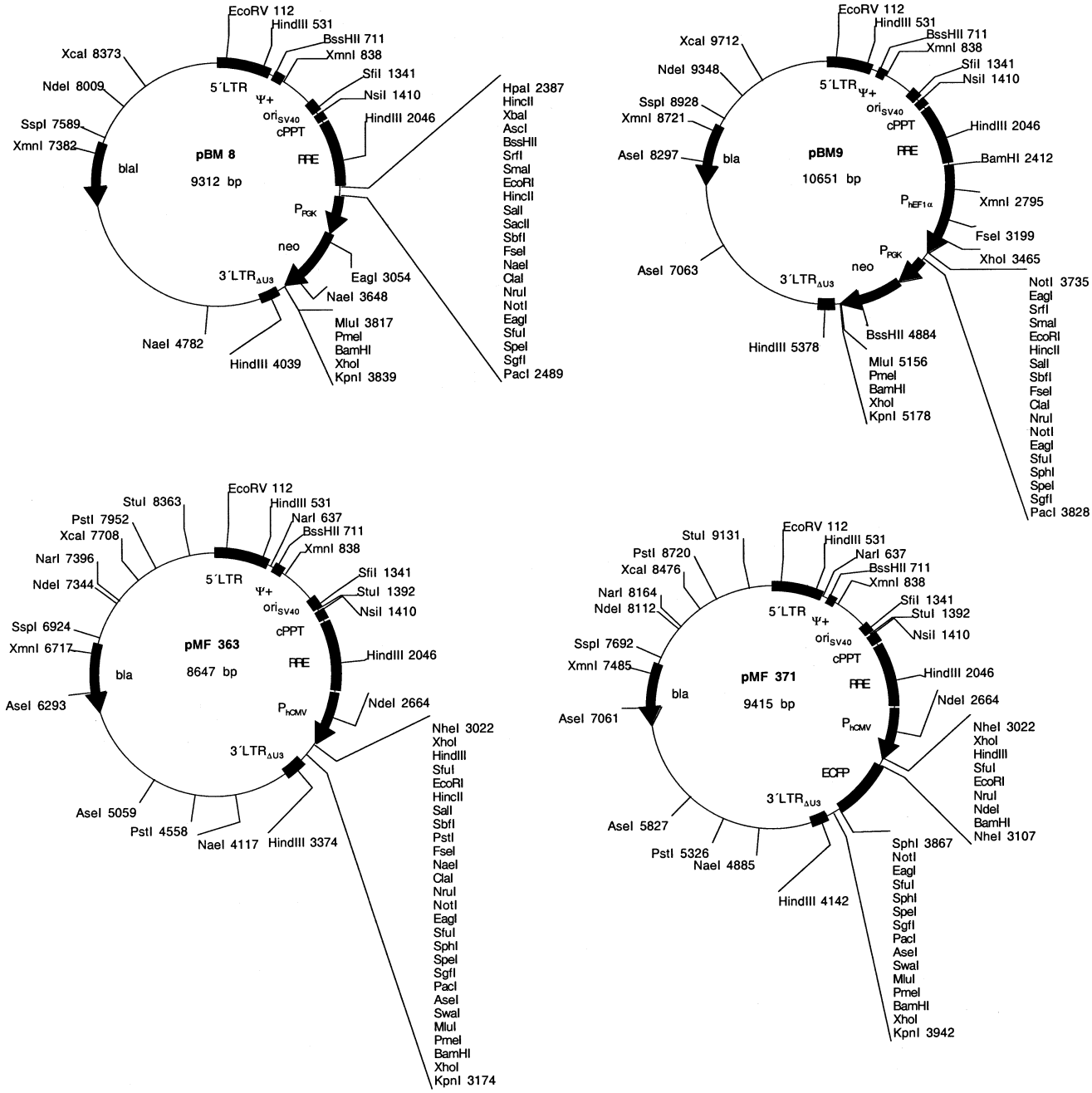



**Figure 1.** (Previous page and above) SIN lentivectors containing optimized MCS. Generic HIV-1-derived lentivectors containing the 5′LTR, the extended packaging signal (ψ+), the Simian virus 40 origin of replication ($ori_{SV40}$), the cPPT, the RRE and the 3′LTR with deleted enhancer sequences ($3'LTR_{\Delta U3}$). The MCS was placed between RRE and $3'LTR_{\Delta U3}$. Besides this basic lentivector configuration a variety of derivatives containing different mammalian/human promoters ($P_{PGK}$, phosphoglycerate kinase promoter; $P_{hEF1\alpha}$, human elongation factor 1α promoter; $P_{hCMV}$, human immediate early cytomegalovirus promoter) and reporter (EYFP and ECFP) or resistance genes (*neo*, G418 resistance gene) have been designed.

sequence in lower case, restriction sites *Hpa*I/*Hinc*II/*Xba*I/*Asc*I/*Srf*I/*Sma*I/*Eco*RI/*Sal*I/*Sac*II/*Sbf*I/*Fse*I/*Cla*I ($G^{m}$ATC sensitive) /*Nru*I/*Not*I are underlined] and OMF180: GATC-GGTACCTCGAGGATCCGTTTAAACGCGTATTTAAA-TTAATTAGCGATCGCACTAGTGCATGCTTCGAAGC-GGCCGCttacttgtacagctcgtc (annealing sequence in lower case, restriction sites *Kpn*I/*Xho*I/*Bam*HI/*Pme*I/*Mlu*I/*Swa*I/*Pac*I/*Sgf*I/*Spe*I/*Sph*I/*Sfu*I/*Not*I are underlined) and ligated as *Hpa*I/*Kpn*I fragment into pNL-EGFPΔU3 resulting in pMF351 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCSI-$P_{hCMV}$-EYFP-MCSII-$3'LTR_{\Delta U3}$). (ii) The $P_{hCMV}$-EYFP cassette was eliminated from pMF351 by *Not*I restriction and religation, which resulted in pMF356 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-$3'LTR_{\Delta U3}$).

pMF356 was further digested with *Pac*I/*Mlu*I and the *Pac*I/*Mlu*I $P_{PGK}$-neo ($P_{PGK}$, promoter of the phosphoglycerate

kinase; neo, G418 resistance gene) cassette of pBM6 was inserted (pBM8; 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$). pBM6 was constructed by amplifying P$_{PGK}$-neo from pMSCVneo (Clontech) using oligonucleotides OBM1: CGACTAGTTTAATTAAaattctaccgggtagggg (annealing sequence in lower case, *Spe*I/*Pac*I sites are underlined) and OBM2: CGGGTACCACGCGTcctcagaagaactcgtcaag (annealing sequence in lower case, *Kpn*I/*Mlu*I sites are underlined) and cloned in sense orientation into pcDNA3.1/V5-His TOPO (Invitrogen, Carlsbad, CA).

pMF356 derivatives containing either P$_{hEF1\alpha}$ (P$_{hEF1\alpha}$, promoter of the human elongation factor 1α; pMF359) or P$_{hCMV}$ (pMF363) promoters were generated as follows.

pMF359 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-MCS-3′LTR$_{\Delta U3}$). (i) P$_{hEF1\alpha}$ was amplified from pEF4/MycHisB (Invitrogen) using oligonucleotides OMF164: GATCGGATCCtgcaaagatggataaagt (annealing sequence in lower case, *Bam*HI site is underlined) and OMF165: GATCAAGCTTTTAATTAAGCGATCGCGCCCGGGCGCGGCCGCCaactagccagcttgggtc (annealing sequence in lower case, *Hin*dIII/*Pac*I/*Sgf*I/*Srf*I/*Sma*I/*Not*I sites are underlined) and cloned in antisense orientation into pcDNA3.1/V5-His TOPO (Invitrogen) to result in pMF320. P$_{hEF1\alpha}$ was excised from pMF320 by *Eco*RV/*Xma*I and cloned into the compatible sites (*Hpa*I/*Xma*I) of pMF356 thereby resulting in pMF359 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-MCS-3′LTR$_{\Delta U3}$). (ii) EYFP was excised from pMF201 (33) by *Eco*RV/*Spe*I and cloned into the compatible sites (*Sma*I/*Spe*I) of pMF359 to result in pMF365 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-EYFP-3′LTR$_{\Delta U3}$). (iii) pMF359 was restricted using *Pac*I/*Mlu*I and the P$_{PGK}$-neo cassette excised from pBM6 by *Pac*I/*Mlu*I was inserted (pBM9; 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$). pMF359 derivatives include: (i) pBM42: pMF359 was restricted with *Eco*RI/*Pme*I and EPO (erythropoietin) excised from pMF242 (33) by *Eco*RI/*Eco*RV was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-EPO-3′LTR$_{\Delta U3}$). (ii) pBM43: pMF359 was restricted with *Sal*I/*Mlu*I and VEGF$_{121}$ (variant 121 of the vascular endothelial growth factor) excised from pWW265 (C.Weber and A.Zisch, unpublished data) by *Sal*I/*Mlu*I was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-VEGF$_{121}$-3′LTR$_{\Delta U3}$). (iii) pBM44: pMF359 was restricted with *Eco*RI/*Pme*I and SEAP (human placental secreted alkaline phosphatase) excised from pSS173 (34) by *Eco*RI/*Eco*RV was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-SEAP-3′LTR$_{\Delta U3}$).

pMF363 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-MCS-3′LTR$_{\Delta U3}$): (i) P$_{hCMV}$ was excised from pLPCX (Clontech) with *Stu*I/*Eco*RI and ligated to the compatible *Hpa*I/*Eco*RI sites of pMF356 to result in pMF363 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-MCS-3′LTR$_{\Delta U3}$). (ii) ECFP was excised from pMF201 (33) by *Eco*RI/*Not*I and ligated to the corresponding sites (*Eco*RI/*Not*I) of pMF363 thereby resulting in pMF371 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-3′LTR$_{\Delta U3}$).

