HUMAN GENE THERAPY 14:1799–1807 (December 10, 2003)

Brief Report

Lentiviral Vector-Mediated Delivery of Short Hairpin RNA Results in Persistent Knockdown of Gene Expression in Mouse Brain

CHRIS VAN DEN HAUTE,[1] KRISTEL EGGERMONT,[1] BART NUTTIN,[1] ZEGER DEBYSER,[2] and VEERLE BAEKELANDT[1]

ABSTRACT

RNA interference (RNAi) is an evolutionarily conserved mechanism of posttranscriptional gene-specific silencing. For *in vivo* applications, RNAi has been hampered until recently by inefficient delivery methods and by the transient nature of the gene suppression. Lentiviral vectors (LVs) hold great promise for gene therapeutic applications, pharmaceutical target validation, and functional genomics because stable gene transfer is mediated both in dividing and nondividing cells. We have used a lentiviral vector-based system for RNAi. We produced human immunodeficiency virus type 1-derived LVs encoding a short hairpin RNA specific for enhanced green fluorescent protein (EGFP) mRNA that were capable of inhibiting EGFP expression in mammalian cells. EGFP knockdown persisted after multiple passages of the cells. Of particular interest, our RNAi LVs were equally effective in suppression and prevention of EGFP expression after stereotactic injection in adult mouse brain. Therefore, we believe that the use of LVs for stable RNAi in brain will become a powerful aid to probe gene function *in vivo* and for gene therapy of diseases of the central nervous system.

INTRODUCTION

POSTTRANSCRIPTIONAL GENE SILENCING, also known as RNA interference (RNAi), was first shown in lower animals and plants (for review see Hannon, 2002). Persistent suppression of gene expression in adult organisms by RNA interference is a potentially powerful tool both to study gene function and to develop gene-specific therapeutics. To avoid the nonspecific cellular responses to double-stranded RNA in mammalian cells, small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) were designed (Elbashir *et al.*, 2001). DNA plasmids were constructed that drive expression of shRNAs from RNA polymerase III promoters to induce suppression in mammalian cells in culture (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002) or in adult mice (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002). However, a prerequisite for developing RNAi-based therapeutics is an efficient delivery vehicle to transfer the RNAi cassette into the target cells. Therefore, combining of the RNAi expression cassette with a viral delivery vector was the logical next step.

We are developing optimized human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vectors for the gene therapy of neurodegenerative diseases (Baekelandt *et al.*, 2000, 2002). In this context, we wanted to explore RNAi as a tool with which to intervene in pathways of neurodegeneration. We have designed a lentiviral vector construct containing a short hairpin sequence targeting the enhanced green fluorescent protein (EGFP) gene driven by a mouse U6 RNA polymerase III promoter. Here we demonstrate that lentivirus-mediated delivery of shRNA against EGFP results in efficient and stable reduction of gene expression in mammalian cells in culture and in the mouse brain.

[1]Gene Therapy Program, Experimental Neurosurgery and Neuroanatomy, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.
[2]Gene Therapy Program, Molecular Virology and Gene Therapy, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.

MATERIALS AND METHODS

Cloning

The plasmid mU6pro containing the mouse U6 promoter (Yu *et al.*, 2002) was a kind gift from Dr. D. Turner (University of Michigan, Ann Arbor, MI). A fragment containing the HIV-1 central polypurine tract (cPPT) and central termination sequence (CTS) was generated by polymerase chain reaction (PCR) with a forward primer (5′-AAAAAGCTTCGCCGAATTCACAAATGGC-3′) and reverse primer (5′-AAAAAGCTTCCCCAAAGTGGATCTCTGCTGTCC-3′), with the combivector (see below) as template. This PCR fragment was cloned immediately upstream from the mouse U6 promoter into the *Hin*dIII restriction site of the mU6pro plasmid. Two oligonucleotides encoding the forward short hairpin EGFP (sh-EGFP) sequence (5′-TTTGGCGATGCCACCTACGGCAAGCTCGAGCTTGCCGTAGGTGGCATCGCCCTTTTT-3′) and the reverse sh-EGFP sequence (5′-CTAGAAAAAGGGCGATGCCACCTACGGCAAGCTCGAGCTTGCCGTAGGTGGCATCGC-3′) were annealed *in vitro* (Yu *et al.*, 2002) and cloned in between the *Bbs*I and *Xba*I sites of the cPPT/CTS-mU6pro plasmid. An *Xho*I sequence (CTCGAG) was introduced as spacer and loop between the sense and reverse complementary sequences. Next, the cPPT/CTS-mU6 promoter-sh-EGFP fragment was obtained by *Pme*I–*Xba*I digestion and cloned into the combivector after removal of the cPPT/CTS and cytomegalovirus (CMV) promoter sequences by *Ppu*MI–*Spe*I digestion. The combivector refers to a pHR′-derived transfer plasmid created in-house and containing a multiple cloning site, the SIN-18 deletion, the woodchuck hepatitis posttranscriptional regulatory element (WPRE), and the cPPT/CTS sequence (Naldini *et al.*, 1996; Baekelandt *et al.*, 2002).

