Histone Deacetylase Inhibition Activates Transgene Expression from Integration-Defective Lentiviral Vectors in Dividing and Non-Dividing Cells

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Abstract

Integration-defective lentiviral vectors (IDLVs) are being increasingly deployed in both basic and preclinical gene transfer settings. Often, however, the IDLV transgene expression profile is muted when compared to that of their integration-proficient counterparts. We hypothesized that the episomal nature of IDLVs turns them into preferential targets for epigenetic silencing involving chromatin-remodeling histone deacetylation. Therefore, vectors carrying an array of *cis*-acting elements and transcriptional unit components were assembled with the aid of packaging constructs encoding either the wild-type or the class I mutant D116N integrase moieties. The transduction levels and transgene-product yields provided by each vector class were assessed in the presence and absence of the histone deacetylase (HDAC) inhibitors sodium butyrate and trichostatin A. To investigate the role of the target cell replication status, we performed experiments in growth-arrested human mesenchymal stem cells and in post-mitotic syncytial myotubes. We found that IDLVs are acutely affected by HDACs regardless of their genetic makeup or target cell replication rate. Interestingly, the magnitude of IDLV transgene expression rescue due to HDAC inhibition varied in a vector backbone- and cell type-dependent manner. Finally, investigation of histone modifications by chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) revealed a paucity of euchromatin marks distributed along IDLV genomes when compared to those measured on isogenic integration-competent vector templates. These findings support the view that IDLVs constitute preferential targets for epigenetic silencing involving histone deacetylation, which contributes to dampening their full transcriptional potential. Our data provide leads on how to most optimally titrate and deploy these promising episomal gene delivery vehicles.

Introduction

GENE DELIVERY SYSTEMS based on the human immunodeficiency virus type 1 (HIV-1) and on other, nonhuman lentiviruses have been undergoing intense development and testing in recent years as a result of their versatility and efficiency in transducing both dividing and non-dividing target cells. These features have led to the permeation of lentiviral vector (LV) technology into many different areas of research and, most notably, gene therapy (Dropulić, 2011). Regarding the deployment of LVs in gene therapy protocols, however, two major safety concerns became readily perceptible. These were related to the pathogenic nature of the parental viruses and to the risk associated with the biased integration of vector genomes into transcriptionally active host genes (Schröder *et al.*, 2002; Wu *et al.*, 2003). The biosafety concerns linked to

the former aspect have been minimized through the introduction of split packaging constructs deleted in accessory viral genes to reduce the chance for recombination-mediated assembly of replication-competent lentiviruses (Dull *et al.*, 1998), whereas those associated with the latter have been approached by the employment of class I integrase (IN) mutants that render the vectors integration-defective (Philpott and Thrasher, 2007; Wanisch and Yáñez-Muñoz, 2009; Banasik and McCray Jr, 2010).

In the present study, to generate these integration-defective LVs (IDLVs), we engineered IN moieties harboring the class I point mutation D116N (IN^{D116N}). This mutation results in the functional disruption of the protein by removing the central aspartic acid of the DDE triad present in the catalytic pocket domain whose residue positions are D64, D116, and E152 (see, for instance, Philpott and Thrasher, 2007). Importantly,

in contrast to class II, class I IN mutants such as IN^{D116N} do not display pleiotropic effects on the viral life cycle since they interfere specifically with DNA cleavage and ensuing insertion of linear genomes into host chromosomes. As a consequence of this molecular uncoupling, key features necessary for the buildup and packaging of vector genomes and for the delivery of the genetic payload into the nucleus remain unhindered in IDLVs. These features include those involved in target cell entry, reverse transcription, and nuclear import of preintegration complexes. Thus, class I IN mutants lead to the accumulation in the nucleus of the characteristic 1-long terminal repeat (LTR) and 2-LTR circular DNA forms that, contrary to the linear form, are not substrates for canonical IN-mediated chromosomal integration.

These 1-LTR and 2-LTR circles are thought to arise from the incoming linear double-stranded DNA through homologous recombination and non-homologous end-joining, respectively, and have in principle all the prerequisites to serve as transcriptionally active templates. Together, these characteristics did provide a strong rationale for the development of IDLVs (Philpott and Thrasher, 2007; Wanisch and Yáñez-Muñoz, 2009; Banasik and McCray Jr, 2010). Indeed, in many gene delivery interventions, such as those not dependent on permanent genetic modification of dividing cell populations or involving post-mitotic target cells, IDLVs present an attractive and potentially safer gene delivery option. Examples of their use include vaccination (Negri *et al.*, 2010) and *in vivo* gene transfer to post-mitotic tissues such as the retina (Loewen *et al.*, 2003; Yáñez-Muñoz *et al.*, 2006), brain (Philippe *et al.*, 2006; Yáñez-Muñoz *et al.*, 2006), skeletal muscle (Apolonia *et al.*, 2007), and the liver (Mátraí *et al.*, 2011). IDLVs have also been tested for the *ex vivo* delivery of transcription factors to reprogram somatic cells into induced pluripotent stem cells (Mali *et al.*, 2008). Moreover, efforts to

adapt IDLVs to stably transduce rapidly proliferating cells using zinc finger nucleases or transposases have also been initiated (Lombardo *et al.*, 2007; Staunstrup *et al.*, 2009). However, from many of these reports it becomes clear that on the basis of transgene expression criteria LVs often outperform, by up to one order of magnitude, their IDLV counterparts (see, for example, Philippe *et al.*, 2006; Cornu and Cathomen, 2007; Wanisch and Yáñez-Muñoz, 2009; Mátraí *et al.*, 2011). This phenomenon does not seem to be restricted to HIV-based systems since several experiments have extended it to IDLVs derived from feline immunodeficiency virus and foamy virus (Loewen *et al.*, 2003; Deyle *et al.*, 2010). That DNA episomes of viral origin (Levero *et al.*, 2009; You 2010; Nevels *et al.*, 2011) or otherwise can become “chromatinized” and thus acquire nucleosome-like properties soon upon entry into the nucleus is well established. Whether this assembly of protein–DNA complexes promotes or represses gene expression through chromatin opening or compaction, respectively, is contingent upon a plethora of variables that are likely to include the nuclear domain localization of the episomes and their *cis*- and *trans*-acting elements, as well as the physiological statuses of the host cells (Bishop *et al.*, 2006; Riu *et al.*, 2007; Kantor *et al.*, 2009; Ross *et al.*, 2009; Ross *et al.*, 2011). Ultimately, these tilt the balance between conducive versus repressive high-order epigenetic mechanisms such as those involving histone post-translational modifications of which acetylation, methylation, and ubiquitination are but a few examples (Cedar and Bergman, 2009; Bannister and Kouzarides, 2011).

Hitherto, a systematic side-by-side investigation of the impact of chromatin-remodeling histone deacetylases (HDACs) on the transduction levels and transgene-product yields, resulting from LVs versus IDLVs in cycling and in non-dividing cells, has not been performed. In the present study, by using a diverse array of vector backbones, target cell types, and