*Tricistronic pLentiTRIDENT expression vectors.* pLentiTRIDENT vectors contain a pTRIDENT-derived tricistronic expression cassette inserted in the MCS of pMF363. pLentiTRIDENT1 (pMF370; 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-MCSI-IRESI-MCSII-IRESII-MCSIII-3′LTR$_{\Delta U3}$) was constructed by excising the MCSI-IRESI-MCSII-IRESII-MCSIII cassette from pTRIDENT1 [pMF125 (35)] by *Eco*RI/*Bgl*II and cloning it into the compatible sites (*Eco*RI/*Bam*HI) of pMF363. pLentiTRIDENT1 was further restricted using *Eco*RI/*Mlu*I and the triple-fluorescent expression cassette (ECFP-IRESI-RFP-IRESII-EYFP) excised from pWW85 (36) by *Eco*RI/*Mlu*I was inserted [pLentiTFT1 (pBM2); 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-IRESI-RFP-IRESII-EYFP-3′LTR$_{\Delta U3}$]. pLentiTRIDENT2 (pMF369; 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-MCSI-IRES-MCSII-CITE*-MCSIII-3′LTR$_{\Delta U3}$) was constructed by excising the MCSI-IRES-MCSII-CITE*-MCSIII cassette from pTRIDENT3 [pMF122 (35)] by *Eco*RI/*Bgl*II and cloning it into the compatible sites (*Eco*RI/*Bam*HI) of pMF363. pLentiTRIDENT2 was further restricted using *Eco*RI/*Asc*I and the ECFP-IRESI-RFP cassette excised from pWW85 (36) by *Eco*RI/*Asc*I was inserted to result in pBM1 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-IRES-RFP-CITE*-MCSIII-3′LTR$_{\Delta U3}$). Subsequently, pBM1 was digested using *Spe*I/*Mlu*I and the EYFP cassette excised from pWW85 by *Spe*I/*Mlu*I was inserted [pLentiTFT2 (pBM3); 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-ECFP-IRES-RFP-CITE*-EYFP-3′LTR$_{\Delta U3}$].

*Highly compact lentiviral expression vectors.* pNL-derived lentiviral expression vectors contain superfluous chromosomal sequences flanking the lentiviral expression unit (32). In order to eliminate these chromosomal sequences, the 5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-MCS-3′LTR$_{\Delta U3}$ cassette was amplified from pMF356 using oligonucleotides OMF199: GATCGACGTCacttacaccaggaaaggc (annealing sequence in lower case, *Aat*II site is underlined) and OMF200: GATCGCTAGCacctctacctcctggggg (annealing sequence in lower case, *Nhe*I site is underlined) and cloned as *Aat*II/*Nhe*I fragment to the compatible, *Aat*II/*Xba*I-restricted pTRIDENT1 [pMF125 (35)] backbone thereby resulting in pMF388 (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-MCS-3′LTR$_{\Delta U3}$). pMF388 derivatives include: (i) pMF394: pMF388 was restricted with *Hpa*I/*Kpn*I and the P$_{hCMV}$-EYFP cassette excised from pMF351 by *Hpa*I/*Kpn*I was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-EYFP-3′LTR$_{\Delta U3}$). (ii) pBM7: P$_{hEF1\alpha}$ was excised from pMF320 by *Eco*RV/*Xma*I and cloned into the compatible *Hpa*I/*Xma*I sites of pMF388 (5′LTR-ψ+-oriSV40-cPPT-RRE-P$_{EF1\alpha}$-MCS-3′LTR$_{\Delta U3}$). (iii) pBM13: pMF388 was restricted with *Pac*I/*Mlu*I and the *Pac*I/*Mlu*I P$_{PGK}$-neo cassette excised from pBM6 was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$). (iv) pBM14: pBM7 was restricted with *Pac*I/*Mlu*I and the *Pac*I/*Mlu*I P$_{PGK}$-neo cassette excised from pBM6 was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{EF1\alpha}$-MCSI-P$_{PGK}$-neo-MCSII-3′LTR$_{\Delta U3}$). (v) pBM40: pBM7 was restricted with *Spe*I/*Mlu*I and EYFP excised from pBM12 by *Nhe*I/*Mlu*I was inserted (5′LTR-ψ+-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-EYFP-3′LTR$_{\Delta U3}$).

*pLentiModule, a modular lentiviral expression vector (all sites indicated correspond to GenBank accession no. AF033819).* pLentiModule consists of four minimal lentiviral modules [U3, R-U5-ψ+-SD, cPPT-RRE-SA, PPT-3′LTR$_{\Delta U3}$ (ΔU3-R-U5)] containing unique restriction sites at their joints. These modules were independently amplified from pNL-EGFPΔU3

and cloned into pcDNA3.1/V5-His TOPO (Invitrogen). (i) pMF352: U3 (8631–9085) was amplified using oligonucleotides OMF183, GATCCTCGAGTCGCGActggaagggctaatttgg (annealing sequence lower case, *Xho*I/*Nru*I sites are underlined); and OMF184, GATCGCCCGGGCCAGTACAGGCAAAAAGCAGCTGCTTATATGTAGCATCTGAGGGCTCGCCACTCCCCAGTCCCGCCCAGGCCACACCTCCCTGCAGGcctggaaagtccccagcg (annealing sequence lower case, *Srf*I and *Sbf*I are underlined; this primer inserts a *Sbf*I at position 9007 of U3) (*Xho*I/*Nru*I-U3$_1$-*Sbf*I-U3$_2$-*Srf*I). (ii) pMF376: R-U5-ψ+-SD (1–793) was amplified from pNL-EGFPΔU3 using oligonucleotides OMF185, GATCGCCCGGGCggtctctctggttagacc (annealing sequence lower case, *Srf*I site is underlined); and OMF193, GATCATTTAAATtttaaagttctaggtgat (annealing sequence lower case, *Swa*I site is underlined) (*Srf*I-R-U5-ψ+-SD-*Swa*I). pMF353: a shorter R-U5-ψ+-SD module (1–377) was amplified using oligos OMF185 and OMF186: GATCATTTAAATtcattaatctaattctcc (annealing sequence lower case, *Swa*I site is underlined). (iii) pMF375: cPPT-RRE-SA [4330–4475 (cPPT), 7200–8024 (RRE)] was amplified using oligonucleotides OMF191, GATCATTTAAATatgcataaaagaaaaggg (annealing sequence lower case, *Swa*I site is underlined); and OMF192, CTAGGATCACGCGTTTAATTAAGCGATCGCACTAGTATCGATGGCGCGCCGGCCGGCCAGGCCTGCGGCCGCGAATTCGACGTCatccgttcactaatcgaa (annealing sequence lower case, *Mlu*I/*Pac*I/*Sgf*I/*Spe*I/*Cla*I/*Asc*I/*Fse*I/*Stu*I/*Not*I/*Eco*RI/*Aat*II are underlined) (*Swa*I-cPPT-RRE-SA-*Mlu*I/*Pac*I/*Sgf*I/*Spe*I/*Cla*I/*Asc*I/*Fse*I/*Stu*I/*Not*I/*Eco*RI/*Aat*II). (iv) pMF355: PPT-3′LTR$_{\Delta U3}$ (ΔU3-R-U5) (8615–9181 and 1–181) was amplified using oligonucleotides OMF189, GATCACGCGTGGATCC**AAAAGAAAAGGGGGGA**CTGGAAGGGCTAATTCACTCCCAAAGAAGACAA*GATG*TTTAAACGTTAACTGATCA*ctg*cttttgcctgtact [annealing sequence lower case, *Mlu*I/*Bam*HI is underlined, PPT is shown in bold; *Pme*I/*Hpa*I/*Bcl*I are underlined and placed between the *Eco*RV/*Pvu*II (GAT … CTG, italics) ligation sites flanking the ΔU3 deletion (corresponding to 8666–9066) of the 3′LTR (25,32)]; and OMF190, GATCTCTAGAGTCGACGCTAGCCATATGctgctagagattttccaca (annealing sequence lower case, *Xba*I/*Sal*I/*Nhe*I/*Nde*I are underlined) (*Mlu*I/*Bam*HI-PPT-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-R-U5-*Nde*I/*Nhe*I/*Sal*I/*Xba*I).

