Factors Affecting the Formation, Stability, and Expression of Unintegrated Lentiviral Vector Genomes

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology.

Chapel Hill
2010

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ABSTRACT

Matthew Bayer: Factors Affecting the Formation, Stability, and Expression of Unintegrated Lentiviral Vector Genomes

(Under the direction of Tal Kafri, M.D, Ph.D)

Lentiviral vectors present an attractive means of delivering therapeutic transgenes, as they deliver a relatively large, stably integrated and expressed genetic payload to both dividing and nondividing cells. However, with the genotoxic hazard of integrating vectors tragically illustrated by recent clinical trials using simple retroviral vectors, the necessity of developing a lentivector with minimal risk of insertional mutagenesis is clear. In fact, integrase-mutated lentivectors, which deliver only unintegrated, episomal vector genomes to target cells, have been in use for a number of years, and offer a means of stably expressing transgenes in nondividing cells. However, the means by which the various types of episomes (linear, 1-LTR circular, 2-LTR circular, and mutant circular) are formed, and the extent to which they are transcriptionally active, have not been thoroughly characterized *in vitro* or *in vivo*. This dissertation investigates the effect of cellular factors, such as DNA-repair proteins and cell-cycle status, vector factors, such as sequences in the vector's U3 region and polypurine tract (PPT), and organismal factors, such as target organ and *in vivo* stability, on the formation, stability, and expression of lentivector episomes.

Interestingly, transduction of cell lines mutant for genes in the homologous-recombination pathway of DSB repair showed no change in 1-LTR

circular-episome formation. Similarly, cells arrested in G1 phase of the cell cycle, which does not support HR, displayed unaffected levels of 1-LTR circles. Furthermore, vectors bearing a large deletion to the U3 region, which strongly increases episomal, though not integrated, transgene expression, saw no significant change in episome formation from that of a vector with a short U3 deletion. Conversely, vectors with a deleted PPT exhibited a dramatic increase in the relative abundance of 1-LTR circles, leading to reduced integrase-mediated and integrase-independent integration. F inally, examination of *in vivo* episome formation and expression indicated that the relative abundance of 1-LTR circular episomes increases over a three-week period in the liver, that episomes express trangenes stably for up to six months in the liver, and that episomes exhibit greater stringency of tissue-specific expression than integrated provirus.

Acknowledgments

I would like to thank:

My advisor, Tal Kafri

The Kafri lab, past and present members:

Boris Kantor, Adam Cockrell, Ping Zhang, Jian Dong, and Thipparat Suwanmanee

Xiangping Li, Brian Zeithaml, Hong Ma, Blair Roszell, Karin Haack, and Adam Richter

Matt Hirsch, Chengwen Li, and the rest of the Samulski lab

Bob Duronio, Cara Marlow, and Sausyty Hermreck with the Curriculum

My committee members:

Steven Bachenheimer
John Olsen
Michael Resnick
Jude Samulski
Jennifer Webster-Cyriaque

My wife, Gigi

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293T	human embryonic kidney cell line, transformed with sheared adenoviral genome and SV40 virus's large T antigen
BDNF	brain-derived neurotrophic factor
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
G1	growth phase 1 of the cell cycle
G2	growth phase 2 of the cell cycle
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HIV	human immunodeficiency virus
HR	homologous recombination
LTR	long terminal repeat
MFI	mean fluorescence intensity
NeuN	neuronal nuclear antigen
NHEJ	nonhomologous end-joining
$p24^{gag}$	HIV capsid protein
PBS	phosphate-buffered saline solution
PCR	polymerase chain reaction
PPT	polypurine tract
qPCR	quantitative polymerase chain reaction

R	sequence repeated at both ends of the lentiviral RNA genome
RNA	ribonucleic acid
RLU	relative light units
RT	reverse transcription
S	DNA synthesis phase of the cell cycle
SIN	self-inactivating
SSA	single-strand annealing
U3	sequence unique to the 3′end of the lentiviral RNA genome, containing most of the transcriptional control elements of the provirus, which include the promoter proper, and multiple enhancer sequences responsive to cellular, and in some cases viral, transcriptional activator proteins
U5	sequence unique to the 5′end of the lentiviral RNA genome; the site of poly(A) addition is at the boundary between R and U5
vTK	prefix designating vectors generated in Tal Kafri's lab
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
X-SCID	X-linked severe combined immunodeficiency

Chapter 1

INTRODUCTION

HIV-1

Human immunodeficiency virus 1 (HIV-1), a human retrovirus of the lentivirus family, is the causative agent of acquired immune deficiency syndrome (AIDS). HIV-1 virion particles consist of a 9.2kb diploid RNA genome and structural (gag), enzymatic (pol), envelope (env) and accessory proteins wrapped inside an envelope derived from the lipid membrane of the host cell, with viral glycoproteins on the envelope surface to mediate target-cell entry [1] (Fig. 1). Briefly, the HIV-1 genome includes a packaging sequence (ψ), two long terminal repeats, at the 5' and 3' ends of the viral genome that each encode promoter/enhancer elements and a polyadenylation sequence, and the gag genes (matrix, capsid, and nucleocapsid), pol genes (reverse transcriptase, integrase, and protease), env genes (surface glycoprotein and transmembrane protein), and accessory genes (tat, rev, nef, vif, vpr, and vpu) (Fig. 2).