FIG. 1. Validation of the integration-defective phenotype of vector particles generated with the aid of packaging construct psPAX2.IN^{D116N}. **(A)** Genetic composition of the HIV-1-based LVs and IDLVs generated for and used in the current study. Lentiviral vectors (LVs) and integration-defective LVs (IDLVs) containing the reporter genes *hrGFP* (humanized *Renilla reniformis* green fluorescence protein) or *eGFP* (*Aequorea victoria* enhanced green fluorescence protein) were generated by using packaging constructs psPAX2 and psPAX2.IN^{D116N}, respectively. These marker genes are under the transcriptional control of the composite viral-cellular CAG regulatory sequence (CAG) or under that of the enhancer/promoter elements from the human cytomegalovirus *immediate-early* (CMV), the human *PGK1* (hPGK), or the human *UBC* (hUbiC) genes. Gray boxes with broken arrow, hybrid HIV-1/Rous sarcoma virus (RSV) or HIV-1/CMV 5' long terminal repeats (LTR); Ψ, HIV-1 packaging signal; solid arrows, open reading frames (ORFs) of *eGFP* or *hrGFP*; gray arrows, above-specified enhancer/promoter *cis*-acting elements; RRE, Rev-responsive element; cPPT, complementary polypurine tract; rβGpA and mMpA, rabbit β-globin and murine metallothionein polyadenylation signals, respectively; HBV PRE and WHV PRE, human hepatitis B virus and Woodchuck hepatitis virus post-transcriptional regulatory elements, respectively. All vectors have a self-inactivating (SIN) U3 3' LTR deletion architecture (gray boxes without broken arrow). **(B)** Monitoring of the frequency of *hrGFP*-positive cells in HeLa cell cultures exposed to three different amounts of psPAX2-based LV.CAG.*hrGFP* or psPAX2.IN^{D116N}-based IDLV.CAG.*hrGFP* vectors (47 ng, 15.7 ng, and 5.2 ng of p24^{gag}/8 × 10⁴ cells). Left panel, flow cytometric analysis was carried out at 3 days post-transduction to measure the initial frequency of *hrGFP*-positive cells (left-hand graph). Middle panel, frequencies of reporter-positive cells in HeLa cell cultures at different time points post-transduction plotted in relation to those measured by flow cytometry at 3 days post-transduction. Right panel, representative direct fluorescence microscopy images corresponding to HeLa cell cultures at 13 days after having been incubated with LV.CAG.*hrGFP* or with IDLV.CAG.*hrGFP* at concentrations, from left to right, of 47 ng, 15.7 ng, or 5.2 ng of p24^{gag}/8 × 10⁴ cells. Size bars in the micrographs of panel B denote 200 μm. **(C)** Flow cytometric analysis of long-term HeLa cell populations that were initially exposed to IDLV.CAG.*hrGFP* or to LV.CAG.*hrGFP* at 47 ng of p24^{gag}/8 × 10⁴ cells. At 25 days post-transduction IDLV.CAG.*hrGFP*- and LV.CAG.*hrGFP*-transduced cells were mock-treated (UNT) or were treated with TSA (4 μM), NaBu (10 mM), or 5-AzaC (4 μM) for 24 h. Measurements of the percentage of reporter-positive cells and mean fluorescence intensities (MFI) were carried out 24 h later. **(D)** LV.CAG.*hrGFP* and IDLV.CAG.*hrGFP* titers quantified by qPCR in HeLa cell cultures at the indicated days post-transduction. At the onset, HeLa cell cultures containing 8 × 10⁴ cells were incubated with 2.6 × 10⁶ vgc/ml of either LV.CAG.*hrGFP* or IDLV.CAG.*hrGFP*.

second- or third-generation packaging constructs, we show that histone deacetylation constitutes a major cellular determinant underlying the transcriptional underperformance of IDLVs. By using chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR), we establish that post-translational histone modifications characteristic of transcriptionally active *loci* do associate preferentially with LV genomes as opposed to IDLV genomes. Upon HDAC inhibition, however, enrichment of these open chromatin marks could be detected on IDLV DNA, which in turn, correlated with an increase in transgene expression as measured at the mRNA and protein levels. Finally, we present a working model for this experimental outcome and briefly discuss the most direct practical consequences emerging from our findings.

Materials and Methods

Cells

293T cells, used for vector production, and HeLa cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Breda, The Netherlands) supplemented with 10 and 5% fetal bovine serum, respectively (FBS; Invitrogen) at 37°C in a humidified-air 10% CO₂ atmosphere. The origin and the culture conditions for the human myoblasts and the human bone-marrow-derived mesenchymal stem cells (hMSCs) used in this study have been described previously (Cudré-Mauroux *et al.*, 2003; Knaän-Shanzer *et al.*, 2005; Gonçalves *et al.*, 2008; Gonçalves *et al.*, 2011).

Plasmids

The four lentiviral vector shuttle plasmids used in this study are described in Figure 1A. The plasmids FUGW and CMVPRES (herein referred to as pLV.hUbiC.eGFP and pLV.CMV.eGFP, respectively) have also been described elsewhere (Lois *et al.*, 2002; Seppen *et al.*, 2002). Detailed information about the plasmids pLV.hPGK.eGFP and pLV.CAG.hrGFP can be found through their GenBank accession numbers JQ627826 and JQ627827, respectively. The second-generation packaging plasmid psPAX2 was provided by Didier Trono (Addgene plasmid 12260; Cambridge, MA), whilst the third-generation packaging plasmid pLP1, the HIV-1 *rev* expression plasmid pLP2, and the vesicular stomatitis glycoprotein-G (VSV-G) pseudotyping construct pLP/VSVG are from Invitrogen. A derivative of packaging plasmid pLP1 encoding IN^{D116N}, pLP1.IN^{D116N}, was used to obtain psPAX2.IN^{D116N}. Briefly, pLP1.IN^{D116N} and psPAX2 were digested with AflIII and Eco32I (both from Fermentas, St. Leon-Rot, Germany). The 1.7-kb insert bearing the mutation for IN^{D116N} was ligated to the 8.9-kb backbone fragment from psPAX2 yielding psPAX2.IN^{D116N}. The presence of the point mutation in the resulting clones was verified by nucleotide sequencing.

Vector production

The above-mentioned vector shuttle plasmids were used for the generation of integration-competent or integration-defective vector stocks. The former were made by co-transfecting psPAX2 and pLP/VSVG, whereas the latter were generated by co-transfecting psPAX2.IN^{D116N} and pLP/VSVG, respectively. The shuttle plasmid pLV.CMV.eGFP was also deployed to make not only second- but also third-generation integration-competent or integration-defective vector stocks by using pLP1, pLP2, and pLP/VSVG or pLP1.IN^{D116N}, pLP2 and pLP/VSVG, respectively. The vector production protocol made use of 25-kDa linear polyethyleneimine (PEI; Polysciences, Warrington, PA) as transfection agent (Askar *et al.*, 2012). Briefly, 17×10^6 293T cells were seeded per 175-cm² culture flask (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). The following day, the cells were transfected by adding to 19 ml of regular medium, 1 ml of a 150 mM NaCl solution containing a mixture of 30 μ g of DNA composed of shuttle, packaging, and pseudotyping plasmids at a ratio of 2:1:1 (size-normalized for molecule copy number) and 90 μ l of PEI at 1 mg/ml. For the production of third-generation LV.CMV.eGFP and IDLV.CMV.eGFP stocks, 293T cells were co-transfected with 30 μ g of a plasmid mixture consisting of pLV.CMV.eGFP, pLP1 (or pLP1.IN^{D116N}), pLP2, and pLP/VSVG at a ratio of 2:1:1:1 (size-normalized for molecule copy number). The final 20-ml transfection mixtures were maintained overnight onto the cells, after which, transfection media were removed and replaced by DMEM supplemented with 5% FBS. Of note, each LV/IDLV pair was generated, processed, and titrated in parallel. Direct fluorescence microscopy on 293T producer cells one day post-transfection served to verify similar transfection efficiencies. Characteristic microscopic fields corresponding to some of these productions are shown in Supplementary Figure S1 (Supplementary Material available online at www.liebertonline.com/hum). Two days later the

conditioned media were collected and the cellular debris were removed by centrifugation. Next, the supernatants were filtrated through 0.45- μ m pore-sized cellulose acetate filters (Pall, Mijdrecht, The Netherlands), and the resulting clarified vector preparations were either stored at -80°C until further utilization or were concentrated as follows. Clarified producer-cell supernatants were laid onto 5-ml 20% (w/v) sucrose cushions in 35.8-ml polyallomer tubes (Beckman Coulter, Woerden, The Netherlands) and were subjected to ultracentrifugation (15,000 rpm for 2 h at 4°C) in an Optima LE-80K ultracentrifuge (Beckman Coulter) using the SW28 rotor. After removing the supernatant, the vector particle-containing pellets were resuspended in 400 μ l ice-cold phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin by rocking overnight at 4°C . The next day, the vector particles were collected, aliquoted, and stored at -80°C until further use.

Vector titrations

Physical particle titers of all vector preparations were determined by using the RETROTEK HIV-1 p24 antigen ELISA kit as specified by the manufacturer (ZeptoMetrix, Eersel, The Netherlands).

A second titration method, based on qPCR, was used to measure vector genome copies (vgc) per ml (vgc/ml) in transduced cells. Briefly, HeLa cells were plated at a density of 8×10^4 cells per well of 24-well plates (Greiner Bio-One). Twenty-four hours later the cells were either mock-transduced or were transduced with six three-fold dilutions of the vector stock. At 24 h post-transduction, the cells were extensively washed with PBS before total cellular DNA was extracted by using the DNeasy Blood & Tissue kit (Qiagen, Venlo, The Netherlands) (Kutner *et al.*, 2009).