Lentiviral vector production

HIV-1-derived vector particles, pseudotyped with the envelope of vesicular stomatitis virus (VSV), were produced by transfecting 293T cells with a packaging plasmid encoding viral Gag and Pol proteins (pCMVΔR8.91), a plasmid encoding the envelope of vesicular stomatitis virus (pMDG), and the transfer plasmid encoding RNAi-EGFP. Transient transfection of 293T cells was carried out in 10-cm dishes. For each plate, a DNA mixture (700 μl) containing 20 μg of transfer plasmid, 10 μg of packaging construct, and 5 μg of envelope plasmid in 150 m*M* NaCl was prepared. A 700-μl volume of polyethylenimine (PEI) solution (110 μl of 10 m*M* PEI stock solution, pH 7.0, diluted with 590 μl of 150 m*M* NaCl) was added to this DNA mixture. After 15 min at room temperature, the DNA–PEI complex was added dropwise to the 293T cells in Opti-MEM I (GIBCO-BRL, Merelbeke, Belgium) with gentamicin (20 μg/ml) and without fetal calf serum (FCS). After overnight incubation, the medium was changed. Supernatants were collected from day 2 to day 5 posttransfection. The vector particles in the supernatant were filtered through a 0.45 μm pore size filter and then sedimented by ultracentrifugation at 15,000 rpm at 4°C for 5 hr. The pellets were redissolved in phosphate-buffered saline (PBS). For *in vivo* experiments, the vectors were further centrifuged for 1 hr at 20,000 rpm and redissolved in PBS, resulting in a final 8,000-fold concentration. p24 antigen content was determined with the HIV-1 p24 core profile ELISA (PerkinElmer Life Sciences, Boston, MA). RNA content of the lentiviral vector (LV) was determined in a two-step real-time reverse transcription (RT)-PCR. RNA was isolated from the vectors with TRIzol LS (Invitrogen, Merelbeke, Belgium). cDNA was synthesized with ThermoScript reverse transcriptase (Invitrogen) and a gene-specific reverse primer complementary to the 5′ end of the *gag* gene. Next, the DNA was quantified by real-time TaqMan PCR with gene-specific primers complementary to the U5 region of the 5′ long terminal repeat (LTR) and the 5′ end of the *gag* gene (Pannecouque *et al.*, 2002). After serial dilution of vector RNA, a linear correlation was obtained between the copy number and the fluorochrome signal intensity over 5 logs.

Cell culture and LV transduction

293T cells were obtained from Dr. O. Danos (Génethon, Evry, France). Chinese hamster ovary (CHO-K1) cells were obtained from the American Type Culture Collection (Manassas, VA). 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM plus GlutaMAX; GIBCO-BRL) supplemented with 10% FCS (Harlan Sera-Lab, Loughborough, UK) and gentamicin (20 μg/ml) (GIBCO-BRL) at 37°C in a 5% CO_2 humidified atmosphere. CHO cells were grown in F-12 nutrient mixture (Ham) plus GlutaMAX (GIBCO-BRL) supplemented with 5% FCS and gentamicin (20 μg/ml). EGFP-overexpressing 293T and CHO cells were generated by transduction or transfection, respectively. 293T cells were transduced with an HIV-1-derived lentiviral vector (pCH-GFP-W) encoding EGFP under the control of the human CMV (hCMV) promoter. A single fluorescent cell was picked and expanded into clonal 293T-GFP cells. CHO cells were cotransfected with the CytoGem plasmid pGFPemd-p[R] (Packard Bioscience/PerkinElmer Life Sciences, Meriden, CT) and pCMV-IRESneo, using Lipofectamine 2000 (Invitrogen). In pGFPemd-p[R] GFP expression is driven from a simian virus 40 (SV40) promoter. Individual CHO-GFP colonies were picked after selection with G418 and clone III_{27} was further cultured (P. Cherepanov, unpublished results). Fluorescence-activated cell sorting (FACS) analysis was performed to confirm the homogeneity of the cultured cells. The 293T and CHO clones that stably overexpress EGFP were plated on 24-well dishes at a density of 10^5 cells per well. After 24 hr, cells were transduced for 5 hr with various amounts of RNAi-EGFP lentiviral vector in the presence of polybrene (4 μg/ml). Before transduction the medium of the cells was replaced by medium with 1% FCS. After washing and addition of fresh medium cells were passaged every 3 to 4 days. FACS analysis was performed at various time intervals after transduction, using 10,000 fixed cells to quantify the number and the mean fluorescence intensity of cells positive for EGFP. 293T or CHO cells negative for EGFP and 293T-EGFP or CHO-GFP cells were used to determine cutoff values.

Quantification of proviral DNA by real-time PCR

Single CHO-GFP cells were picked 1 week after transduction with the RNAi-EGFP LV and cultured into clonal cell lines. DNA was extracted with a DNeasy tissue kit (Qiagen, Venlo, The Netherlands) 7, 9, and 11 weeks after transduction and the number of proviral DNA copies was determined by quantita-

tive real-time PCR as described (Van Maele *et al.*, 2003). The number of GFP-positive cells and mean fluorescence intensity were determined by FACS analysis at the same time points.

Northern blotting

293T and CHO cells from a 24-well plate were harvested 10 days after transduction and total RNA was isolated with TRIzol LS reagent (GIBCO-BRL) according to the manufacturer's protocol. Five micrograms of total RNA was fractionated by electrophoresis in a 1.4% formaldehyde agarose gel and transferred to positively charged nylon membranes (Roche, Mannheim, Germany). Blots were hybridized with a digoxigenin-labeled GFP RNA probe. The signals were visualized on X-ray film, using an alkaline phosphatase-conjugated anti-digoxigenin antibody with CDP-Star (Roche Applied Science, Basel, Switzerland) as substrate.

Immunoblotting

Cells were centrifuged for 10 min at 1,000 rpm and the pellet was washed twice with PBS to remove the medium. After centrifugation cells were boiled for 5 min in 1% sodium dodecyl sulfate (SDS), sheared by passage through a needle (29 gauge), and boiled again for 3 min. Total protein concentration of the extract was determined (Pierce Biotechnology, Rockford, IL) and 10 μg of protein was loaded on 4–12% Bis-Tris gels (Invitrogen). The primary antibodies used were the following: mouse anti-α-tubulin (clone B-5-1-2; Sigma, St. Louis, MO), rabbit anti-GFP (Invitrogen), and rabbit anti-amyloid precursor protein (kind gift of Dr. B. De Strooper, Leuven, Belgium). Detection was performed by subsequent incubation with a horseradish peroxidase (HRP)-labeled secondary antibody and development with an ECL$^+$ kit (Amersham Biosciences, Uppsala, Sweden).