pLentiModule was assembled on a pTRIDENT1 backbone following a four-step procedure. (i) U3 was excised from pMF352 using *Xho*I/*Srf*I and ligated to the corresponding sites (*Xho*I/*Srf*I) of pTRIDENT1 to result in pMF357. (ii) R-U5-ψ+-SD was excised from pMF353 and pMF376 by *Srf*I/*Swa*I and ligated into the corresponding sites (*Srf*I/*Swa*I) of pMF357 to result in pMF358 and pMF358x. (iii) cPPT-RRE-SA was excised from pMF375 using *Swa*I/*Mlu*I and ligated into the corresponding sites (*Swa*I/*Mlu*I) of pMF358 and pMF358x to result in pMF360 and pMF360x. (iv) PPT-3′LTR$_{\Delta U3}$ was excised from pMF355 by *Mlu*I/*Xba*I and ligated into the corresponding sites (*Mlu*I/*Xba*I) of pMF360 and pMF360x to result in pMF402 and pLentiModule1 (pBM4; *Xho*I/*Nru*I-U3$_1$-*Stu*I / *Sbf*I-U3$_2$-*Srf*I-R-U5-ψ + -SD-*Swa*I-cPPT-RRE-SA-*Aat*II/*Eco*RI/*Not*I/*Eag*I/*Stu*I/*Fse*I/*Eag*I/*Asc*I/*Bss*HII/*Cla*I/*Spe*I/*Sgf*I/*Pac*I/*Mlu*I/*Bam*HI-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I). plentiModule1 derivatives include the following. (i) pLentiModule2: pLentiModule1 was cut with *Eco*RI/*Mlu*I

**Table 2.** EPO, VEGF$_{121}$ and SEAP production in CHO-K1 cells following lentiviral transduction

| Lentivector | Production (ng/ml) | | |
|---|---|---|---|
| | EPO | VEGF$_{121}$ | SEAP |
| pBM42 | 65.4 ± 9.2 | | |
| pBM43 | | 20.0 ± 0.5 | |
| pBM44 | | | $2.1 \times 10^3 \pm 80.6$ |
| pLentiModule7 | 37.1 ± 5.5 | | |
| pLentiModule6 | | 11.3 ± 1.1 | |
| pLentiModule8 | | | $1.1 \times 10^3 \pm 23.6$ |

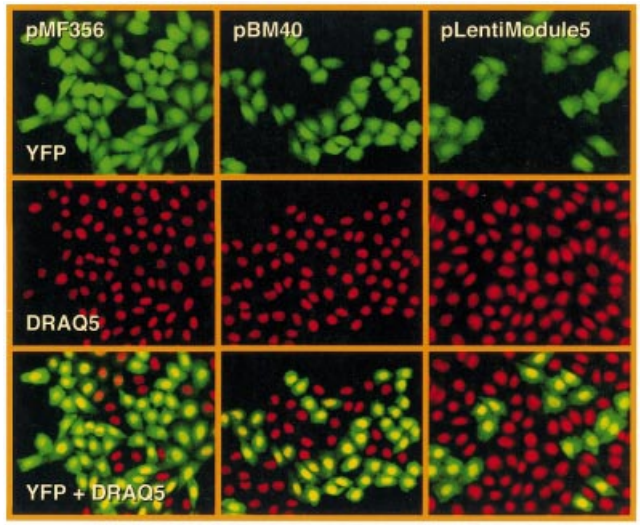
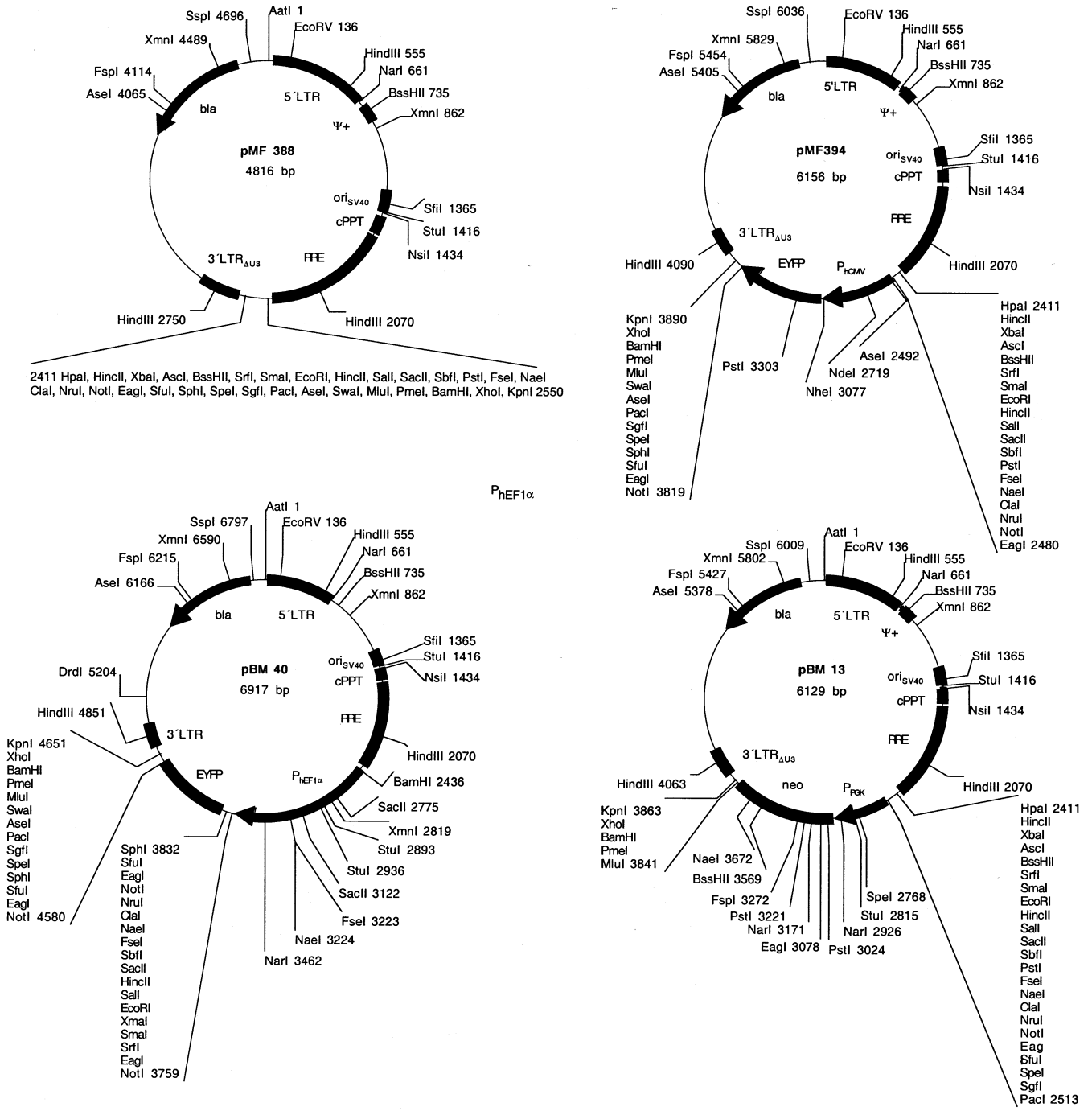



**Figure 2.** Confocal microscopy analysis of transduction efficiencies of key lentivectors. CHO-K1 were transduced with $2 \times 10^6$ EYFP-encoding pMF365-, pBM40- and pLentiModule-derived lentiviruses. Transduced cells were stained with the DNA-specific DRAQ5 dye and analyzed for YFP- and DRAQ5-mediated fluorescence by confocal microscopy (magnification 200×).

**Table 3.** Titer and p24 production of various lentivectors

| Lentivector | Titer (c.f.u./ml)[a] | p24 (ng/ml) |
|---|---|---|
| pNL-EGFP[b] | $2 \times 10^7$ | 95.0 ± 5.3 |
| pNL-EGFPΔU3 | $4 \times 10^7$ | 165.3 ± 5.1 |
| pMF351 | $2.5 \times 10^7$ | 104.8 ± 6.4 |
| pMF365 | $2 \times 10^7$ | 97.3 ± 8.5 |
| pBM2 | ND | 158.7 ± 7.3 |
| pBM40 | $2 \times 10^7$ | 97.3 ± 9.6 |
| pBM42 | ND | 66.1 ± 8.3 |
| pBM43 | ND | 70.6 ± 7.8 |
| pBM44 | ND | 63.5 ± 8.0 |
| pLentiModule1 | $2.5 \times 10^7$ | 110 ± 7.2 |
| pLentiModule2 | $2.5 \times 10^7$ | 110 ± 7.3 |
| pLentiModule4 | $2 \times 10^7$ | 97.2 ± 12.3 |
| pLentiModule5 | $1.2 \times 10^7$ | 48.6 ± 5.4 |
| pLentiModule6 | ND | 64.9 ± 6.7 |
| pLentiModule7 | ND | 58.8 ± 8.6 |
| pLentiModule8 | ND | 72.3 ± 8.1 |

ND, not determined.
[a]Determined as described in Reiser *et al*. (17).
[b]Determined on NIH/3T3 cells, see Reiser *et al*. (17).