To detect the various lentiviral vector forms, primers were designed to hybridize to sequences present within the LTR U5 region and the packaging signal. PCR amplifications with previously published primers (van Tuyn *et al.*, 2005) targeting the human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) locus provided for an internal control to normalize, on a per sample basis, vector to chromosomal DNA copy numbers. In addition, we ruled out the carryover of significant amounts of shuttle plasmid DNA into target cells by performing qPCR with a primer set designed to amplify the prokaryotic β -lactamase. The oligodeoxyribonucleotides and detailed reaction mixture compositions deployed in these assays are listed in Supplementary Table 1. The PCR amplifications were performed by using 1 $\times$ Colorless GoTaq Flexi Buffer (Promega, Leiden, The Netherlands), 200 nM deoxynucleoside triphosphates (dNTPs) (Invitrogen), 200 nM of each primer (Supplementary Table 1), 0.1 $\times$ SyBR Green (Invitrogen), and 2 U of GoTaq Flexi DNA polymerase (Promega).

The cycling steps were carried out in a CFX Connect (Bio-Rad, Veenendaal, The Netherlands) with the parameters set as follows. After a 1-min denaturation step at 95°C , the samples were subjected to 35 cycles consisting of 20 sec at 95°C , 20 sec at the annealing temperature specified in Supplementary Table 1, and 20 sec at the elongation temperature of 72°C . The amount of vector DNA molecules within a cell and the amount of cell genomes per sample were determined on the basis of calibration curves using 10-fold serial dilutions of plasmids containing the target sequences. These

DNA templates consisted of the shuttle plasmids used to produce the corresponding lentiviral vector stocks and the pCRII-TOPO cloning vector (Invitrogen) carrying a human *GAPDH* fragment. Vector genome copies per ml were calculated according to the following formula: normalized vector genome copies $\times$ number of transduced cells/volume of lentiviral vector stock (ml) (Charrier *et al.*, 2005).

The vector doses as measured by ELISA and qPCR and expressed in terms of ng of p24^{gag} antigen and vector genome copies per ml, respectively, are shown in Supplementary Table 2.

Reverse transcription qPCR analysis

To quantify transgene expression at the RNA level in the absence or in the presence of trichostatin A (TSA), experiments were conducted as follows. HeLa cells were plated at a density of 4×10^5 cells per well of 6-well plates (Greiner Bio-One). Twenty-four hours later, the cells were either mock-transduced or were transduced with 2.4×10^6 vgc/ml of LV.CMV.eGFP or of IDLV.CMV.eGFP or with 1.3×10^6 vgc/ml of LV.hPGK.eGFP or of IDLV.hPGK.eGFP. The HeLa cell transductions were performed in the absence or in the presence of TSA at $4 \mu\text{M}$. One day post-transduction the cells were extensively washed with PBS after which they were trypsinized and divided. Half of the cells were used to determine, by qPCR, vector genome copies per ml as described in the previous section, whereas the other half were processed for reverse transcription (RT)-qPCR analysis. To this end, total RNA was extracted by applying the NucleoSpin RNA II kit protocol (BioKé, Leiden, The Netherlands) and reverse transcription of $1 \mu\text{g}$ of total RNA was carried out at 50°C for 1 h by using 200 ng of random primers, 200 nM dNTPs, $1 \times$ First-Strand Buffer, 5 mM dithiothreitol, and 200 U of SuperScript III Reverse Transcriptase (all from Invitrogen). Subsequently, a 10-fold dilution of the resulting cDNA was used for the amplification of *eGFP* and endogenous human *GAPDH* transcripts using the primers and PCR conditions listed in Supplementary Table 1, whereas the general cycling parameters are described under the previous section. The quantification of transgene and housekeeping *GAPDH* gene expression was calculated on the basis of 10-fold serial dilutions of the shuttle plasmid used to produce the respective lentiviral vector stock and the pCR4-TOPO cloning vector (Invitrogen) containing the human *GAPDH* target sequence, respectively.

Functional assay to establish the integration competency of vectors with wild-type IN or class I mutant IN^{D116N}

To validate the integration-defective phenotype conferred by the IN mutant IN^{D116N}, HeLa cells were seeded at a density of 8×10^4 cells per well of 24-well plates (Greiner Bio-One). Twenty-four hours later the cells were transduced with 5.2, 15.7, or 47 ng of p24^{gag} of either LV.CAG.hrGFP or IDLV.CAG.hrGFP. The *hrGFP* expression was quantified by flow cytometry at 3, 8, 14, 20, and 25 days post-transduction. HeLa cells transduced with LV.CAG.hrGFP or IDLV.CAG.hrGFP at a dose of 47 ng of p24^{gag} were also treated 25 days post-transduction with $4 \mu\text{M}$ of TSA, 10 mM of sodium butyrate (NaBu), or $4 \mu\text{M}$ of 5-azacytidine (5-AzaC) for 24 h before flow cytometry (LSR-II; BD Biosciences, Breda, The Netherlands). To confirm the loss of IDLV genomes, similar

experiments were performed on HeLa cells seeded at a density of 8×10^4 cells per well of 24-well plates and transduced with 2.6×10^6 vgc/ml of either LV.CAG.hrGFP or IDLV.CAG.hrGFP. Total cellular DNA was extracted at 1, 5, and 26 days post-transduction and used to quantify the amount of lentiviral vector DNA by qPCR as described in the section "Vector titrations."

Transduction experiments and treatments with HDAC inhibitors

HeLa cells and human myoblasts were plated at a density of 8×10^4 and 2×10^5 cells per well of 24-well plates (Greiner Bio-One), respectively. Twenty-four hours later the cells were transduced with either the LVs or the IDLVs. The following amounts of p24^{gag} physical particle doses were applied: 50 ng of LV.CAG.hrGFP or IDLV.CAG.hrGFP, 93 ng of LV.CMV.eGFP or IDLV.CMV.eGFP (2nd generation), 93 ng of LV.hPGK.eGFP or IDLV.hPGK.eGFP, 290 ng of LV.hUbiC.eGFP or IDLV.hUbiC.eGFP, and 93 ng of LV.CMV.eGFP.3rd or IDLV.CMV.eGFP.3rd (3rd generation). Transductions were performed in the absence or in the presence of the HDACs inhibitors, NaBu and TSA. NaBu was used at a final concentration of 10 mM, whereas TSA was applied at final concentrations of 1 or $4 \mu\text{M}$ in experiments involving HeLa cells and $8 \mu\text{M}$ in those using human myoblasts. One day post-transduction, the medium was removed from HeLa cells and was replaced by regular medium, whereas myoblasts were kept in their initial transduction medium for another 24 h. At 2 days post-transduction, *hrGFP* or *eGFP* expression was first assessed by direct fluorescent microscopy and subsequently quantified by flow cytometry.

To investigate the role of cell replication status, transduction experiments were also performed in post-mitotic myotubes and in growth-arrested hMSCs. Skeletal myotubes were obtained by seeding 2×10^5 human myoblasts per well of 24-well plates (Greiner Bio-One). These cells were used for transduction experiments and 5-bromo-2'-deoxyuridine (BrdU) DNA incorporation assays.

One day later, the cells were washed twice with PBS and maintained for 5 days in differentiation medium (DM) (Cudré-Mauroux *et al.*, 2003; Gonçalves *et al.*, 2008; Gonçalves *et al.*, 2011). The resulting myotubes were then transduced with either LV.CMV.eGFP or IDLV.CMV.eGFP at 93 ng of p24^{gag} per well in the absence or in the presence of either $8 \mu\text{M}$ TSA or 10 mM NaBu. Twenty-four hours later, the medium was removed and replaced by fresh DM. Two days post-transduction, the *eGFP* expression was monitored by direct fluorescence microscopy. hMSCs were plated at 1×10^5 or 5×10^4 cells per well of 24-well plates (Greiner Bio-One). The following day, the higher-density seeded hMSCs were washed twice with PBS and were maintained for 6 days in DM, whereas the lower-density seeded hMSCs remained in regular growth medium (GM). At the day of transduction, cells kept in GM or in DM were used for the BrdU DNA incorporation assay specified below. hMSCs cultured for 6 days in DM were transduced with LV.CMV.eGFP or IDLV.CMV.eGFP. Each vector was applied at 784 ng of p24^{gag} per well in the absence or in the presence of either $8 \mu\text{M}$ TSA or 8 mM NaBu. The cells were maintained in their transduction medium for 4 days. At this time point, the *eGFP* expression was quantified by flow cytometry.