Surgery

We used adult C57BL/6 mice. The animal experiments were approved by the bioethics committee of the Katholieke Universiteit Leuven (Leuven, Belgium). All surgical procedures were performed with chloral hydrate anesthesia (400 mg/kg, administered intraperitoneally), using aseptic procedures. The mice were placed in a stereotactic head frame, and after midline incision of the skin one or two small holes were drilled in the skull in the appropriate location, using bregma as reference. Coordinates used for the striatum were anteroposterior (AP) 0.5, lateral (LAT) 2.0, dorsoventral (DV) 3.0–2.0. Highly concentrated vector (10^8 pg of p24 per milliliter) supplemented with polybrene (4 μg/ml) was injected at a rate of 0.5 μl/min with a 30-gauge needle on a 10-μl Hamilton syringe. During the 4-min injection, the needle was raised slowly in the dorsal direction over the distance indicated by the 2 DV coordinates. After the injection, the needle was left in place for another 4 min before being slowly withdrawn from the brain. We used an LV encoding α-synuclein as a control (Lauwers *et al.*, 2003). For some experiments animals received a second injection into the striatum 1 week after the first injection. Mice were killed with an overdose of pentobarbital, and intracardial perfusion with 4% paraformaldehyde in PBS was performed. After postfixation overnight, 50-μm-thick coronal brain sections were made with a Vibratome. The position of the needle tract in each hemisphere was determined by examination of the sections with a light microscope.

Quantification of gene expression

We analyzed EGFP expression by direct fluorescence, using an inverted microscope. Images were taken with a digital camera (DC300F; Leica Microsystems, Bensheim, Germany). The volume of the transduced brain area was quantified by a stereological procedure based on the Cavalieri principle. For each animal serial sections (minimum five) with an interval of 200 μm centered around the injection site were analyzed. A point-counting grid was placed over the screen on which the entire transduced brain region was displayed from a low-power objective. Points overlying EGFP-positive cells and fibers were counted. The transduced volume was calculated by multiplying the sum of the counted points with the distance between the counted sections and the area associated with each point on the grid (Baekelandt *et al.*, 2002).

RESULTS

As a first step, we have designed an HIV-1-derived lentiviral vector encoding a short hairpin RNA specific for EGFP mRNA. We cloned a fragment containing the mouse U6 promoter in front of an sh-EGFP sequence into an HIV-1-derived transfer plasmid containing the central polypurine tract/central termination site (cPPT/CTS) sequence, the SIN-18 deletion, and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Fig. 1).

Lentiviral vectors were produced by triple transient transfection of 293T cells with the RNAi-EGFP transfer plasmid, the pMDG plasmid encoding the VSV-G envelope, and a second-generation packaging plasmid (8.91) as described (Baekelandt *et al.*, 2002). The concentration of vector particles after ultracentrifugation of the supernatant was in the range (10^8 pg of p24 per milliliter) typically obtained with this vector system. Because lentiviral vectors are RNA vectors and the RNAi mechanism is based on the degradation of specific mRNA, the possibility existed that the viral genomic RNA would be degraded by RNAi during vector production. We therefore quantified the RNA content of the produced RNAi-EGFP LV by real-time RT-PCR. The RNAi-EGFP LV preparation contained about 10^{11} RNA copies per milliliter, which was again comparable to the amount of RNA present in other LV preparations.

Next, we determined whether the RNAi-EGFP LVs were capable of inhibiting EGFP expression in mammalian cells in culture. Individual clones of a CHO cell line and a 293T cell line, that both stably express EGFP, were picked and transduced with the RNAi-EGFP LV. A titer-dependent decrease in the number of EGFP-expressing cells, up to 90%, was observed in both cell types (Fig. 2A and B). In the CHO cells that remained positive there was no downregulation of EGFP expression levels (measured as mean fluorescence intensity by FACS analysis). The extent of inhibition was maximal 6 to 7 days after transduction. Transduction with an unrelated LV did not inhibit EGFP expression (data not shown). Reduction of EGFP mRNA and pro-