and the $P_{hCMV}$-EYFP cassette excised from pMF351 by *Eco*RI/*Mlu*I was inserted to result in pLentiModule2 (pBM5) (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-$P_{hCMV}$-EYFP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I). (ii) pLentiModule5: pLentiModule1 was cut with *Bam*HI and the $P_{hEF1\alpha}$-EYFP-encoding *Bam*HI cassette of pMF365 was inserted (pBM45) (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-$P_{hEF1\alpha}$-EYFP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I). (iii) pLentiModule6: pLentiModule1 was cut with *Bam*HI and the $P_{hEF1\alpha}$-VEGF$_{121}$-encoding *Bam*HI cassette of pBM43 was inserted (pBM46) (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-$P_{hEF1\alpha}$-VEGF$_{121}$-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I). (iv) pLentiModule7: pLentiModule1 was cut with *Bam*HI and the $P_{hEF1\alpha}$-EPO-encoding *Bam*HI cassette of pBM42 was inserted (pBM47) (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-$P_{hEF1\alpha}$-EPO-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I). (v) pLentiModule8: pLentiModule1 was cut with *Bam*HI and the $P_{hEF1\alpha}$-SEAP-

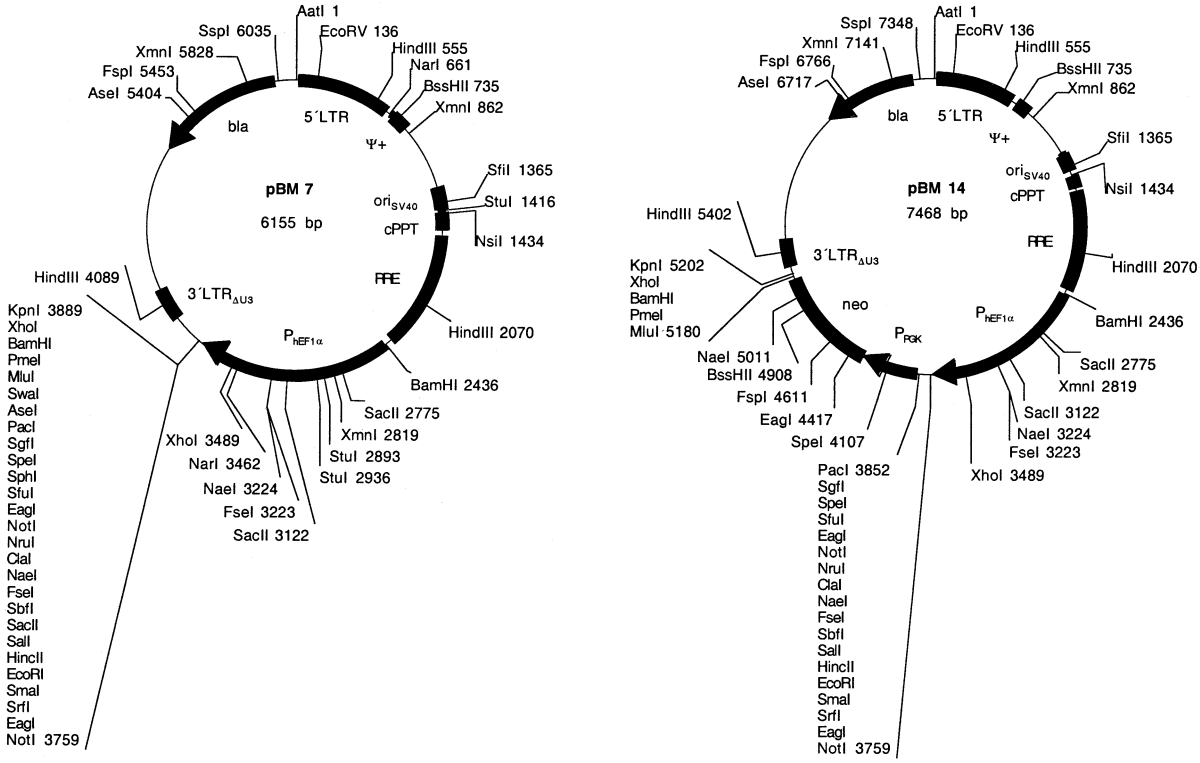



**Figure 3.** (Previous page and above) Size-optimized SIN lentivectors. These lentivectors are derivatives of the ones shown in Figure 1 and are devoid of any chromosomal sequences flanking the lentiviral expression vectors.

encoding *Bam*HI cassette of pBM44 was inserted (pBM48) *Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hEF1\alpha}$-SEAP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I).

In order to place the ori$_{SV40}$ on pLentiModule1, this plasmid was restricted with *Xba*I and the ori$_{SV40}$ *Xba*I/*Nhe*I fragment excised from pMF378 was inserted to result in pLentiModule3 (pBM15) (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-MCS-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I-ori$_{SV40}$). pMF378 was constructed by amplifying ori$_{SV40}$ from pNL-EGFPΔU3 using oligonucleotides OMF195: GATCTCTAGAtatgactccgcccatccc (annealing sequence lower case, *Xba*I site is underlined) and OMF196: GATCGCTAGCttgcaaaagcctaggcc (annealing sequence lower case, *Nhe*I sequence is underlined) and ligating it into pcDNA3.1/V5-His TOPO. Furthermore, pLentiModule2 was restricted with *Xba*I and the ori$_{SV40}$ *Xba*I/*Nhe*I fragment excised from pMF378 was inserted to result in pLentiModule4 (pBM12) (*Xho*I/*Nru*I-U3$_1$-*Stu*I/*Sbf*I-U3$_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I-cPPT-RRE-SA-P$_{hCMV}$-EYFP-ΔU3$_1$-*Pme*I/*Hpa*I/*Bcl*I-ΔU3$_2$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I-ori$_{SV40}$).

### Lentivirus production and infection

For production of replication-incompetent SIN lentiviruses, a mixture containing 94 µl DMEM, 6 µl FUGENE (Roche Diagnostics AG, Rotkreuz, Switzerland), 25 µM chloroquine, 1 µg pLTR-G [encoding the pseudotyping envelope protein VSV-G of the vesicular stomatitis virus (17)], 1 µg of the helper construct pCD/NL-BH* (32) and 1 µl of the desired transgene-encoding lentiviral expression vector were transfected into human embryonic kidney cells (HEK293-T, kindly provided by Andreas Zisch). The medium was replaced after 12–15 h and virus particles were produced for another 48 h. Viral particles were collected from the HEK293-T supernatant by filtration through a 0.45 µm filter (Schleicher & Schuell GmbH, Dassel, Germany: FP 030/2) yielding typical titers of $2 \times 10^7$ c.f.u./ml when titrated on CHO-K1 cells. p24 assays were performed using a commercial kit (NEK-050, Perkin Elmer, Boston, MA) [pMF351, 104.8 ± 6.4 ng/ml p24; pLentiModule1, 109.9 ± 7.25 ng/ml p24 (triplicate mean values)]. 60 000 target cells per 6-well were infected with 200 µl viral supernatant. Hippocampal slice cultures were infected as described by Ehrengruber and co-workers (37).

## RESULTS

### Design of multi-purpose lentiviral expression vectors containing extensive multiple cloning sites

Most lentiviral expression vectors currently available from the research groups, which have pioneered lentiviral transduction technologies are lacking extensive MCS rendering the straightforward design of HIV-1-based expression vectors complicated (16). We have constructed a versatile lentiviral expression vector family derived from the HIV-1-based SIN pNL-EGFPΔU3 (J.Reiser, unpublished data; 38) which contains up to 29 unique sites for restriction endonucleases, many of which are rare-cutting 8 bp-recognizing enzymes. pNL-EGFPΔU3 has a classical lentiviral expression vector set-up

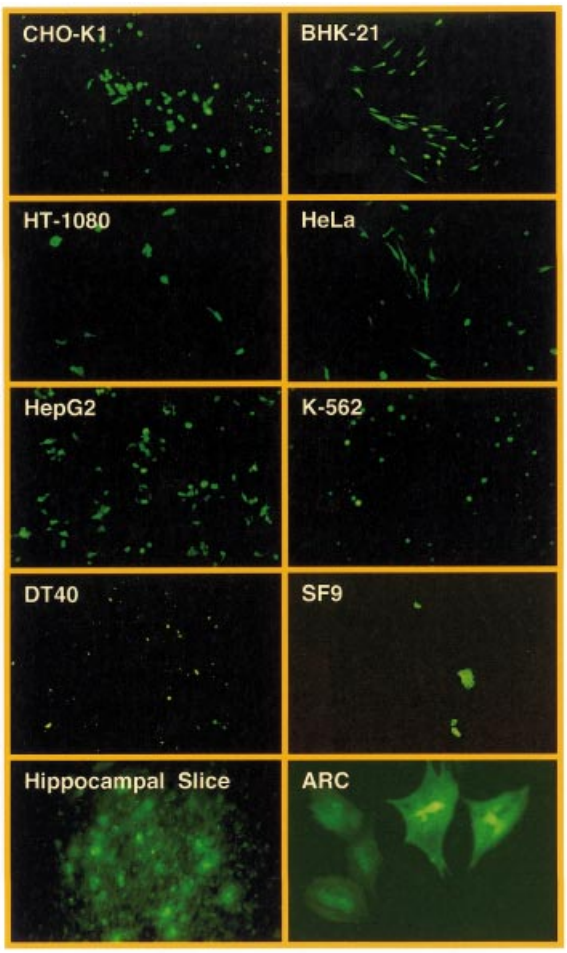



**Figure 4.** Transduction of optimized lentivectors into various insect, chicken, murine and human cell types as exemplified by VSV-G-pseudotyped pMF351-derived lentiviruses (Fig. 1). The gene encoding the EYFP was successfully transduced into Chinese hamster ovary cells (CHO-K1), baby hamster kidney cells (BHK-21), human fibrosarcoma cells (HT-1080), human cervial adenocarcinoma cells (HeLa), human hepatocellular carcinoma cells (HepG2), human chronic myelogenous leukemia cells (K-562), bursal chicken cells (DT40) as well as insect cells (SF9). pMF351-derived lentiviruses also transduced glial cells in hippocampal slice cultures (hippocampal slice) and primary ARC (magnification 100×, hippocampal slice 50×; ARC 400×).