Vector dose response

Vector dose response experiments on HeLa cells in the absence or in the presence of TSA were done by serial dilutions of the vector stocks followed by flow cytometry. In brief, HeLa cells were seeded at a density of 8×10^4 cells per well of 24-well plates (Greiner Bio-One). Twenty-four hours later, these cells were transduced with three-fold vector dilutions either in the absence or in the presence of $4 \mu\text{M}$ of TSA. The following day the cell monolayers were washed once with PBS and fresh regular medium was added. At 2 days post-transduction, frequencies of reporter-positive cells and the mean fluorescence intensity (MFI) values were determined by flow cytometry.

BrdU DNA incorporation assay

The proliferative status of cells in myotube and hMSC cultures was assessed by the incorporation of BrdU into chromosomal DNA. These cultures were incubated at 37°C for 3 h in the absence or in the presence of this thymidine analog at $30 \mu\text{g}/\text{ml}$. Subsequently, nuclei displaying BrdU incorporation were visualized by immunofluorescence microscopy using a fluorescein-conjugated anti-BrdU antibody (Roche, Woerden, The Netherlands). Briefly, after the 3-h BrdU incubation period, the BrdU-containing medium was removed and the cells were washed twice with PBS. The cells were subsequently fixed by exposing them to ice-cold methanol for 10 min at 4°C , after which they were air-dried for a few minutes and rehydrated with PBS. The DNA denaturation step was accomplished by adding 0.07% NaOH in 70% ethanol for 10 min. Sequential dehydration steps consisting of adding 70%, 90%, and 100% ethanol onto the specimens over a 10-min period were followed by air-drying and a 10-min blocking step in the presence of 0.5% (w/v) Boehringer Milk Powder (BMP; Roche) in PBS. A mixture of $5.3 \mu\text{l}$ of the anti-BrdU antibody diluted in $100 \mu\text{l}$ PBS containing 0.5% BMP was then added to the cells that were kept in the dark for 1 h at room temperature before being washed five times with PBS. Finally, the nuclei were stained with $10 \mu\text{g}/\text{ml}$ Hoechst 33342 (Invitrogen) in PBS, followed by four washes with PBS. Frequencies of BrdU-positive nuclei were determined using an Olympus IX51 inverse fluorescence microscope (Olympus, Zoeterwoude, The Netherlands). Images were captured by a ColorView II Peltier-cooled charge-coupled device camera (Olympus) and were archived using Cell[^]F software (Olympus).

ChIP analysis

HeLa cells were plated at a density of 4×10^6 cells per 10-cm^2 culture dish ($100 \times 20 \text{ mm}$; Greiner Bio-One). Twenty-four hours later, the cells were transduced with 1.3×10^7 vgc/ml of LV.hPGK.eGFP or of IDLV.hPGK.eGFP. Transductions with IDLVs were performed in the absence or in the presence of TSA at $4 \mu\text{M}$. Two days later, the cells were exposed to a 1% solution of the cross-linking agent formaldehyde, after which their DNA was collected and sheared by sonication to an average size of 900 bp. Next, the DNA was subjected to ChIP by using the EpiQuick Chromatin Immunoprecipitation Kit (Epigentek, Uithoorn, The Netherlands). The ChIP assay was validated by deploying as positive control an RNA polymerase II-specific antibody and, as negative con-

trol, a mouse IgG antibody together with GAPDH-specific primers (Millipore, Amsterdam, The Netherlands) targeting the 3' end of the GAPDH promoter (Supplementary Table 1). The antibodies used for the ChIP analysis were raised against histone 3 (H3; Millipore) or against H3 protein variants acetylated at lysines 9 and 14 (H3K9/K14ac; Epigentek) or tri-methylated at lysine 4 (H3K4me3; Millipore). The resulting immunoprecipitated DNA and corresponding input DNA were quantified by qPCR using the primers and reaction conditions specified in Supplementary Table 1 and the general cycling parameters given under the section "Vector titrations." The qPCR data were presented as percent input and were calculated according to the following formula (%) (where Ct is the cycle threshold): $2^{-(\text{Ct}(\text{ChIP}) - \text{Ct}(\text{Input}))} \times 100 \times \text{DNA input dilution factor}$, where Ct(ChIP) is the Ct value of the immunoprecipitated DNA using the antibody of interest and Ct(Input) is the Ct value of the input DNA. All PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining to confirm their identity.

Statistics

Data were analyzed by using the GraphPad Prism 5 software package and evaluated for significance by applying unpaired two-way Student's *t*-tests ($P < 0.05$ considered significant).

Results

Generation of lentiviral vectors harboring wild-type or catalytic site mutant IN moieties and validation of their phenotypes

Vector particles were produced by co-transfecting 293T cells with shuttle plasmid pLV.CAG.hrGFP (Fig. 1A, upper diagram), a VSV-G expression plasmid, and the packaging construct psPAX2 or its derivative psPAX2.IN^{D116N}, encoding wild-type IN or mutant IN^{D116N} peptides, respectively. The resulting VSV-G-pseudotyped vectors LV.CAG.hrGFP and IDLV.CAG.hrGFP were titrated using an HIV-1 p24^{gag} ELISA assay and were incubated with human cervix carcinoma HeLa cells at the following doses: 47 ng, 15.7 ng, and 5.2 ng of p24^{gag}/ 8×10^4 cells. The frequency of hrGFP-positive cells was monitored at 3 and the indicated days post-transduction by flow cytometry and direct fluorescence microscopy (Fig. 1B). This functional assay served as an indicator for the insertion of vector DNA into the chromosomes of transduced cells. The vector LV.CAG.hrGFP, made with the aid of psPAX2, gave rise to a fraction of reporter-positive cells that did not significantly diminish upon extensive subculturing, whereas the vector IDLV.CAG.hrGFP, generated by using psPAX2.IN^{D116N}, led to a time-dependent decline in the frequency of hrGFP-positive cells (Fig. 1B, middle and left panels). In fact, at 25 days post-transduction, and thereafter (not shown), the proportion of hrGFP-positive cells in cultures initially exposed to IDLV.CAG.hrGFP was on average 320-fold lower than in those originally incubated with LV.CAG.hrGFP with absolute frequencies of reporter-marked cells of 0.01, 0.01, and 0.03% for the populations exposed to the lowest, intermediate, and highest IDLV doses, respectively (Fig. 1B, middle panel). These residual frequencies of stably transduced cells are within the range reported in

other studies deploying vectors harboring class I IN mutants and result from IDLV host chromosomal integration through a non-canonical, IN-independent process, presumably at sporadic double-stranded DNA breaks (Wanisch and Yanez-Munoz, 2009; Matrai *et al.*, 2011).

Exposure of long-term cultures stably transduced with IDLV.CAG.hrGFP or LV.CAG.hrGFP to the HDAC inhibitors NaBu or TSA or to the DNA methyltransferase inhibitor 5-AzaC had no effect on the fraction of hrGFP-positive cells (Fig. 1C, upper graphs) and, depending on the small-molecule drug applied, no impact or only a *circa* 1.5-fold increase on the MFI values (Fig. 1C, middle graphs). Of note, this slight increase in MFI upon TSA or NaBu treatment was similar in cells stably transduced with IDLV.CAG.hrGFP or with LV.CAG.hrGFP (Fig. 1C, lower graph). These data reveal that NaBu, TSA, and 5-AzaC do not overtly alter the intrinsic transcriptional activity of the recombinant expression unit. These results further suggest that the decline in the frequency of hrGFP-positive cells reflects IDLV.CAG.hrGFP episomal loss due to ongoing cell division as opposed to being the result of epigenetic promoter silencing of chromosomally integrated transgenes. By deploying qPCR to measure vgc/ml in HeLa cell cultures at early and late time-points post-transduction, we established that the drop in the frequency of reporter-positive cells is indeed the result of IDLV.CAG.hrGFP-specific DNA loss (Fig. 1D). We conclude that vectors made with the aid of psPAX2.IN^{D116N} display an integration-defective phenotype.

HDAC inhibition sharply induces transgene expression in human cells transduced with integration-defective IDLV.CAG.hrGFP

We asked whether HDACs play a role in modulating IDLV transgene expression and whether these chromatin-remodeling cellular factors regulate IDLVs and LVs in a differential manner. To this end, we started by carrying out transduction experiments in human cervix carcinoma HeLa cells and in human myoblasts using the VSV-G-pseudotyped vectors IDLV.CAG.hrGFP and LV.CAG.hrGFP (Fig. 1) together with the HDAC inhibitors NaBu and TSA. These small-molecule drugs constitute commonly used probes to study the epigenetic regulation of gene expression involving HDACs. TSA in particular inhibits specifically HDACs from class I, II, and IV (Keedy *et al.*, 2009), whereas NaBu is known to display a more pleiotropic effect.