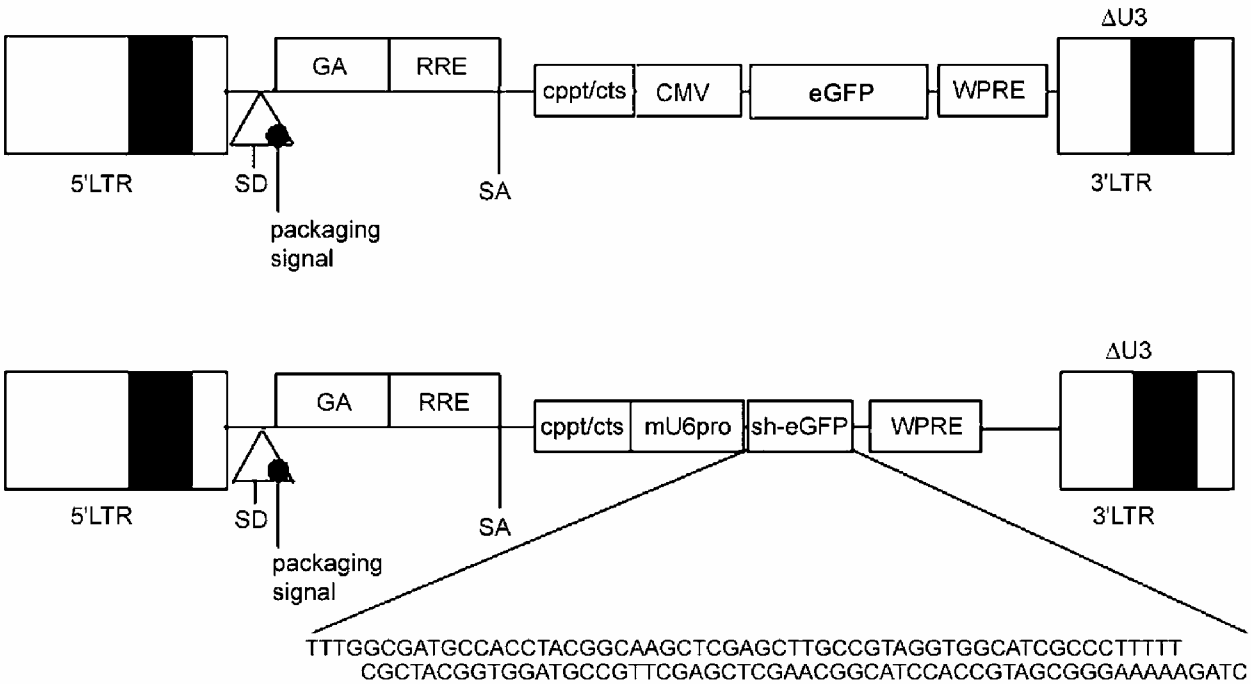


FIG. 1. LV constructs. Graphical representation of the LV transfer plasmid containing EGFP driven by the CMV promoter and the new transfer plasmid containing a short hairpin-EGFP sequence driven by the mouse U6 promoter. LTR, long terminal repeat; SD, splice donor site; GA, 5′ end of *gag*; RRE, Rev-responsive element; SA, splice acceptor site; cppt/cts, central polypurine tract/central termination sequence; CMV, cytomegalovirus promoter; eGFP, enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3, a deletion of 400 bp including the enhancer and promoter from U3; mU6pro, mouse U6 promoter; sh-EGFP, short hairpin-EGFP.

tein was evidenced by Northern and Western blotting (Fig. 2C and D). The suppression of gene expression was specific for EGFP and did not affect other cellular proteins (Fig. 2D). The transduced cells were kept in culture and passaged 1:10 every 3 to 4 days. At regular time intervals, EGFP-positive cells were counted by FACS analysis. EGFP knockdown was evident for at least 2 weeks (four passages), demonstrating the stable character of the RNA interference mediated by LV transduction. At the highest concentrations of LV used, there was a trend toward decreased inhibition after 1 week (Fig. 2B). To investigate this further, individual clones of the CHO-GFP cells were picked after transduction with the RNAi-EGFP LV. These clonal, transduced cell lines showed a stable reduction of EGFP expression for at least 4 months (Table 1). This suggests that the transduced cells may have a slight growth disadvantage compared with nontransduced cells. DNA analysis by quantitative real-time PCR of the clonal and transduced CHO cell lines showed a copy number of integrated proviral DNA of between one and six per cell (Table 1). The highest inhibition of EGFP expression, as determined by mean fluorescence intensity, corresponded with the highest copy number of proviral DNA. No proviral DNA was detected in nontransduced cells.

To test the performance of our RNAi-EGFP LVs *in vivo*, we performed stereotactic injections in mouse brains ($n = 12$). In a first experiment, we coinjected 1 μl of an LV encoding EGFP together with 2 μl of the RNAi-EGFP LV into mouse striatum. As a control, 1 μl of the LV encoding EGFP was injected in the other hemisphere together with 2 μl of physiological saline. The brains were analyzed for EGFP expression 1 week ($n = 1$) and 2 weeks ($n = 2$) after transduction. There was a nearly complete (98 ± 2%) inhibition of EGFP expression at the side where the RNAi-EGFP LV was injected (Fig. 3A). In a second experimental setting, LVs encoding EGFP were injected in both the left and the right striatum. One week later, these mice were injected with RNAi-EGFP LV in the right hemisphere and with an unrelated LV at the other side. EGFP expression was examined 2 weeks ($n = 2$) and 5 weeks ($n = 2$) after the second injection. Again, efficient silencing of EGFP expression was observed after transduction with RNAi-EGFP LV (Fig. 3B). Inhibition ranged between 51 and 90% in the different animals (average, 75 ± 17%). Incomplete inhibition of EGFP expression was most likely due to incomplete overlap of the two consecutive injection sites. To unequivocally prove the persistent nature of EGFP knockdown, we injected a separate group of mice, first with RNAi-EGFP LV and a control LV in the contralateral hemisphere, followed by an injection with the LV encoding EGFP at both sides 1 week later. Mice were analyzed for EGFP expression 1 week ($n = 1$), 3 weeks ($n = 2$), and 6 months ($n = 2$) after transduction. Again, EGFP expression was suppressed by 78.8 ± 12% in brain areas coinjected with RNAi-