HIV-1 primarily infects human CD4+ T lymphocytes and macrophages [2]. When an HIV-1 virion enters a target cell and uncoats the structural proteins, reverse transcriptase converts its genome from single-stranded RNA to a double-stranded DNA molecule, which then enters the nucleus, where the viral integrase protein may mediate the viral genome's integration into the host-cell chromatin [3]. Viral RNA, transcribed from either integrated or unintegrated lentiviral genomes, is then either packaged into a new virion as a

full-length viral genome or serves as mRNA to translate viral proteins, with or without prior splicing of the viral genome [3] (Fig. 1). Once the viral proteins are assembled in the infected cell's cytoplasm, the HIV-1 particle's env proteins fuse the viral membrane with the cellular membrane and the virus "buds" out of the cell [3]. The ability of HIV-1 to use its cell-derived envelope to evade a strong immune response, as well as its persistence in infected cells, made it attractive to researchers looking for a novel virus to adapt for gene-transfer applications.

HIV-1-Derived Vectors

Human immunodeficiency virus-1 (HIV-1) was first adapted for use as a gene-therapy vector over 13 years ago [4,5], and the use of vectors derived from HIV-1, as well as other lentiviruses, has steadily increased in sophistication and popularity since then [6]. Lentiviruses' large (~9kb) genetic payload, low immunogenicity, and ability to transduce nondividing cells make them an attractive vehicle for gene therapy [6]. To that end, researchers have modified lentiviruses to make them nonpathogenic and replication-incompetent. In brief, they adapted the virus by deleting the accessory genes nef, vpr, and vif, which mediate such pathogenic activities as inducing cell death, inducing G2 cell-cycle arrest, and facilitating virus secretion, respectively[7-9]. Also, researchers split the viral genome into at least three reading frames on separate plasmids, with the transgene and all necessary *cis* elements expressed from one plasmid and the packaging and envelope proteins expressed from two or more other plasmids [6]. Virus particles made by this method can transduce target cells, but do not contain the packaging and envelope RNA necessary to replicate in them [4,5,10,11] (Fig. 2).

Beyond the modifications necessary to make lentiviral gene-therapy vectors safe

for researchers and patients, researchers have modified lentiviral vectors to improve their transduction efficacy. For instance, they have replaced the wild-type lentiviral envelope protein with one from a heterologous virus, such as the G protein of the vesicular stomatitis virus (VSV-G), which imparts on the vector broader tropism; whereas wild-type virus particles, as noted above, transduce primarily CD4+ T cells and macrophages, VSV-G-pseudotyped vector particles target cells bearing a ubiquitous phospholipid receptor [4,11] (Fig. 2). Another modification to largely delete the vector's native promoter, located in the LTR, such that, after reverse transcription, only an engineered internal promoter remains to express RNA while full-length RNA expression is prevented; vectors with this modification are known as self-inactivating, or SIN [12]. Indeed, with modifications to prevent pathogenesis and replication [12-14], lentiviral vectors have demonstrated excellent biosafety, coupled with the ability to deliver a relatively large genetic payload to both dividing and nondividing cells. Interestingly, research on wild-type HIV-1 has revealed viral sequences in the LTR that function to inhibit expression [15-18], and, therefore, when deleted from SIN vectors, may allow enhanced transgene expression from the internal promoter [19]. Furthermore, the ability of lentiviral vectors to insert their genomes into target-cell chromosomes gives lentivectors the advantage of mediating stable gene expression in dividing cells, but presents the risk of inducing insertional mutagenesis, which recent studies with retroviral vectors have shown to be a tragically real possibility [20,21]. Therefore, reducing the risk of oncogenic events, either by directing vector integration to predetermined “safe” chromosomal loci or by preventing integration itself, is a critical issue facing the field of gene therapy [22-24].