Thus, HeLa cells and human myoblasts were exposed to equivalent amounts of LV.CAG.hrGFP and IDLV.CAG.hrGFP in the absence (UNT) or in the presence of TSA (1 μ M and 4 μ M) or of NaBu (10 mM). At 48 h post-transduction, the parameters evaluated through flow cytometry were the transgene product yields as reflected by the MFI values and the frequency of hrGFP-positive cells. In untreated cells, both parameters, that is, the transcriptional activity (Fig. 2A and C) and the percentage of reporter-positive cells (Fig. 2B and D) were clearly higher in the cell cultures exposed to LV.CAG.hrGFP than in those incubated with IDLV.CAG.hrGFP. These data are consistent with the results presented in the left panel of Figure 1B as well as with the aforesaid studies reporting on the underperformance of IDLVs. Interestingly, the transgene activity in HeLa cells and in human myoblasts that were treated with the HDAC

inhibitors and transduced with IDLV.CAG.hrGFP (solid bars in upper panels of Fig. 2A and C, respectively) was significantly higher than that measured in their respective untreated cultures. Of note, a TSA dose-effect on the increment of MFI values could also be discerned.

Conversely, this drug-dependent transgene activation was much less pronounced in the parallel cell cultures that were treated with the same dosages of HDAC inhibitors but were transduced with LV.CAG.hrGFP instead (gray bars in upper panels of Fig. 2A and C). Indeed, presentation of these data in terms of the fold increase in MFI values over those corresponding to the respective untreated controls unambiguously shows IDLV.CAG.hrGFP as being the most drug-responsive of the two vector classes (lower panels of Fig. 2A and C). It is also noteworthy mentioning that, in contrast to those measured in the human myoblast cultures, the frequencies of reporter-positive cells were virtually unchanged by the use of HDAC inhibitors in HeLa cell cultures (Fig. 2B and D). Finally, the conducive role of TSA on IDLV.CAG.hrGFP-mediated transgene expression can also be grasped by inspecting the flow cytometry dot plots and micrographs depicted in Figure 2E and F, respectively.

Inhibition of HDACs induces IDLV-mediated transgene expression regardless of vector genetic makeup

Next, we sought to investigate the extent to which the above findings hold in vectors with different backbones. To this end, we generated integration-defective vectors IDLV.CMV.eGFP, IDLV.hPGK.eGFP, and IDLV.hUbiC.eGFP in parallel with their respective integration-competent counterparts LV.CMV.eGFP, LV.hPGK.eGFP, and LV.hUbiC.eGFP (Fig. 1A). These vectors contain, amongst other *cis*-acting sequences, regulatory elements from the human cytomegalovirus *immediate-early* enhancer/promoter (CMV) or from the human housekeeping *PGK1* (hPGK) or ubiquitin C-encoding *UBC* (hUbiC) genes. Transduction experiments with the vector set harboring the CMV-based expression unit revealed again that the integration-proficient version led to significantly higher transgene expression levels and functional gene delivery activities than its integration-defective counterpart in HeLa cells (upper panel of Fig. 3A and B, compare the gray with the solid bars labeled UNT) and in human myoblasts (upper panel of Fig. 3C and D, compare the gray with the solid bars labeled UNT). Interestingly, in these experiments, the transcriptional activity from the LV.CMV.eGFP genomes was also induced by treatment of target cells with NaBu or with TSA. Nonetheless, in conformity with the previous results obtained with IDLV.CAG.hrGFP and LV.CAG.hrGFP, the IDLV.CMV.eGFP vector was the most responsive to HDAC inhibition in HeLa cells (lower panel of Fig. 3A and E) and in human myoblasts (lower panel of Fig. 3C). In fact, IDLV.CMV.eGFP transductions carried out in the presence of 4 and 8 μ M of TSA or of 10 mM of NaBu fully rescued transgene expression levels as well as percentages of reporter-positive cells. Data presented in Supplementary Figure S2 further shows that HDACs also modulate to a higher extent the transcriptional activity of IDLVs made with the aid of a third-generation packaging system.

As of yet, all transduction experiments involved vectors whose transcription units comprise enhancer/promoter

FIG. 2. Testing the effect of histone deacetylase (HDAC) inhibition on the transgene product yields and transduction levels achieved by LV.CAG.hrGFP versus IDLV.CAG.hrGFP. Flow cytometric analysis at 48 h post-transduction of HeLa cells (**A**, **B**, and **E**) exposed for 24 h to LV.CAG.hrGFP or to IDLV.CAG.hrGFP at 50 ng of p24^{gag}/8×10⁴ cells in the absence (UNT) or in the presence of TSA (1 μM or 4 μM) or NaBu (10 mM). Flow cytometry at 48 h post-transduction of human myoblasts (**C** and **D**) incubated for 48 h with LV.CAG.hrGFP or with IDLV.CAG.hrGFP at 50 ng of p24^{gag}/2×10⁵ cells in the absence (UNT) or in the presence of TSA (4 μM or 8 μM) or NaBu (10 mM). The flow cytometry results are derived from a minimum of three independent experiments and are expressed in terms of the percentage of reporter-positive cells and corresponding MFI values with bars and error bars representing means and standard deviations, respectively. The data presented in the lower panels of (**A**) and (**C**) represent the fold increment in MFI resulting from HDAC inhibition and were computed from the results shown in their respective upper panels. Numerals between brackets correspond to MFI fold increase values that are not statistically significant. (**F**) Characteristic hrGFP direct fluorescence microscopy photographs at 48 h post-transduction of untreated (UNT) or one-day TSA-treated HeLa cell cultures transduced with LV.CAG.hrGFP (LV) or with IDLV.CAG.hrGFP (IDLV) at 50 ng of p24^{gag}/8×10⁴ cells. Bars=100 μm.

FIG. 3. Impact of HDAC inhibition on the transgene product yields and transduction levels obtained by LV.CMV.eGFP versus IDLV.CMV.eGFP. Flow cytometric analysis at 48 h post-transduction of HeLa cells (**A** and **B**) untreated (UNT) or treated for 24 h with 4 μ M TSA or 10 mM NaBu and exposed to LV.CMV.eGFP or to IDLV.CMV.eGFP at 93 ng of p24^{gag}/8 $\times$ 10⁴ cells. Flow cytometry at 48 h post-transduction of human myoblasts (**C** and **D**) incubated for 48 h with LV.CMV.eGFP or with IDLV.CMV.eGFP at 93 ng of p24^{gag}/2 $\times$ 10⁵ cells in the absence (UNT) or in the presence of TSA (4 μ M or 8 μ M) or NaBu (10 mM). The flow cytometry data are derived from a minimum of three independent experiments and are expressed in terms of the percentage of reporter-positive cells and corresponding MFI values with bars and error bars representing means and standard deviations, respectively. The data presented in the lower panels of (**A**) and (**C**) represent the fold increment in MFI resulting from HDAC inhibition and were calculated from the results shown in their respective upper panels. Numerals between brackets correspond to MFI fold increase values that are not statistically significant. (**E**) Representative eGFP direct fluorescence microscopy photographs of untreated (UNT) or one-day TSA- or NaBu-treated HeLa cell cultures (4 μ M or 10 mM, respectively) transduced with LV.CMV.eGFP (LV) or with IDLV.CMV.eGFP (IDLV) at 93 ng of p24^{gag}/2 $\times$ 10⁵ cells, 48 h post-transduction. Bars = 100 μ m.

elements with nonhuman DNA sequences. To discard the possibility that the low level of transgene expression from IDLV episomes is the result of these foreign, nonhuman, transcriptional elements, we next performed transduction experiments with the IDLV and LV set harboring the human *PGK1* or the human *UBC* regulatory sequences (Fig. 1A). Transduction experiments with these two sets of vectors confirmed that, in the absence of HDAC inhibitors, class I IN mutant particles lead to lower transcriptional and transductional activities when compared to those bearing wild-type IN moieties (compare the gray with the solid bars labeled UNT in the upper panels of Fig. 4A, C, G, and I, and in Fig. 4B, D, H, and J).