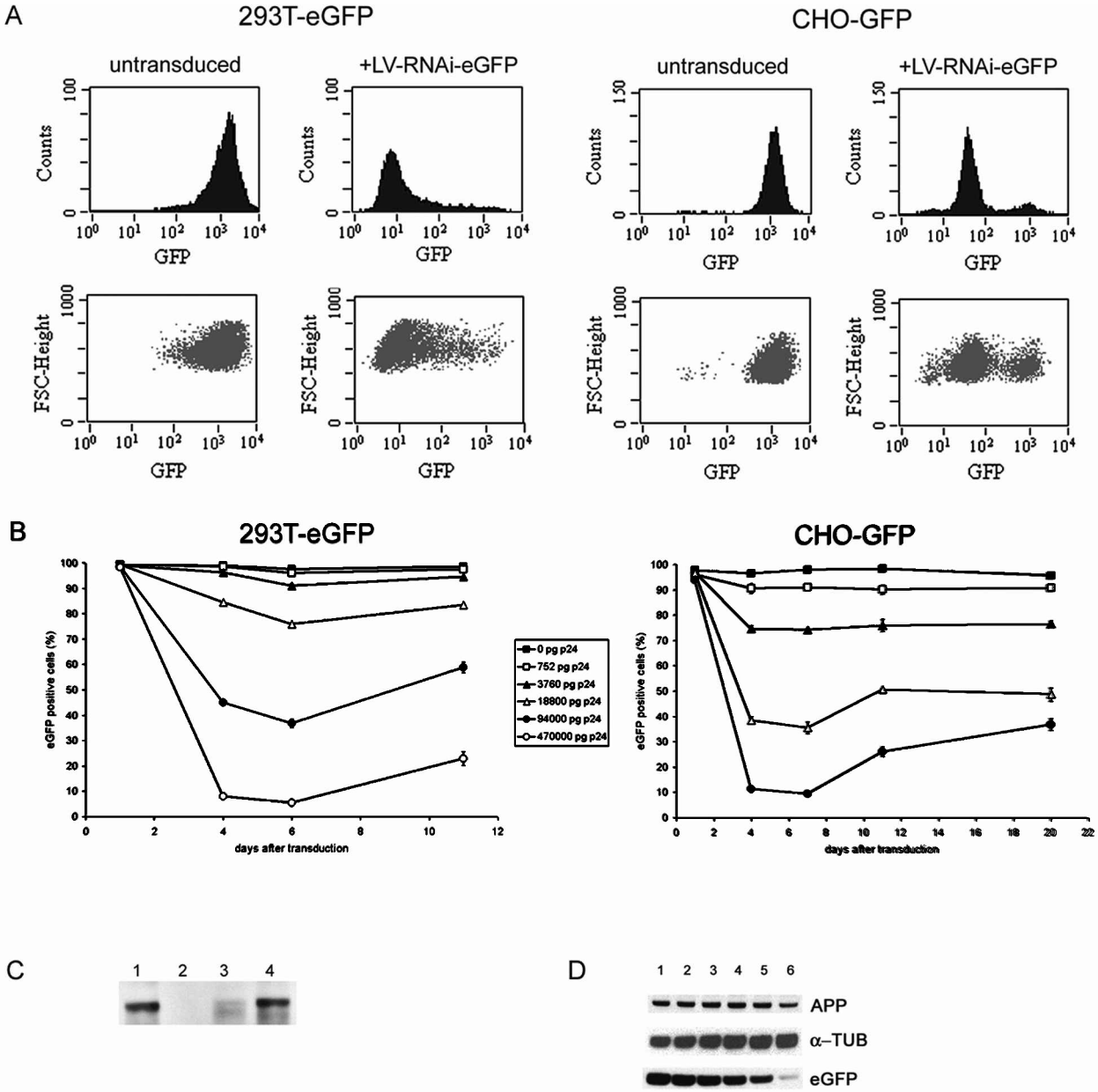


FIG. 2. Lentiviral vectors containing an RNAi-EGFP sequence stably reduce EGFP expression in mammalian cells in culture. (**A**) FACS analysis of human 293T and CHO cells stably expressing EGFP after transduction with RNAi-EGFP LV. FACS analysis of untransduced 293T-EGFP or CHO-GFP cells and of cells transduced with 470,000 or 94,000 pg p24 of RNAi-EGFP LV are shown. *Top*: Representative examples of histograms of FACS analysis 6 days (293T-EGFP) and 7 days (CHO-GFP) after transduction. *Bottom*: Corresponding dot plots. (**B**) Time course of suppression of EGFP expression by RNAi-LV in CHO and 293T cells as shown by the percentage of EGFP-positive cells. Cells were transduced with 0, 752, 3,760, 18,800, 94,000, and 470,000 (not for CHO-GFP) pg p24 of RNAi-EGFP LV. FACS analysis was performed at various time intervals after transduction. Graphs show a dose-dependent inhibition (up to 90%) of EGFP expression for more than 2 weeks in culture. An average of three different experiments is shown with standard error bars. (**C**) Northern blot of 293T cells 10 days after transduction with an RNAi-EGFP LV shows a corresponding decrease in EGFP mRNA. Lane 1, untransduced EGFP-expressing 293T cells; lane 2, EGFP-negative 293T cells; lanes 3 and 4, EGFP-expressing cells transduced with 470,000 and 3,760 pg p24 of RNAi-EGFP LV, respectively. (**D**) Western blot shows a specific and dose-dependent decrease in EGFP protein in 293T-EGFP cells 11 days after transduction. Lanes 1, 2, 3, 4, 5, and 6 correspond to 0, 752, 3,760, 18,800, 94,000, and 470,000 pg p24 of RNAi-EGFP LV. As an internal control for each sample we used antibodies against amyloid precursor protein (APP) and α-tubulin (α-TUB).

TABLE 1. QUANTITATIVE REAL-TIME PCR ON CLONAL CHO-EGEP CELLS AFTER TRANSDUCTION WITH RNAi-EGFP LENTIVIRAL VECTOR

Cell line[a]	*Integrated proviral DNA (copies/cell)*[b]	*Fluorescence (MFI)*[c]
CHO	0.004 ± 0.0007	3.59
CHO-EGFP	0.003 ± 0.001	874
A1	0.007 ± 0.004	938
A3	6.263 ± 2.790	16.47
A5	2.068 ± 1.676	26.64
A7	0.008 ± 0.008	819.22
C1	0.006 ± 0.004	927.4
C3	0.002 ± 0.001	844.93
C5	6.106 ± 3.181	15.42
C7	4.780 ± 2.069	18.44
E1	1.040 ± 0.594	77.56
E3	0.002 ± 0.0007	842.64
E5	6.016 ± 1.192	15.14

[a]Of the 11 clones cultured (A1 to E5), 6 (A3, A5, C5, C7, E1, and E5) contained detectable integrated proviral DNA and a concomitant prominent reduction in EGFP expression as determined by FACS analysis. The five other clones (A1, A7, C1, C3, and E3) were not transduced and showed a high MFI. CHO cells were used as negative controls.