consisting of 5′LTR, ψ+, splice donor (SD), origin of replication of the simian virus 40 ($ori_{SV40}$), central polypurine tract (cPPT), RRE, splice acceptor (SA) and a $3'LTR_{\Delta U3}$. The $P_{hCMV}$-EGFP expression cassette is inserted between the SA and the $3'LTR_{\Delta U3}$ using the unique *Hpa*I and *Kpn*I sites (5′LTR-ψ+-SD-$ori_{SV40}$-cPPT-RRE-SA-*Hpa*I-$P_{hCMV}$-EGFP-*Kpn*I-$3'LTR_{\Delta U3}$). The $ori_{SV40}$ is boosting the vector copy number in transiently transfected cells producing SV40 large T antigen, such as HEK293-T cells (17). A 400 bp *Eco*RV/*Pvu*II deletion within the 3′LTR ($3'LTR_{\Delta U3}$) inactivates the 5′LTR promoter upon reverse transcription and integration when the ΔU3 deletion is transferred to the 5′LTR in a self-sufficient manner, a process also referred to as SIN (25,26).

In order to extend the MCS of pNL-EGFPΔU3, a PCR-amplified $P_{hCMV}$-EYFP cassette flanked by 26 unique restriction sites was cloned into the *Hpa*I/*Kpn*I sites of pNL-EGFPΔU3 and resulted in pMF351 (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCSI-$P_{hCMV}$-EYFP-MCSII-$3'LTR_{\Delta U3}$; Fig. 1, Table 1). The $P_{hCMV}$-EYFP was subsequently excised by a *Not*I deletion resulting in pMF356, a lentiviral expression vector containing 28 unique restriction sites (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-$3'LTR_{\Delta U3}$; Fig. 1, Table 1). As non-exhaustive examples for the construction kit-like lentiviral expression vector platform a variety of pMF356 derivatives have been designed which contain: (i) a $P_{PGK}$-driven neomycin resistance gene (*neo*) (pBM8; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCSI-$P_{PGK}$-*neo*-MCSII-$3'LTR_{\Delta U3}$), (ii) a strong constitutive human elongation factor 1α promoter ($P_{hEF1\alpha}$; pMF359; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-MCS-$3'LTR_{\Delta U3}$), (iii) a $P_{hEF1\alpha}$-driven EYFP cassette (pMF365; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-MCSI-EYFP-MCSII-$3'LTR_{\Delta U3}$), (iv) a $P_{hEF1\alpha}$ promoter plus a $P_{PGK}$-neo cassette (pBM9; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-MCSI-$P_{PGK}$-*neo*-MCSII-$3'LTR_{\Delta U3}$), (v) a human cytomegalovirus immediate early promoter ($P_{hCMV}$; pMF363; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hCMV}$-MCS-$3'LTR_{\Delta U3}$) or (vi) a $P_{hCMV}$-ECFP cassette (pMF371; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hCMV}$-MCSI-ECFP-MCSII-$3'LTR_{\Delta U3}$) (see Fig. 1 and Table 1 for all plasmids).

In order to validate this class of multi-purpose lentivectors, a variety of pMF359 derivatives were constructed which encode EPO (pBM42), the vascular endothelial growth factor ($VEGF_{121}$, pBM43) or the SEAP (pBM44) under control of $P_{hEF1\alpha}$. pBM42-, pBM43- and pBM44-derived lentiviruses were able to drive high-level protein secretion following transduction of CHO-K1 (Table 2). Also, pMF351- and pMF365-based lentivectors displayed similar transduction efficiencies compared with their parental constructs of the pNL-EGFP series (38) (Fig. 2, Table 3).

Although the basic lentiviral expression units contained in these expression vectors (pMF351, pMF356, pBM8, pMF359, pMF365, pBM9, pBM363, pMF371; Fig. 1) are only ~3.5 kb in size, they are flanked by superfluous chromosomal regions that are remnants of the integration sites of the first HIV proviruses cloned from the human chromosome [molecular clone HXB2 (39)]. As these chromosomal sites are not packaged and transferred to the target cells, they are not limiting the cargo size of the lentiviral expression units. However, the entire expression vectors may reach the replicative limit when amplified in *Escherichia coli*.

Elimination of any chromosomal sequences was achieved by PCR-mediated amplification of the lentiviral expression units (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-$3'LTR_{\Delta U3}$). *Aat*II and *Nhe*I sites engineered in the primer extensions enabled ligation to the pTRIDENT-derived minimal high-copy number vector backbone fragment generated by *Aat*II/*Xba*I digestion (35,40). This cloning procedure resulted in pMF388, a 4.8 kb vector containing a lentiviral expression module with 29 unique restriction sites for convenient integration of desired transgenes (Fig. 3, Table 1; pMF388, 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-$3'LTR_{\Delta U3}$). Several exemplary pMF388 derivatives have been designed which contain in addition to the basic lentiviral expression unit: (i) a $P_{hCMV}$-driven EYFP cassette (pMF394; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCSI-$P_{hCMV}$-EYFP-MCSII-$3'LTR_{\Delta U3}$), (ii) a $P_{hEF1\alpha}$ promoter (pBM7; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-MCS-$3'LTR\Delta_{U3}$), (iii) a $P_{hEF1\alpha}$-driven EYFP cassette (pBM40; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-EYFP-

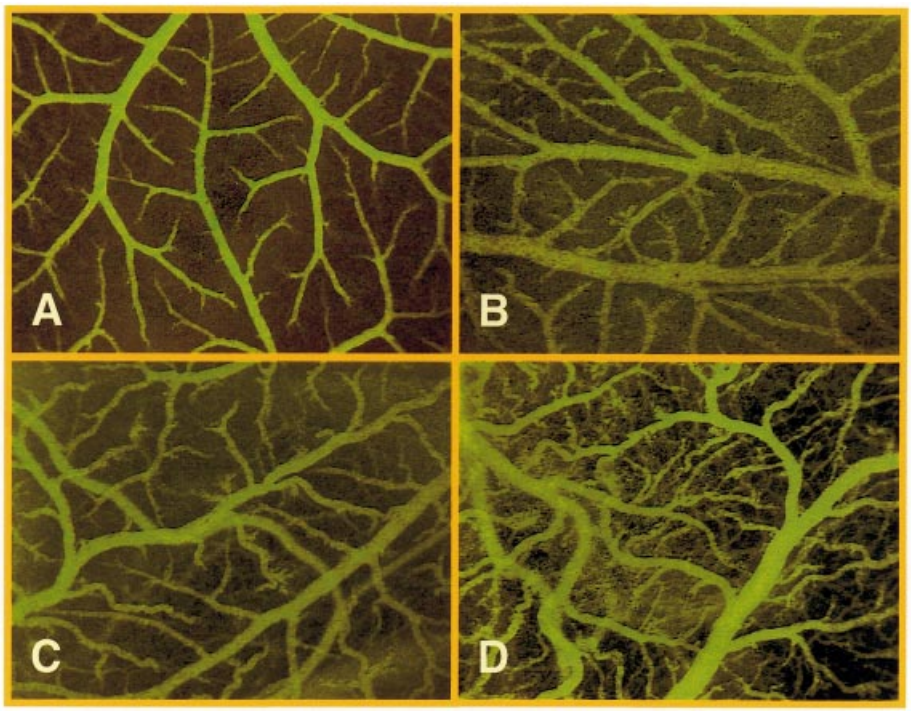



**Figure 5.** *In vivo* examination of microvascular growth in the CAM of 11 day old chicken embryos 48 h after pBM43-based lentiviral $VEGF_{121}$ transduction. Whereas the regular vessel pattern observed on the CAM remained intact in mock transductions (**A**), dose-dependent angiogenic response resulted in increased vascular density and atypical (brush- and delta-like) endpoint patterns following spotting of $0.5 \times 10^6$ c.f.u./ml (producing 3.4 ± 0.3 ng/ml $VEFF_{121}$ in 48 h) (**B**), $3 \times 10^6$ c.f.u./ml (producing 21.2 ± 1.7 ng/ml $VEGF_{121}$ in 48 h) or i.v. injection of $1.5 \times 10^6$ c.f.u./ml (producing 10.6 ± 0.9 ng/ml $VEGF_{121}$ in 48 h) (**C**) (magnification 25×).