Importantly, equally in agreement with the previous data, the increase in transgene expression levels resulting from HDAC inhibition was in general higher in cultures exposed to IDLV.hPGK.eGFP (solid bars in Fig. 4A and C) or to IDLV.hUbiC.eGFP (solid bars in Fig. 4G and I) than in those incubated with LV.hPGK.eGFP (gray bars in Fig. 4A and C) or with LV.hUbiC.eGFP (gray bars in Fig. 4G and I). These differential drug-dependent relative increases in the MFI values are plotted in the lower graphs of Figure 4A, C, G, and I. This general drug-dependent enhancement of IDLV-born transgene expression has most likely in turn contributed to a measurable increase in the frequency of reporter-positive cells in HeLa and in human myoblast cultures transduced

with IDLV.hPGK.eGFP (solid bars in Fig. 4B and D) or with IDLV.hUbiC.eGFP (solid bars in Fig. 4H and J). Typical flow cytometry dot plots and direct fluorescence microscopy images corresponding to these experiments are shown in Fig. 4E and F, respectively.

Cumulative analysis of the differential transcriptional activities resulting from HDAC inhibition in HeLa cells and in human myoblasts transduced with each of the two vector classes are depicted in Figure 5. These cumulative data show that TSA or NaBu treatments result in similar levels of IDLV-specific transgene activation (Fig. 5A). Crucially, IDLVs are invariably the most affected by HDAC activity in myoblasts and in HeLa cells regardless of vector genetic makeup (Fig. 5B and C, respectively). Nonetheless, often, the extent of HDAC-dependent IDLV transgene expression inhibition varied according to the target cell type and the vector backbone used (Fig. 5A–C).

To further investigate the relationship between vector chromosomal integration competencies on the one hand and the impact of HDACs on transgene expression parameters on the other, we performed vector dose-response experiments in HeLa cells treated or not treated with TSA. These experiments revealed a higher susceptibility of IDLVs to HDAC-mediated transgene repression across a wide, over two-order of magnitude, range of vector amounts with different IDLV backbone-specific trend lines reflecting variation in TSA-dependent transgene activation levels (Fig. 6). The latter finding strengthens the assertion that the magnitude of IDLV responsiveness to HDAC inhibition depends, to some extent, on their particular genetic makeup. Conversely, with the exception of the modest upregulation of transgene expression from the CMV-containing vector LV.CMV.eGFP, integration-competent LVs were remarkably unresponsive to HDAC inhibition (Fig. 6).

To complement the data on the quantification of transgene expression at the reporter protein level, we set up transduction experiments similar to those carried out previously but used a RT-qPCR read-out system instead in order to measure transgene mRNA levels. To that end, HeLa cells were exposed or not exposed to TSA and were transduced with vectors harboring the regulatory elements hPGK (Fig.

7A) or CMV (Fig. 7B). Consistent with the flow cytometric data, *eGFP* transcript numbers were augmented to a greater extent by TSA in IDLV- than in LV-transduced cells. Furthermore, the highest *eGFP* mRNA expression levels generated by the IDLV pair in TSA-treated cultures were similar to those measured in TSA-treated cultures exposed to their respective LV counterparts (Fig. 7A and B, dashed line in upper graphs). Finally, in these experiments we also took the opportunity to confirm by qPCR that the amounts of LV and IDLV genomes in the target cells were equivalent (Fig. 7A and B, lower graphs).

Inhibition of HDACs induces IDLV-mediated transgene expression in post-mitotic and non-dividing cells

As aforesaid, some of the experimental and clinical set-ups in which IDLVs are deemed advantageous involve the transduction of post-mitotic or non-dividing cells. Therefore, we sought to investigate whether the preferential modulation of IDLVs by HDACs also takes place in non-dividing target cells. To this end, we started by performing transduction experiments in skeletal myotubes. These myotubes arise after cell-cycle exit and fusion of mononucleated precursor myoblasts exposed to mitogen-poor DM (Walsh and Perlman, 1997). The post-mitotic character of the resulting syncytial structures is well established. Nonetheless, we confirmed this assertion by using an immunofluorescence-based assay that identifies nuclei in the S phase of the cell cycle via the detection of BrdU incorporation in nascent DNA chains. Dividing myoblasts readily incorporated BrdU, whilst differentiated myotubes identified by large clusters of Hoechst 33342-labeled nuclei did not (Fig. 8A, compare upper with lower panels). Transduction of these post-mitotic structures with IDLV.CMV.eGFP or with LV.CMV.eGFP in the presence or in the absence of HDAC inhibitors revealed a clear drug-dependent enhancement of transgene expression. Importantly, this enhancement was most prominent in the myotube cultures exposed to the IDLV.CMV.eGFP vector (Fig. 8B). Taken together, these results are in line with those previously obtained in dividing myoblasts (Fig. 3 and Supplementary Fig. S2), suggesting that the post-mitotic status of

FIG. 4. Effect of HDAC activity on the transcriptional and transductional levels achieved by LVs versus IDLVs harboring human *PGK1*- or human *UBC*-derived regulatory elements. Flow cytometric analysis at 48 h post-transduction of HeLa cells (A and B) and human myoblasts (C and D) transduced with LV.hPGK.eGFP (gray bars) or with IDLV.hPGK.eGFP (solid bars) in the absence (UNT) or in the presence of TSA or NaBu at the indicated concentrations. The vector concentrations applied onto HeLa and human myoblast cultures were 93 ng p24^{gag}/8 × 10⁴ cells and 93 ng p24^{gag}/2 × 10⁵ cells, respectively. Typical flow cytometry dot plots and direct fluorescence microscopy fields of HeLa cells and human myoblasts incubated with LV.CMV.eGFP (LV) or with IDLV.CMV.eGFP (IDLV) particles are shown (panels E and F, respectively). Bars = 100 μm. The upper and lower figures shown within each dot plot correspond to MFI values and frequency of reporter-positive cells, respectively (E). Vector-exposed HeLa cell and human myoblast cultures were kept in regular medium (UNT) or in medium supplemented with TSA or NaBu at the indicated final concentrations. Flow cytometry data and micrographs were both acquired at 48 h post-transduction. Flow cytometric analysis at 48 h post-transduction of HeLa cells (G and H) and human myoblasts (I and J) transduced with LV.hUbiC.eGFP (gray bars) or with IDLV.hUbiC.eGFP (solid bars) in the absence (UNT) or in the presence of TSA or NaBu at the indicated concentrations. The vector doses applied onto HeLa and human myoblast cultures were 290 ng p24^{gag}/8 × 10⁴ cells and 290 ng p24^{gag}/2 × 10⁵ cells, respectively. The flow cytometry data correspond to a minimum of 3 independent experiments and are expressed in terms of the percentage of eGFP-positive cells and respective MFI values. Bars and error bars represent means and standard deviations, respectively. The data presented in the lower panels of (A), (C), (G), and (I) represent the fold increment in MFI resulting from HDAC inhibition and were calculated from the results shown in their respective upper panels. Numerals between brackets correspond to MFI fold increase values that are not statistically significant.

FIG. 5. Cumulative flow cytometric analysis of the impact of HDACs on the transcriptional activity of LVs versus IDLVs. **(A)** The ratios between the transgene activation levels of LVs versus IDLVs caused by TSA or NaBu (gray and solid bars, respectively) in human myoblasts and in HeLa cells was computed from the data presented in panels **(B)** and **(C)**, respectively. Relative activation of transgene expression from LVs and IDLVs (gray and solid bars, respectively) caused by the inhibition of cellular HDACs with TSA or NaBu in human myoblasts **(B)** or in HeLa cells **(C)**. The various vector backbones used in these experiments (Fig. 1A) are indicated by the regulatory elements of their respective expression units, that is, hUbiC, hPGK, CAG, and CMV.

these cells does not alter the higher susceptibility of IDLV templates to HDAC-mediated silencing.

In another experimental system we deployed primary hMSCs. hMSC cultures that were kept for 6 days in DM contained a strongly reduced frequency of dividing cells when compared to parallel cultures exposed to GM instead (Fig. 8C). Transduction experiments performed under these growth-arrested conditions with IDLV.CMV.eGFP or with LV.CMV.eGFP revealed that the process leading to transgene expression from the former templates was clearly the most affected by HDACs (Fig. 8D, upper and lower panels).

Histone modifications associated with open chromatin are underrepresented throughout IDLV genomes.