[b]Data represent average values ± standard deviation of three separate measurements.

[c]Mean fluorescence intensity (MFI) was determined by FACS analysis.

EGFP LV and EGFP LV (Fig. 3C). The silencing effect persisted up to 6 months after transduction.

DISCUSSION

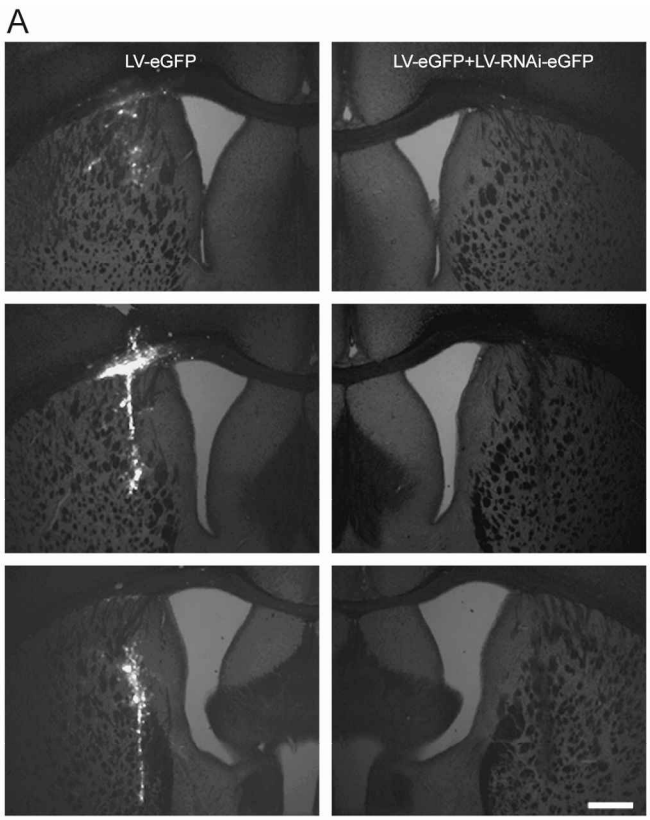
To expand the applicability of gene therapy beyond substituting for dysfunctional proteins in monogenetic diseases, downregulation of proteins involved in pathogenic pathways is required. Current technologies based on antisense, intrabodies, or transdominant negative protein inhibition have unpredictable outcomes. RNA interference is widely acclaimed as a potent technology to knock down protein expression. Lack of efficient delivery vectors for *in vivo* use prompted us to investigate whether lentiviral vectors could be suitable carriers.

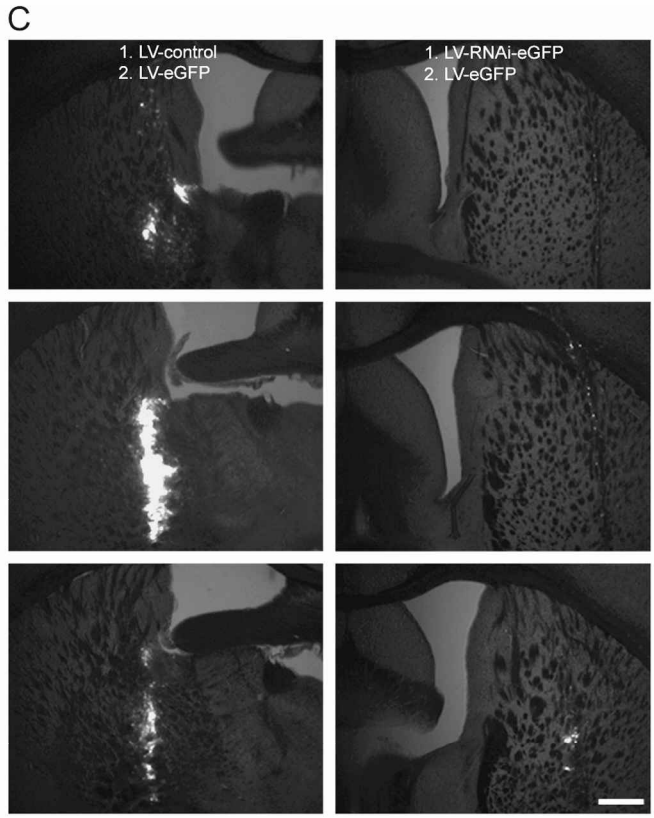
Lentiviral vectors have the advantage of transducing both nondividing and dividing cells, and integration of the transgene into the chromosome ensures stable expression. A potential disadvantage is the RNA nature of the vector genome. Until recently, it was unclear whether the RNA genome of the vector would be degraded because it contains the sense EGFP RNA recognized by the degradation-inducing hairpin. Alternatively, if a hairpin consisting of sense and antisense EGFP RNA fragments were formed, it was unclear whether this would interfere with the packaging of the RNA genome by the vector. We were able to produce LVs encoding an shRNA against EGFP that were capable of knocking down EGFP expression in cell culture and in mouse brain up to 6 months after transduction, excluding a gross defect in vector production. In fact, RNA quantification of LV preparations yielded RNA concentrations similar to those produced by classic EGFP-encoding lentiviral vectors. It is possible that the RNA polymerase II promoter (HIV LTR) interferes with transcription at the mouse U6 promoter during vector production. After transduction, promoter activity at the viral LTR is annihilated by the SIN configuration used, allowing transcription of hairpins at the RNA polymerase III promoter.

Quantitative PCR analysis of selected RNAi-EGFP LV-transduced clonal cell lines revealed an efficient knockdown of EGFP expression. One copy of integrated proviral DNA was sufficient to reduce the mean fluorescence intensity by more than 90% in a CHO-GFP cell line. A higher copy number corresponded with an even stronger inhibition of EGFP expression (up to 98%).