3′$LTR_{\Delta U3}$), (iv) a $P_{PGK}$-driven neomycin resistance cassette ($P_{PGK}$-*neo*; pBM13; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCSI-$P_{PGK}$-*neo*-MCSII-3′$LTR_{\Delta U3}$) or (v) a $P_{hEF1\alpha}$ promoter plus a $P_{PGK}$-neo cassette (pBM14; 5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-MCSI-$P_{PGK}$-*neo*-MCSII-3′$LTR_{\Delta U3}$) (see Fig. 3 and Table 1 for all plasmids).

Besides the great flexibility to accommodate almost any desired transgene, the extended MCS of the lentiviral expression vectors shown in Figures 1 and 3 are fully compatible with pQuattro, pTRIDENT, pRetroTRIDENT, pTWIN, pRetroTWIN and pDuoRex type constructs and enable one-step exchange of multicistronic and regulated expression units among these different expression vector families (35,40–42). In addition, these vectors facilitate straightforward swapping of *cis*-acting elements and transgene expression units between each other using: (i) remaining polylinker sites; (ii) unique restriction sites within the (5′) β-lactamase coding region (*bla*; *Ase*I, *Fsp*I, *Xmn*I, *Ssp*I, *Aat*II); and/or (iii) corresponding *cis*-acting elements (*Eco*RV, *Nar*I, *Bss*HII, *Xmn*I, *Sfi*I, *Stu*I, *Nsi*I).

We have tested the construction kit-like lentiviral expression vector platform by infecting various insect (SF9), chicken (DT40), murine (BHK-21, CHO-K1) and human (HeLa, HT-1080, HepG2, K-562) cell lines as well as ARC and hippocampal slices using the pMF351-derived lentivirus (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-MCS-$P_{hCMV}$-EYFP-MCSII-3′$LTR_{\Delta U3}$). All cell lines transduced with pMF351-derived lentiviruses show high-level expression of the EYFP while EYFP expression is absent in mock-transduced control cultures. Even ARCs, which are known to be refractory to a variety of transfection/transduction technologies, were readily transduced by the pMF351-derived lentivirus providing an alternative to the recently described Sindbis-based transduction systems (28). Also, this lentivirus transduced glial cells in rat hippocampal slices (Fig. 4). In CHO-K1 cells the transduction efficiency of pBM4-derived lentiviruses was similar compared with the parental pNL-EGFP and pMF351 vector series (38) (see above and Fig. 2, Table 3).

The biological potential of lentiviral transduction was further exemplified by expression of VEGF in chicken embryos. Infection of the chick embryos' CAM with increasing concentrations of pBM43-derived lentiviruses (5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hEF1\alpha}$-$VEGF_{121}$-3′$LTR_{\Delta U3}$) resulted in a dose-dependent $VEGF_{121}$-mediated local induction of new blood vessels demonstrated by increased vascular density, enhanced formation of numerous arterioles and venules as well as by a boost in vessel endpoint and branching density (Fig. 5). When overdosing $VEGF_{121}$ ($5 \times 10^6$ c.f.u./ml producing 35.5 ± 3.8 ng/ml $VEGF_{121}$ in 48 h; not shown) or following i.v. application of pBM43-derived lentivirus ($1.5 \times 10^6$ c.f.u./ml producing 10.6 ± 0.9 ng/ml $VEGF_{121}$ in 48 h), the hierarchical, tree-like structure of the supplying vessels disappears and the multitude of arterioles and venules adopt an irregular tortuous shape associated with atypical delta- or brush-like vessel endpoints (Fig. 5D).

## Construction of pLentiTRIDENT vectors for tricistronic expression of up to three transgenes

Multicistronic expression technologies for coordinated expression of several transgenes from a single artificial eukaryotic operon have generated unprecedented impact in

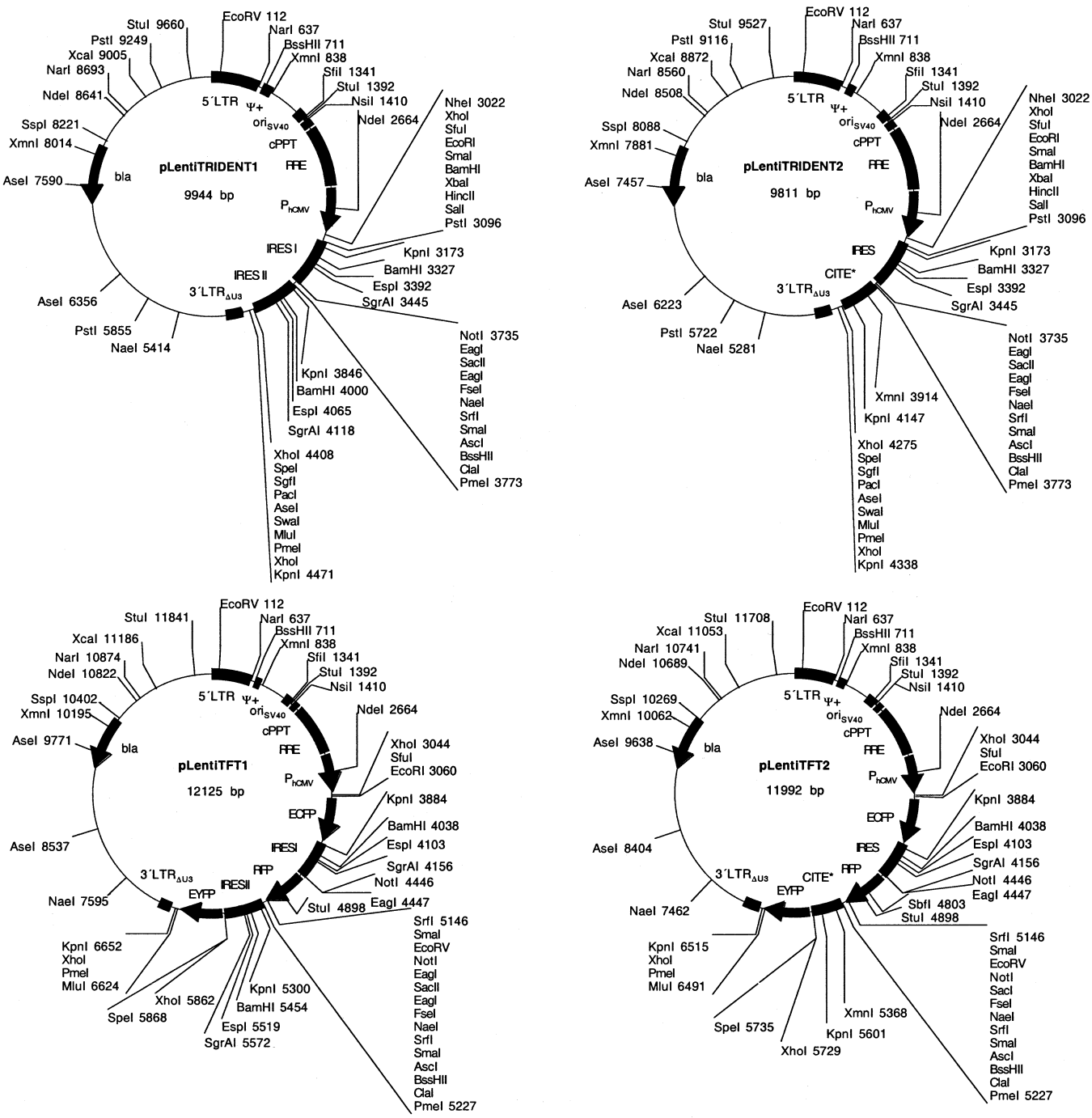



**Figure 6.** pLentiTRIDENT, tricistronic lentiviral expression vectors for simultaneous expression of up to three transgenes. Besides the basic lentiviral *cis*-acting elements (see Fig. 1) pLentiTRIDENT contain a tricistonic expression unit consisting of two IRES or encephalomyocarditis viral origin (CITE*) flanked by extensive MCS for integration of desired transgenes, for example the ones encoding the cyan (ECFP), red (RFP) and yellow (EYFP) fluorescent genes (pLentiTFT1 and pLentiTFT2).

rational reprogramming of mammalian production cell lines and anti-inflammation engineering (43,44). Particularly in a therapeutic setting when the therapeutic proteins consist of multiple subunits or multiple target/strategy approaches involving ribozymes, antisense RNA, transdominant proteins and intracellular antibodies, multicistronic expression technologies are highly desirable (45). We have recently pioneered a multicistronic expression vector family, pTRIDENT, which enables tricistronic expression of up to three transgenes from a single constitutive or regulatable promoter. Whereas the first cistron is translated in a classical cap-dependent manner, translation initiation of the second and third cistrons rely on viral cap-independent internal ribosome entry sites of poliovirus (IRES) or encephalomyocarditis virus

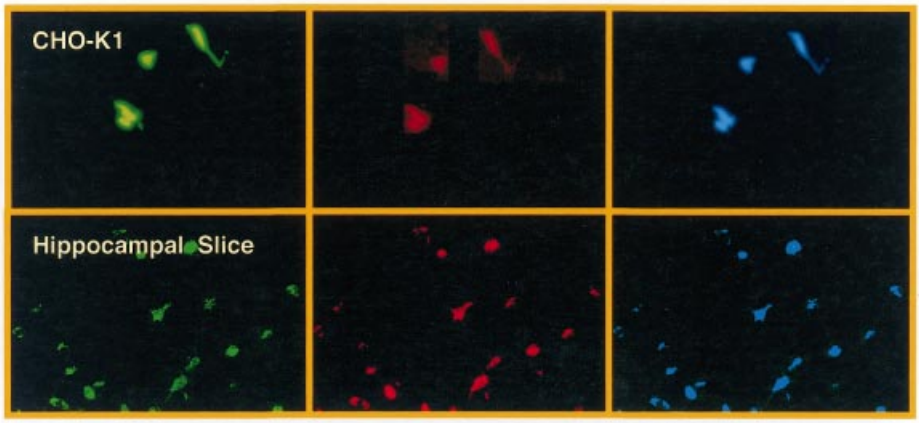



**Figure 7.** Transduction of Chinese hamster ovary (CHO-K1) cell and glial cells in rat hippocampal slice cultures using pLentiTRIDENT1-derived lentiviruses. Simultanous expression of the genes encoding the cyan, red and yellow fluorescent proteins could be observed (magnification 200×).