Finally, we asked whether the transgene expression profiles observed between the two different vector classes correlate with their differential acquisition of specific epigenetic marks. In this regard, it is known that various post-translational histone modifications, whose patterns have been postulated to constitute a “histone code” (Rando, 2012), underlie the transcriptional activity of endogenous genes by regulating the chromatin structure in which they are embedded. Thus, we hypothesized that although LV and IDLV genomes both undergo chromatinization in target cells, the latter templates display in relation to the former a scarcity of histone modifications associated with transcription-conducive open chromatin. To this end, we performed ChIP-qPCR analysis on DNA from HeLa cells transduced with the vector pair harboring the human *PGK1* regulatory sequences. In these experiments, we used primer sets spanning six vector genomic regions together with antibodies raised against H3K9/K14ac or H3K4me3. Both H3K9/K14ac and H3K4me3 are well-established hallmarks of euchromatic DNA (see, for example, Bannister and Kouzarides, 2011). Vector DNA sequences studied in these experiments were those derived from the human *PGK1* locus and the non-human *eGFP* ORF as well as those present in the viral *cis*-acting elements: 5′ LTR, packaging signal (Ψ), Rev-responsive element (RRE), and central polypurine tract (cPPT). We found that, all viral *cis*-acting elements displayed higher amounts of H3K9/K14ac and H3K4me3 on chromatinized LV.hPGK.eGFP than on IDLV.hPGK.eGFP DNA (Fig. 9, compare panel A with panel B). Differing from the above, the 3′ end of the human *PGK1*-derived sequence in LV.hPGK.eGFP and IDLV.hPGK.eGFP exhibited a similar accumulation of H3K4me3 and H3K9/K14ac, with their levels being inferior to those observed at the viral *cis*-acting elements of LV.hPGK.eGFP.

Moreover, the H3K9/K14ac mark was present at substantially lower amounts at the targeted human *PGK1* sequence when compared to those of H3K4me3 (Fig. 9A and B). Interestingly, the level of this epigenetic mark at the 5′ end of *eGFP* in LV.hPGK.eGFP was shown to be much higher than that of H3K4me3. For IDLV.hPGK.eGFP templates, however, this was not found to be the case since the quantities of both H3K9/K14ac and H3K4me3 were similarly low. Significantly, TSA treatment rescued the amounts of H3K4me3 linked to the viral *cis*-acting elements and the 5′ end of the *eGFP* ORF of IDLV to values equivalent to, or higher than, those measured on their LV counterparts (Fig. 9, compare panel A with panel C). Of note, the effect of TSA on the deposition of the H3K9/K14ac mark at the viral and

FIG. 6. Relationship between vector dose and the amplitude of TSA-dependent modulation of transgene expression parameters resulting from LV versus IDLV transductions. The LVs and IDLVs whose structure is shown in Fig. 1A were applied at different doses onto HeLa cells either not treated or treated with 4 μ M of TSA. In all instances, quantification of the transgene expression parameters was done by flow cytometry at 48 h post-transduction. For each vector dose applied (x-axis), the ratios (variation) between the percentages of reporter-positive cells, and MFI values measured in cultures exposed to TSA versus those measured in cultures not treated with TSA, are plotted (y-axis) yielding the various trend lines shown.

nonhuman DNA portions of IDLV templates was either negligible or very modest (Fig. 9). Finally, in contrast to the above, the presence of TSA led to a significant increase in the association of both epigenetic marks with the human *PGK1*-derived DNA in IDLV.hPGK.eGFP genomes. Together, these data correlate well with that depicted in Figure 4A and B and Figure 7A corresponding to TSA-dependent up-regulation of IDLV.hPGK.eGFP transgene expression. Finally, this epigenetic profiling shows that the effect of HDAC inhibition on the levels of IDLV chromatin marking by specific histone modifications can vary between very modest (H3K9/K14ac) to highly robust (H3K4me3).

We conclude that histone modifications normally linked to euchromatin are scarcely distributed along IDLV genomes, which is likely to contribute to their restrained transcriptional activity.

Discussion

IDLVs offer transient and, potentially, long-term transgene expression in dividing and non-dividing cells, respectively, while displaying a much-reduced genotoxic risk (Philpott and Thrasher, 2007; Wanisch and Yáñez-Muñoz, 2009; Banasik and McCray Jr, 2010). At matched genome

copies or physical particle titers, their transcriptional activity is notably diminished when compared to that of their integration-proficient counterparts (see, for instance, Loewen *et al.*, 2003; Philippe *et al.*, 2006; Cornu and Cathomen, 2007; Wanisch and Yáñez-Muñoz, 2009; Deyle *et al.*, 2010; Mátrai *et al.*, 2011). Our experiments in HeLa cells and hMSCs, as well as in a cellular differentiation model based on skeletal muscle progenitors and on post-mitotic myotubes differentiated from them, support the view that histone deacetylation constitutes a major cellular determinant behind the differential transgene expression profiles of LVs and IDLVs. In addition, we showed that the higher transcriptional activation of IDLVs upon HDAC inhibition occurs regardless of the vector backbone, the packaging system deployed, and the tissue source and replication rate of the target cells. Nonetheless, we did observe that the magnitude to which transgene expression from IDLVs is upregulated upon HDAC inhibition can vary according to their composition and target cell type, with rescue levels varying from partial to complete. The former aspect possibly relates to particular regulatory elements or their arrangement, whereas the latter presumably stems from the portfolio and the relative activity of HDACs in the different cell types. In this regard, HeLa cells are enriched in HDAC classes I and IV, whose members

FIG. 7. qPCR-based quantification of transgene expression and vgc/ml in HeLa cells transduced with LVs or IDLVs in the presence or in the absence of TSA. **(A)** HeLa cells were transduced with 1.3×10^6 vgc/ml of LV.hPGK.eGFP or of IDLV.hPGK.eGFP and were untreated (UNT) or treated with 4 μ M TSA. Twenty-four hours post-transduction, transcriptional levels were quantified by RT-qPCR and the input of similar amounts of vector genomes was confirmed by qPCR (upper and lower graph, respectively). **(B)** HeLa cells were transduced with 2.4×10^6 vgc/ml of LV.CMV.eGFP or of IDLV.CMV.eGFP and were untreated (UNT) or treated with 4 μ M TSA. Twenty-four hours post-transduction, transcriptional levels were quantified by RT-qPCR and the input of similar amounts of vector genomes was confirmed by qPCR (upper and lower graph, respectively).

are ubiquitous, whilst skeletal muscle cells contain, in addition to these, tissue-specific class II HDACs (Yahi *et al.*, 2006). In addition, it is also noteworthy mentioning that through the epigenetic profiling experiments we have found that, upon HDAC inhibition, the rescue levels of specific euchromatin marks on IDLV genomes can differ considerably.

The current findings, based on investigating LV versus IDLV transduction end-point parameters in dividing and non-dividing cells exposed to NaBu or TSA are consistent with those of Kantor and colleagues, resulting from experiments in replicating cell lines (Kantor *et al.*, 2009). In particular, by using NaBu and NaBu-like short-chain fatty acids, these authors showed that in cycling hematopoietic cell lines and transformed 293T cells, IDLV genomes become rapidly organized into chromatin structures that acquire general histone marks broadly associated with silent chromosomal loci. We have complemented and expanded their results by measuring histone marks characteristic of transcription-competent chromatin at a broader array of sites located in transgenic sequences and in most LV and IDLV viral *cis*-acting elements. We discovered that in comparison to LV proviral DNA, histone modifications normally linked to euchromatin are scarcely distributed along IDLV genomes and that, consistent with the transgene expression end-points, HDAC inhibition often results in the accumulation of these marks on IDLV templates. Interestingly, the TSA-dependent distribution profile of these marks on IDLV DNA appeared

to be different from that observed on LV genomes. Indeed, a preferential accumulation of H3K4me3 over H3K9/K14ac could be detected on the viral *cis*-acting elements and at the 5' end of the *eGFP* ORF with the quantities of H3K9/K14ac approaching those of H3K4me3 only within the 3' end of the human *PGK1*-derived sequence. The presence of these two marks in this region containing the transcription start site (TSS) of the human *PGK1* is significant. Indeed, high levels of H3K4me3 and H3H9/K14ac have been shown to mark the vicinity of the TSSs of active genes (Liang *et al.*, 2004; Barski *et al.*, 2007; Wang *et al.*, 2008). Moreover, the critical role of these acetylated lysines for the recruitment of TFIID, a key component of the RNA polymerase II transcriptional machinery, at the human *IFN- β* promoter has been previously demonstrated (Agalioti *et al.*, 2002).