The applicability of our LV-based approach for shRNA-mediated RNA interference *in vivo* was demonstrated in three different experimental settings. The RNAi LVs were capable of preventing EGFP expression from LVs injected at the same location and at the same time. To exclude that the knockdown was limited to inhibition of *de novo* expression, RNAi LVs were also used to inhibit EGFP expression after 1 week. Furthermore, LV-mediated delivery of shRNA can be used for preventive applications: 1 week after injection of the RNAi-EGFP LV, *de novo* expression from a newly injected EGFP LV was inhibited. In all experimental settings the requirement for double transduction of cells may even underestimate the potency of the gene silencing. So far, we have only demonstrated knockdown of exogenous LV-mediated transgene expression. We have started the construction of LVs encoding hairpins directed against endogenous genes.

While this work was in progress, other groups have reported viral vector-based systems for RNAi. Using retroviral delivery of siRNA, gene silencing was achieved in mammalian cells in culture (Barton and Medzhitov, 2002;





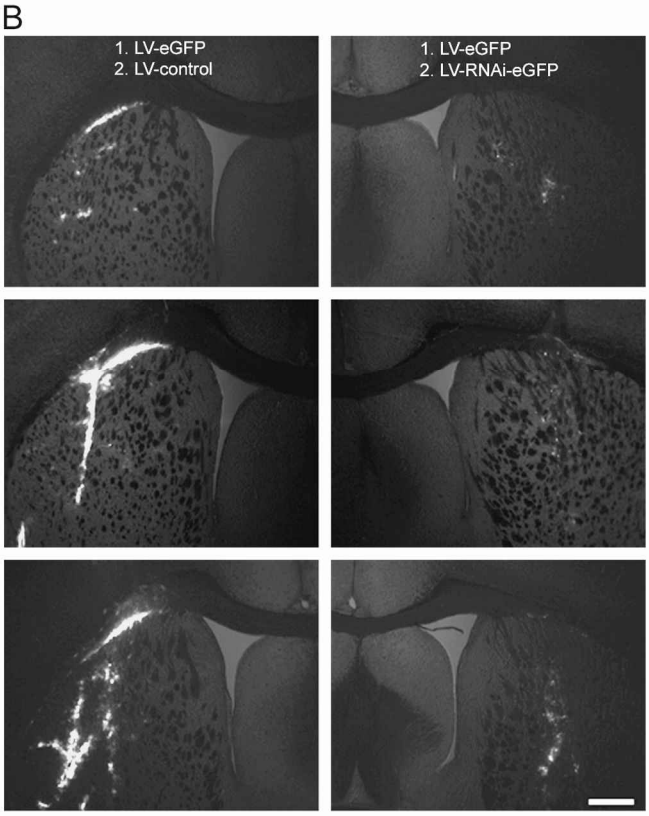


FIG. 3. Lentiviral vectors encoding an RNAi-EGFP sequence mediate efficient and stable inhibition of transgene expression after transduction of mouse brain. Serial sections through mouse striatum are shown spaced 200 μm apart around the site of injection (middle panels). (**A**) LVs encoding EGFP were coinjected stereotactically with an RNAi-EGFP LV in the right striatum and with physiological saline as control in the contralateral hemisphere. The brains were analyzed 1 or 2 weeks after transduction for direct EGFP fluorescence. In the left hemisphere, clear EGFP fluorescence is visible, whereas in the right hemisphere almost no fluorescent signal can be detected. (**B**) RNAi-EGFP LV efficiently suppresses a previously expressed transgene in mouse brain. One week after injecting the LV encoding EGFP we injected an RNAi-EGFP LV in the right hemisphere and a control LV expressing an unrelated protein (α-synuclein) at the contralateral side. Two or 5 weeks later the mouse brains were analyzed for EGFP expression. In the left hemisphere, clear EGFP fluorescence is visible. In the right hemisphere some residual fluorescent signal is detected, probably due to incomplete overlap of the two consecutive injections. (**C**) RNAi-EGFP LV can efficiently prevent transgene expression in mouse brain. RNAi-EGFP LV was stereotactically injected in the right striatum and a control LV was stereotactically injected into the contralateral side. One week later we injected an LV encoding EGFP in both hemispheres. One week, 3 weeks, or 6 months later the mouse brains were analyzed for EGFP expression. In the left hemisphere, clear EGFP fluorescence is visible, whereas in the right hemisphere a major reduction of the fluorescent signal is obtained. Scale bars: 500 μm.

Devroe and Silver, 2002). An adenovirus-mediated delivery system was developed to diminish gene expression *in vitro* and *in vivo* in mouse liver and brain (Xia *et al.*, 2002). And, more recently, lentiviral vector-mediated RNAi was demonstrated in human embryonic kidney cell lines (Abbas-Terki *et al.*, 2002), in human primary T cells (Qin *et al.*, 2003; Rubinson *et al.*, 2003), in mouse embryos (Rubinson *et al.*, 2003; Tiscornia *et al.*, 2003), and in hematopoietic stem cells (Rubinson *et al.*, 2003). In most cases an RNA polymerase III promoter (either H1 or U6) was used to drive expression from the short hairpin sequence. Only in the study by Xia *et al.* (2002) was the siRNA hairpin placed under control of the CMV promoter. Despite these promising results with viral vector-based RNAi, some parameters important for the performance of RNAi LVs remain to be determined, such as the optimal promoter choice, effect of additional genetic elements (WPRE), length of vector genome, stability of knockdown over time, required copy number per cell, and so on.

We are using lentiviral vectors to develop animal models for neurodegenerative diseases based on regional expression of disease-associated genes, as an alternative to classic transgenesis. Such animal models will be useful to investigate the mechanisms of neurodegeneration, to develop (gene) therapeutics and diagnostic tools, and for functional genomics in the central nervous system. In this context, the possibility to silence disease-associated genes would represent a powerful tool for this approach.