(CITE) which had been optimized for maximum translation enhancement (CITE*) (35,40,42).

In order to merge multicistronic expression and lentiviral transduction technology, we have designed the pLentiTRIDENT vector series, which contains a $P_{hCMV}$-driven tricistronic eukaryotic operon within a pMF356-derived lentiviral expression cassette (Fig. 6, Table 1). pLentiTRIDENT1/(pLentiTRIDENT2) was constructed by excising the MCSI-IRESI-MCSII-IRESII/(CITE*)-MCSIII cassette of pTRIDENT1/(pTRIDENT3) by *Eco*RI/*Bgl*II and ligating it into the lentiviral transduction unit of pMF363 (*Eco*RI/*Bam*HI) [5′LTR-ψ+-$ori_{SV40}$-cPPT-RRE-$P_{hCMV}$-MCSI-IRESI-MCSII-IRESII/(CITE*)-MCSIII-3′$LTR_{\Delta U3}$]. The pLentiTRIDENT vectors were validated by inserting the genes encoding the (enhanced) cyan, red and yellow fluorescent proteins into the first, second and third cistrons, respectively, which resulted in the triple-fluorescent pLentiTRIDENT vectors (pLentiTFT1/(pLentiTFT2). CHO-K1 cells and glial cells in hippocampal slices transduced with pLentiTFT vectors showed simultaneous high-level expression of all three fluorescent marker proteins (Fig. 7).

## pLentiModule, an advanced highly modular lentiviral expression system

Previous lentiviral expression vectors have been designed for optimal integration of desired transgenes by engineering of extended MCS. However, lentiviral transduction units have been successfully modified in the past years to optimize specific *cis*-acting modules for: (i) Tat-independent production of lentiviruses by replacement of the 5′LTR enhancer region (19,23,26,46); (ii) optimization of packaging signal and Rev-responsive elements (47,48); and (iii) replacement of the enhancer sequences of the 3′LTR by LoxP sequences for conditional site-specific excision of transduced expression units (49) or by antibiotic-responsive operator modules for conditional proviral gene expression (46).

In order to combine minimal lentiviral transduction modules with extended polylinkers and the highest flexibility for advanced modification of the *cis*-acting elements we designed a novel modular lentivector (pLentiModule) which consists of four modules: (i) *Xho*I/*Nru*I-$U3_1$-*Stu*I/*Sbf*I/*Pst*I-$U3_2$-*Srf*I, (ii) *Srf*I-R-U5-ψ+-SD-*Swa*I/*Nsi*I, (iii) *Swa*I/*Nsi*I-cPPT-RRE-SA-MCSI and (iv) MCSI-3′$LTR_{\Delta U3}$(1)-MCSII-3′$LTR_{\Delta U3}$(2)-MCSIII. These modules are flanked by unique restriction sites and were sequentially assembled into a minimal pTRIDENT-derived prokaryotic vector backbone to result in pLentiModule1 [*Xho*I/*Nru*I-$U3_1$-*Stu*I/*Sbf*I/*Pst*I-$U3_2$-*Srf*I-R-U5-ψ+-SD-*Swa*I/*Nsi*I-cPPT-RRE-SA-MCSI-3′$LTR_{\Delta U3}$(1)-MCSII-3′$LTR_{\Delta U3}$(2)-MCSIII; Fig. 8, Table 1]. In contrast to the previously described lentivectors pLentiModule is devoid of the $ori_{SV40}$ since transfer of this simian virus element into target cells is incompatible with current therapeutic modalities.

pLentiModule1 displays a construction kit-like highly modular structure and enables design of the following lentivector characteristics. (i) Transfer of the entire lentiviral expression cassette using the flanking unique sites *Xho*I/*Nru*I and MCSIII, (ii) exchange of the 5′U3 region by *Xho*I/*Nru*I and *Srf*I, (iii) switching of the 5′U3 enhancer (Tat-binding) sequences by *Xho*I/*Nru*I and *Stu*I/*Sbf*I/*Pst*I, (iv) substitution of the R-U5-ψ+-SD module using Sr*f*I and *Swa*I/*Nsi*I, (v) replacement of the cPPT-RRE-SA module by modified counterparts using the *Swa*I/*Nsi*I and MCSI sites, (vi) cloning of desired transgenes, promoters and resistance cassettes into MCSI which is also compatible with the pLentiTRIDENT vectors and all of the previously described lentivectors (Figs 1, 3 and 6), (vii) swapping of the 3′$LTR_{\Delta U3}$ element using sites contained in MCSI and MCSIII and (viii) integration of functional units (for example, LoxP, tissue-specific or regulatable operators, insulator sequences) into MCSII which are copied into the 5′LTR upon reverse transcription and integration. In addition, (ix) cassette swapping with all other vectors described here is further facilitated using any of the sites within the *bla* region (*Ase*I/*Fsp*I/*Xmn*I/*Ssp*I/*Aat*II) and the R-U5-ψ+-SD module (*Nar*I/*Bss*HII/*Xmn*I).

pLentiModule1 was tested by insertion of a $P_{hCMV}$-EYFP cassette into its MCS (pLentiModule2; Fig. 6, Table 1) and transduction into insect (SF9), chicken (DT40), murine (BHK-21, CHO-K1) and human (HeLa, HT-1080, HepG2, K-562) cell lines as well as into ARCs and glial cells of hippocampal slice cultures (Fig. 9). Furthermore, pLentiModule1-derived lentivectors encoding EPO (pLentiModule7), $VEGF_{121}$ (pLentiModule6) or SEAP (pLentiModule8) under control