In future experiments, it will be interesting to investigate other post-translational histone modifications such as trimethylated lysine 36 and phosphorylated serine 10 on histone H3 (H3K36me3 and H3S10P, respectively). Indeed, H3K36me3 has been shown to be enriched toward the 3' end of actively transcribed regions (Bannister *et al.*, 2005), and its role in preventing cryptic initiation of transcription within coding regions has, as of yet, only been established in yeast (Bannister and Kouzarides, 2011). Concerning H3S10P, it would be of great interest to investigate its association with H3K9/K14ac since in its phosphoacetylated state the methylation of H3K9 cannot occur, which in turn, impairs HP1 α

FIG. 8. Testing the effect of HDAC inhibition on transgene expression levels achieved by LV versus IDLV particles in post-mitotic and non-dividing cells. **(A)** Detection of nuclei in the S phase of the cell cycle by 5-bromo-2'-deoxyuridine (BrdU) immunofluorescence microscopy. Cultures of dividing human myoblasts (upper panel) or of differentiated human myotubes (lower panel) were subjected to Hoechst 33342 staining and to immunofluorescence microscopy using a fluorescein-conjugated anti-BrdU antibody. Hoechst 33342- and BrdU-specific signals are overlaid in the rightward micrographs. The bars in the upper and lower rows of the panels corresponding to myoblast and myotube cultures are 100 and 50 μ m, respectively. **(B)** Cultures of differentiated myotubes incubated with IDLV.CMV.eGFP (upper panel) or with LV.CMV.eGFP (lower panel) at 93 ng p24^{gag}/2 $\times 10^5$ cells. The cultures, untreated or treated with TSA or NaBu, were subjected to live-cell imaging through eGFP and Hoechst 33342 direct fluorescence microscopy at 48 h post-transduction (leftward and central micrographs, respectively). Bars = 100 μ m. **(C)** Detection of nuclei in S phase by BrdU immunofluorescence microscopy. Cycling or growth-arrested hMSCs kept for 6 days in growth medium (GM) or in differentiation medium (DM), respectively, were stained with the DNA dye Hoechst 33342 and were subjected to immunofluorescence microscopy using a fluorescein-conjugated anti-BrdU antibody (upper panel). The frequency of BrdU-positive, replicating cells under each of these two experimental conditions is shown in the lower panel. Bars = 100 μ m. **(D)** Upper graphs, flow cytometric analysis of the frequencies of eGFP-positive cells and MFI values corresponding to hMSCs maintained for 6 days in DM and subsequently transduced with LV.CMV.eGFP (gray bars) or with IDLV.CMV.eGFP (solid bars) at 784 ng of p24^{gag}/1 $\times 10^5$ cells. The flow cytometry was performed at 4 days post-transduction. The vector-exposed cells were either not treated with HDAC inhibitors or were incubated in the presence of 8 μ M of TSA or 8 mM of NaBu. Lower graphs, variation in the frequencies of reporter-positive cells and in the MFI values between drug-treated and mock-treated hMSCs.

FIG. 9. Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) profiling of the euchromatin marks H3K4me3 and H3K9/K14ac on LV.PGK.eGFP versus IDLV.PGK.eGFP genomes. HeLa cells were transduced with LV.hPGK.eGFP (**A**) or with IDLV.hPGK.eGFP in the absence (**B**) or in the presence of 4 μ M TSA (**C**). Total cellular DNA was immunoprecipitated with antibodies specific for histone H3 or for the H3 protein variants H3K4me3 or H3K9/K14ac. The immunoprecipitated DNA and their respective input DNA were subjected to qPCR to quantify the percent input corresponding to the different vector genome-associated proteins. The vector DNA sequences targeted were located within the viral *cis*-acting elements; 5' LTR (LTR), packaging signal (Ψ), Rev-responsive element (RRE), and central poly-purine tract (cPPT), or at the 3' end of the *hPGK* promoter or the 5' end of the *eGFP* ORF.

recruitment and subsequent transcriptional repression (Mateescu *et al.*, 2004).

It is generally considered that in the context of integration-competent vectors, the deployment of regulatory sequences from mammalian genes is preferable to the use of strong

viral enhancer/promoter elements as the latter are more prone to partial or complete silencing. Our data is in line with this view since, of the four integration-competent vectors tested, LV.CMV.eGFP tended to be the most responsive to HDAC inhibition independently of the dose applied (Fig. 6). In the context of IDLVs, however, the use of human DNA-derived regulatory elements did not prevent HDAC-mediated silencing, suggesting that their episomal nature overrides their current designs. Thus, the investigation of new IDLV constructions based on the incorporation of DNA elements such as insulators or matrix attachment regions (Moltó *et al.*, 2009; Barkess and West, 2012) is warranted before their intrinsic performance is improved.

Taken together, the data gathered herein and elsewhere (Kantor *et al.*, 2009) strongly supports the proposition that the net result of the operative epigenetic mechanisms on IDLV templates includes transcriptional silencing involving HDAC activity. Thus, at this stage, a broad-stroke working model for the acute susceptibility of IDLVs to HDAC-mediated transcriptional repression can be submitted as follows (Fig. 10). Integration-competent LVs, by "piggybacking" on the parental HIV-1 nonrandom proviral integration process, insert their genomes preferentially into transcriptionally active genes (Schröder *et al.*, 2002; Wu *et al.*, 2003). As corollary, this increases the likelihood that LV templates home into euchromatin-rich nuclear domains, whose features include high and low concentrations of histone acetyltransferases (HATs) and HDACs, respectively. Low HDAC levels, plus a transcription-permissive milieu, effectively "shields" most proviral sequences from acquiring heterochromatin marks. The net result is high-level transgene expression (Fig. 10, left-hand half). In contrast, although incoming linear IDLV genomes can escape exonucleases through DNA repair-assisted build-up of 1- and 2-LTR circles, the latter forms do not possess any active mechanism(s) to home into transcription-permissive nuclear domains. This, coupled with the relatively high concentrations of HDACs (Downes *et al.*, 2000; Finlan *et al.*, 2008), turns the IDLV viral *cis*-acting and transgenic sequences "easy prey" for the cellular processes involved in foreign DNA silencing. The net result is, in this case, no or low-level transgene expression (Fig. 10, right-hand half).

The physical association of HDACs with viruses such as avian sarcoma virus-derived vectors has been previously described (Katz *et al.*, 2007). This interaction has been shown to occur rapidly after transduction of HeLa cells, which supports the rationale for applying HDACs inhibitors at the moment of transduction. Although our data supports the inclusion of HDAC inhibitors in transduction protocols involving IDLVs, in the context of gene therapy translational research, however, these protocols will have to be based on compounds that are clinically compatible. In this regard, it is noteworthy mentioning that a wealth of information is being gathered on the clinical use of HDAC inhibitors, most notably as anti-cancer agents (Bolden *et al.*, 2006; Lane and Chabner, 2009). Finally, equally from a utilitarian point of view, we propose that IDLV titrations based on functional transgene expression end-points are expanded to include titers resulting from HDAC inhibition. Ideally, these titrations should be performed on the intended target cells of choice using specific inhibitors of HDACs such as TSA. These TSA-derived functional titers can complement those based on

FIG. 10. Working model for the differential susceptibility between LVs and IDLVs to cellular HDACs. Upon active translocation into the target cell nucleus, host factors together with HIV-1 pre-integration complexes harboring wild-type IN moieties bias the insertion of proviral DNA into genes located within transcription permissive domains. These domains being enriched in histone acetyltransferases (HATs) and poor in histone deacetylases (HDACs), provide for an environment in which high-level transgene expression is favored. Conversely, following active translocation into the target cell nucleus, HIV-1 pre-integration complexes containing the class I IN mutant IN^{D116N} peptide cannot catalyze the host chromosomal insertion of linear IDLV genomes. The processing of these molecules by cellular DNA repair pathways ensues, resulting in the formation of circular 2-LTR or 1-LTR episomes that, for the most part, accumulate in transcription repressive domains. These domains, being enriched in histone deacetylases (HDACs) and poor in acetyltransferases (HATs), constitute a milieu in which HDAC-mediated transgene silencing is favored, which ultimately results in low-level transgene expression.

concentrations of vgc or physical particles and should constitute a better approximation to the actual maximum transgene expression potential of any given IDLV preparation.

In summary, we found that HDACs constitute major cellular determinants underlying the poor transcriptional activity of IDLVs in target cells regardless of their replication rate. The activation of transgene expression following HDAC inhibition generally correlated with the accumulation of open chromatin marks on IDLV genomes. Finally, the degree of transgene expression upon HDAC inhibition varied in a vector backbone-, vector dose-, and cell type-dependent manner.

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