In conclusion, we have demonstrated that lentiviral vectors can be used as efficient carriers of shRNA to specifically knock down gene expression in the brain. The availability of an efficient viral vector system increases the likelihood for future clinical applications of the already promising RNAi technology.

ACKNOWLEDGMENTS

We thank Martine Michiels and Sofie Willems for excellent technical assistance with vector production and stereotactic injections. We acknowledge Martine Geraerts and Bénédicte Van Maele for Q-PCR analysis. The original lentiviral vector constructs were a kind gift from Dr. D. Trono (University of Geneva, Geneva, Switzerland) and Dr. L. Naldini (University of Turin, Turin, Italy), and the TRIP vectors were a generous gift from Dr. P. Charneau (Institut Pasteur, Paris, France). The construct containing the U6 promoter was kindly provided by Dr. D. Turner (University of Michigan, Ann Arbor, MI). C.V.d.H. has a postdoctoral grant from the Flemish Institute supporting Scientific-Technological Research in Industry (IWT). Z.D. and V.B. are postdoctoral fellows of the Flemish Fund for Scientific Research (FWO Vlaanderen). This work was funded by IDO grant 98/006, IDO 01/004, and VIS grant 00/010 from the KULeuven Research Council and by an STWW grant (980339) of the Flemish Institute supporting Scientific-Technological Research in Industry (IWT).

REFERENCES

ABBAS-TERKI, T., BLANCO-BOSE, W., DEGLON, N., PRALONG, W., and AEBISCHER, P. (2002). Lentiviral-mediated RNA interference. Hum. Gene Ther. **13,** 2197–2201.

BAEKELANDT, V., DE STROOPER, B., NUTTIN, B., and DEBYSER, Z. (2000). Gene therapeutic strategies for neurodegenerative diseases. Curr. Opin. Mol. Ther. **2,** 540–554.

BAEKELANDT, V., CLAEYS, A., EGGERMONT, K., LAUWERS, E., DE STROOPER, B., NUTTIN, B., and DEBYSER, Z. (2002). Characterization of lentiviral vector-mediated gene transfer in adult mouse brain. Hum. Gene Ther. **13,** 841–853.

BARTON, G.M., and MEDZHITOV, R. (2002). Retroviral delivery of small interfering RNA into primary cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 14943–14945.

BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science **296,** 550–553.

DEVROE, E., and SILVER, P.A. (2002). Retrovirus-delivered siRNA. BMC Biotechnol. **2,** 15.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–498.

HANNON, G.J. (2002). RNA interference. Nature **418,** 244–251.

LAUWERS, E., DEBYSER, Z., VAN DORPE, J., DE STROOPER, B., NUTTIN, B., and BAEKELANDT, V. (2003). Neuropathology and neurodegeneration in rodent brain induced by lentiviral vector-mediated overexpression of α-synuclein. Brain Pathol. **13,** 364–372.

LEWIS, D.L., HAGSTROM, J.E., LOOMIS, A.G., WOLFF, J.A., and HERWEIJER, H. (2002). Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat. Genet. **32,** 107–108.

MCCAFFREY, A.P., MEUSE, L., PHAM, T.T., CONKLIN, D.S., HANNON, G.J., and KAY, M.A. (2002). RNA interference in adult mice. Nature **418,** 38–39.

NALDINI, L., BLÖMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F.H., VERMA, I.M., and TRONO, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272,** 263–267.

PADDISON, P.J., CAUDY, A.A., BERNSTEIN, E., HANNON, G.J., and CONKLIN, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16,** 948–958.

PANNECOUQUE, C., PLUYMERS, W., VAN MAELE, B., TETZ, V., CHEREPANOV, P., DE CLERCQ, E., WITVROUW, M., and DEBYSER, Z. (2002). New class of HIV integrase inhibitors that block viral replication in cell culture. Curr. Biol. **12,** 1169–1177.

PAUL, C.P., GOOD, P.D., WINER, I., and ENGELKE, D.R. (2002). Effective expression of small interfering RNA in human cells. Nat. Biotechnol. **20,** 505–508.

QIN, X.F., AN, D.S., CHEN, I.S., and BALTIMORE, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. U.S.A. **100,** 183–188.

RUBINSON, D.A., DILLON, C.P., KWIATKOWSKI, A.V., SIEVERS, C., YANG, L., KOPINJA, J., ZHANG, M., MCMANUS, M.T., GERTLER, F.B., SCOTT, M.L., and VAN PARIJS, L. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. **33,** 401–406.

SUI, G., SOOHOO, C., AFFAR EL, B., GAY, F., SHI, Y., and FORRESTER, W.C. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 5515–5520.

TISCORNIA, G., SINGER, O., IKAWA, M., and VERMA, I.M. (2003). A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc. Natl. Acad. Sci. U.S.A. **100,** 1844–1848.

VAN MAELE, B., DE RIJCK, J., DE CLERCQ, E., and DEBYSER, Z. (2003). Impact of the central polypurine tract on the kinetics of human immunodeficiency virus type 1 vector transduction. J. Virol. **77,** 4685–4694.

XIA, H., MAO, Q., PAULSON, H.L., and DAVIDSON, B.L. (2002). siRNA-mediated gene silencing in vitro and in vivo. Nat. Biotechnol. **20,** 1006–1010.

YU, J.Y., DERUITER, S.L., and TURNER, D.L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 6047–6052.

Address reprint requests to:
Dr. Veerle Baekelandt
Gene Therapy Program
Experimental Neurosurgery and Neuroanatomy
Kapucijnenvoer 33, VCTB+5
B-3000 Leuven, Belgium

E-mail: Veerle.Baekelandt@med.kuleuven.ac.be
Zeger.Debyser@med.kuleuven.ac.be.

Received for publication April 8, 2003; accepted after revision October 25, 2003.

Published online: November 17, 2003.