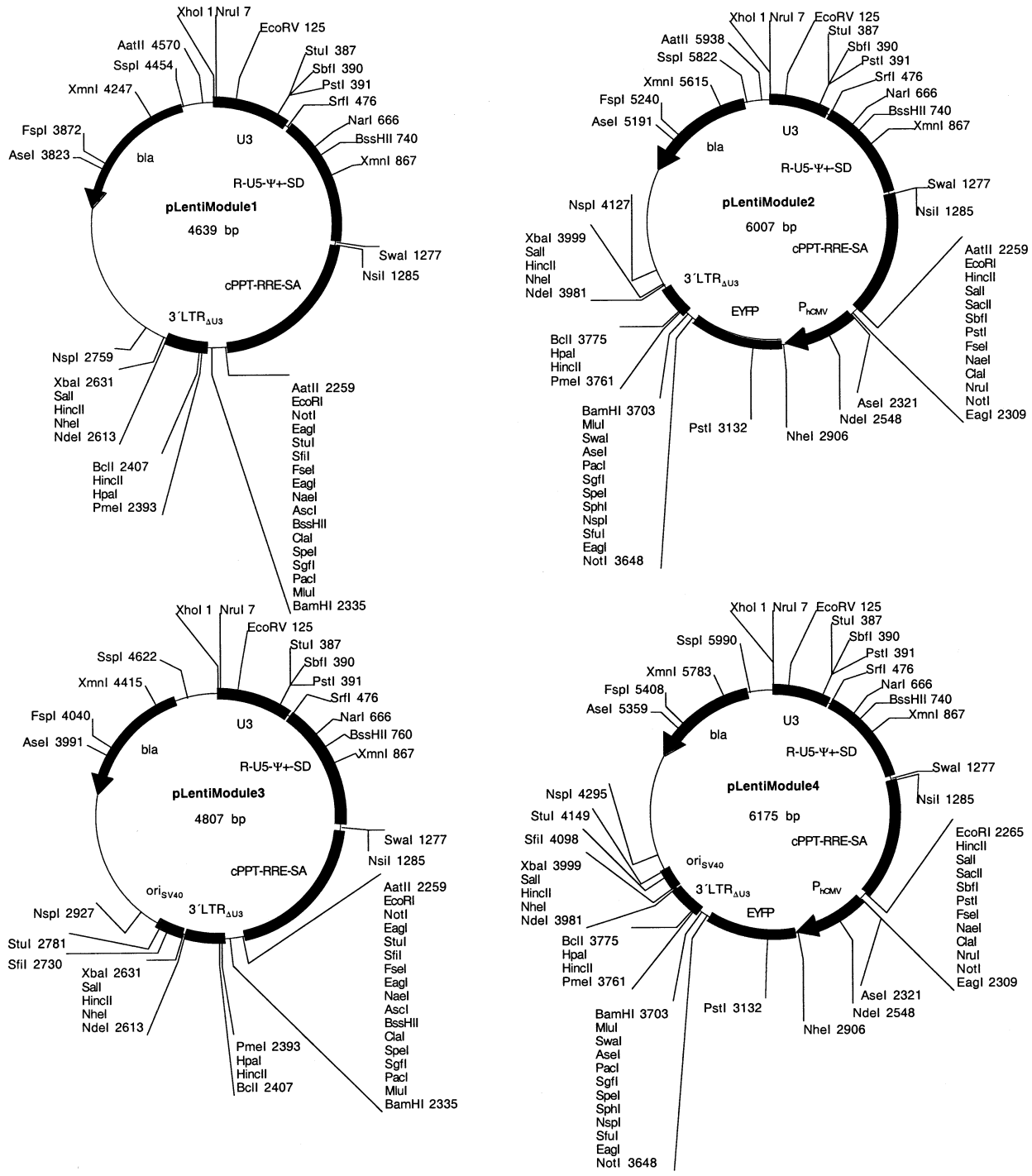



**Figure 8.** Modular SIN lentiviral expression vectors. In order to facilitate the design of advanced lentiviral expression vectors, all *cis*-acting lentiviral sequences are flanked by unique restriction sites in pLentiModule vectors (*Xho*I/*Nru*I-U3-*Srf*I; *Srf*I-R-U5-ψ+-SD-*Swa*I/*Nsi*I; *Swa*I/*Nsi*I-cPPT-RRE-SA-MCS; MCS-3′LTR$_{\Delta U3}$-*Nde*I/*Nhe*I/*Sal*I/*Xba*I). Unique restriction sites have also been placed within U3 (*Stu*I, *Sbf*I, *Pst*I) and at the site of the deletion within 3′LTR$_{\Delta U3}$ (*Pme*I, *Hpa*I, *Bcl*I). In order to enhance virus production an ori$_{SV40}$ (origin of replication of the Simian virus 40) has been placed 3′ of the 3′LTR outside of the lentiviral expression unit. Test lentivectors pLentiModule2 and pLentiModule3 contain a P$_{hCMV}$-driven EYFP expression cassette in the central MCS.

P$_{hEF1\alpha}$ display high-level expression of these therapeutic/reporter proteins in CHO-K1 cells (Tables 2 and 3).

As pLentiModule1 and pLentiModule2 are devoid of ori$_{SV40}$, the virus titer is significantly reduced to the ones generated using an intact ori$_{SV40}$/large T antigen replication system (Table 3). We have therefore inserted a 168 bp fragment spanning the ori$_{SV40}$ into MCSIII of pLentiModule1 and pLentiModule2, respectively, resulting in pLentiModule3 and pLentiModule4. As quantified from CHO-K1 transductions, ori$_{SV40}$-containing pLentiModule derivatives achieve

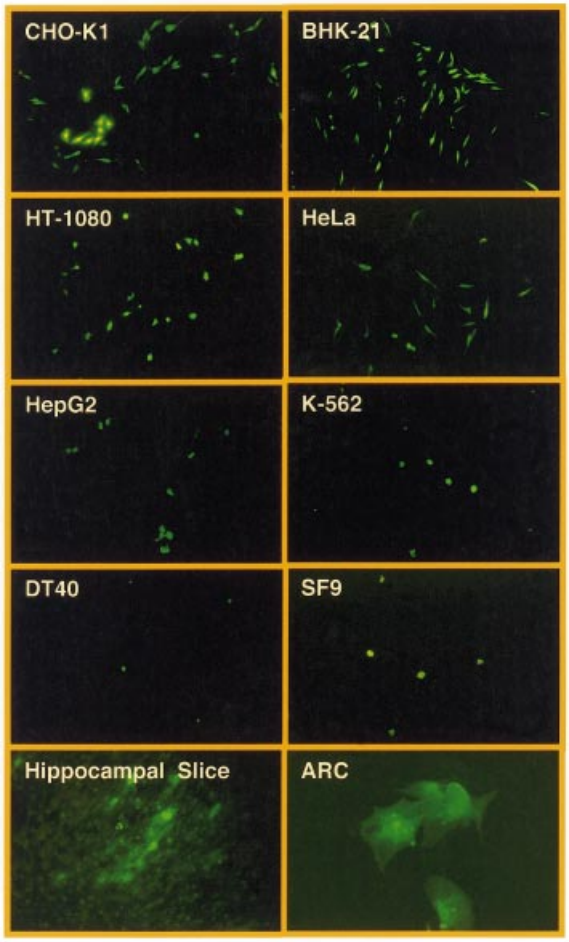



**Figure 9.** Transduction of pLentiModule-derived lentiviruses into various insect, chicken, murine and human cell types as exemplified by a VSV-G-pseudotyped pLentiModule3-derived lentivirus (pLentiModule4). The gene coding for the EYFP was successfully transduced into Chinese hamster ovary cells (CHO-K1), baby hamster kindney cells (BHK-21), human fibrosarcoma cells (HT-1080), human cervial adenocarcinoma cells (HeLa), human hepatocellular carcinoma cells (HepG2), human chronic myelogenous leukemia cells (K-562), bursal chicken cells (DT40) as well as insect cells (SF9). pLentiModule4-derived lentiviruses also transduced glial cells in hippocampal slice cultures (hippocampal slice) and primary ARC (magnification 100×, hippocampal slice 50×; ARC 400×).

virus titers, which compares to aforementioned lentivectors (Fig. 2, Table 3).

## DISCUSSION

Since their discovery in the early 1980s, HIV-1 lentiviruses were known to cause one of the most devastating epidemics in industrialized countries in recent history (50,51). However, the unique characteristic of HIV-1 to transduce and integrate into the human genome owing to a set of accessory genes managing nuclear import has brought this deadly virus into the limelight of the gene therapy arena as most genetic defects in humans reside in non-proliferating terminally differentiated cells. As the first development of replication-incompetent HIV-1-based vectors to transduce mitotically inert rat neurons, human skin fibroblasts and CD34$^+$ cells (16,17), lentiviral transduction technologies have witnessed a progress to the top of currently most promising gene therapy initiatives.

Elimination of some 60% of its genome (including all accessory genes) and re-assembly in multiply attenuated split-genome packaging systems successfully addressed legitimate initial safety concerns. The most advanced lentiviral transduction systems include a third-generation packaging system consisting of a (i) gag-pol, (ii) rev and (iii) a vesicular stomatitis virus-derived envelope protein (VSV-G) as well as (iv) a SIN lentiviral expression vector. The split-genome strategy dramatically reduces the emergence of replication-competent recombinants and pseudotyping eliminates targeted infection of CD4$^+$ T lymphocytes as well as extension of the cell tropism for therapeutic interventions (19). However, for successful infection of some target tissues including the liver some accessory proteins such as Vpr and Vif may be essential and should be considered for the packaging set-up (9). Typically, replication-incompetent lentiviral particles are produced by co-transfecting a helper cell line with all packaging and the lentiviral expression vector. However, with pharmaceutical production of therapeutic lentiviruses in mind a variety of different stable packaging cell lines have been designed which produced replication-incompetent transgenic lentiviruses in a tetracycline-responsive manner (20–23).

The lentiviral expression unit is the only genetic material transferred to the target cells and encodes the desired therapeutic transgene. This expression vector contains only *cis*-acting sequences and is devoid of any lentiviral coding sequences, which prevents significant humoral immune responses. Major improvements of lentiviral expression vectors include: (i) elimination of enhancer sequences from the 3′LTR which results in complete loss of the 5′LTR's transcriptional capacity and the viral mobilization competence upon integration of the provirus in the target chromosome thereby increasing safety and minimizing interferences with internal and chromosomal promoters *in vivo* and (ii) replacement of 5′LTR promoters by heterologous promoters to achieve Tat-independent and/or tetracycline-responsive virus production (23,25,26,49). Many of these state-of-the-art lentivectors have been successfully used in pioneering gene therapy studies for the treatment of many prominent human diseases in animal models including Parkinson's disease and metachromatic leukodystrophy (8,10).

Despite the dramatic progress in lentivector development in recent years lentiviral expression configurations available from the pioneering research groups have been rather specialized and inconvenient for straightforward design of multi-purpose lentivectors by a broad scientific community. We have therefore designed a highly modular construction kit-like expression platform based on the latest generation SIN lentivectors for mono- and multicistronic expression in a variety of cell lines and primary cells including, for the first time, ARCs. Also, to our knowledge, we report here the first lentiviral transduction into chicken embryos. This simple-to-use multi-purpose lentivector expression platform, which is compatible with any of the aforementioned packaging concepts is expected to greatly facilitate the use of lentiviral transduction technologies by a wider scientific community to accelerate the advancement in gene therapy and tissue engineering.

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