Generating Nonintegrating Lentiviral Vectors

Of the above-mentioned methods for minimizing insertional genotoxicity, eliminating integration is the best understood to date. The simplest way to prevent lentiviruses from inserting their genomes into target-cell chromatin is by mutating the vector genome, either at the integrase binding sites, known as *att* sites, located at the 5' and 3' ends of the vector genome [25], or at the integrase gene. Indeed, several effective, nonpleiotropic integrase-disabling mutations have been identified at the D64, D116, or E152 residues of its catalytic-core domain [26-30], resulting in a virus that cannot integrate into target-cell chromosomes, but can still import its cDNA genome into the nucleus. Recent studies have shown that integrase mutations are more effective than *att*-site mutations in preventing integration, and that integrase-*att*-site double mutants do not reduce integration frequency below that of integrase mutations alone [31,32].

Though nonintegrating lentiviral vectors appear to reduce significantly the risk of retrovirus-mediated insertional mutagenesis, which was demonstrated in recent clinical trials [20,21,33,34] and replicated *in vitro* [23,35] and *in vivo* [36,37], they do not prevent integration completely. Indeed, a relatively low level of integration (1 integrated copy per ~5 x 10^3-4 x 10^5 transducing vector particles) is observed in transductions with integrase-defective viruses or vectors [29,38]. Due to the irregular nature of vector-chromosome junctions observed in these integration events, they are believed to be caused not by residual integrase activity, but by illegitimate integration, most likely through recombination or nonhomologus end-joining between linear unintegrated vector genomes and chromosomal DNA [32]. The genotoxic hazard of illegitimate integration, though seemingly slight, increases as the multiplicity of infection or as the scale of transduction increases, and so

any methodological advances to reduce the level of illegitimate integration would be a useful contribution to vector safety.

Episome Formation

While the formation and maintenance of integrated lentiviral provirus have been studied extensively, the factors affecting the formation of lentiviral episomes are not thoroughly understood. Entering the nucleus as a linear molecule of DNA, the lentiviral genome would be expected to induce a cellular response to its open, double-stranded ends. Indeed, the DNA damage-sensing machinery of infected cells appears to interact with at least some linear unintegrated lentiviral genomes. An earlier study has established that the nonhomologous end-joining (NHEJ) proteins Ligase IV and Xrcc4 are necessary for the formation of 2-LTR circular episomes [39] (Fig. 3), suggesting that NHEJ mediates 2-LTR circle formation by bringing together the ends of unintegrated linear viral genomes. On the other hand, little is known about how linear unintegrated vector genomes, the most common episomal species [40], are maintained without inducing an apoptotic or arrest response to their double-stranded open ends, nor have the mechanisms governing the formation of mutant and 1-LTR circular episomes been fully elucidated. Mutant circles appear to form either by intramolecular recombination or autointegration of linear unintegrated lentiviral cDNA molecules [41]. Importantly, 1-LTR circular episomes may form through homologous recombination (HR) between the LTRs [42, 43, 44-46] (Fig. 4), or as products of prematurely terminated reverse transcription [47-49] (Fig. 5). The hypothesis that 1-LTR circles are formed during reverse transcription was advanced in the early years of retrovirus research, when it was found that retroviruses incubated in a cell-free extract can form 1-LTR circles, suggesting that viral proteins, such as reverse transcriptase, are

sufficient for 1-LTR circle formation[48]. Furthermore, another study found that, during retroviral infection, 1-LTR circles are found not only in the nucleus, where HR proteins are active, but in the cytoplasm, where reverse transciptase is active and HR factors are not active, again suggesting that reverse transciptase mediates the formation of 1-LTR circles [49].

However, more recent studies suggest that 1-LTR circles might, in fact, be generated by HR proteins. One investigation showed that linear viral DNA genomes, after being purified from viral proteins and incubated in a cellular extract, form 1-LTR circles without viral factors [42]. Another group found that an siRNA-induced knockdown of the HR protein Rad52 reduced the formation of 1-LTR circles in HIV-infected cells, also suggesting that HR proteins are involved in 1-LTR circle formation [44]. Specifically, HR factors in the single-strand annealing (SSA) pathway of HR would be expected to play a role, as the SSA pathway, not requiring an unbroken template strand to effect recombination of the HIV genome's LTRs, would be the logical mechanism for HR-mediated 1-LTR circle formation [50-52].

Episome Stability and Expression

Lentiviral episomes were long considered a short-lived, transcriptionally inert product of reverse transcription [29,30,53,54], and only in recent years have they been shown to mediate significant, sustained transgene expression, both in the context of lentivirus [26,28,55-61] and lentiviral vectors [32,62-67], in keeping with results obtained with other extrachromosomal gene-therapy vectors [68-72]. In one example, Saenz and colleagues showed that nonintegrating vectors, though diluted out of dividing cells, could stably transduce growth-arrested cells *in vitro* and could transduce postmitotic neurons at levels

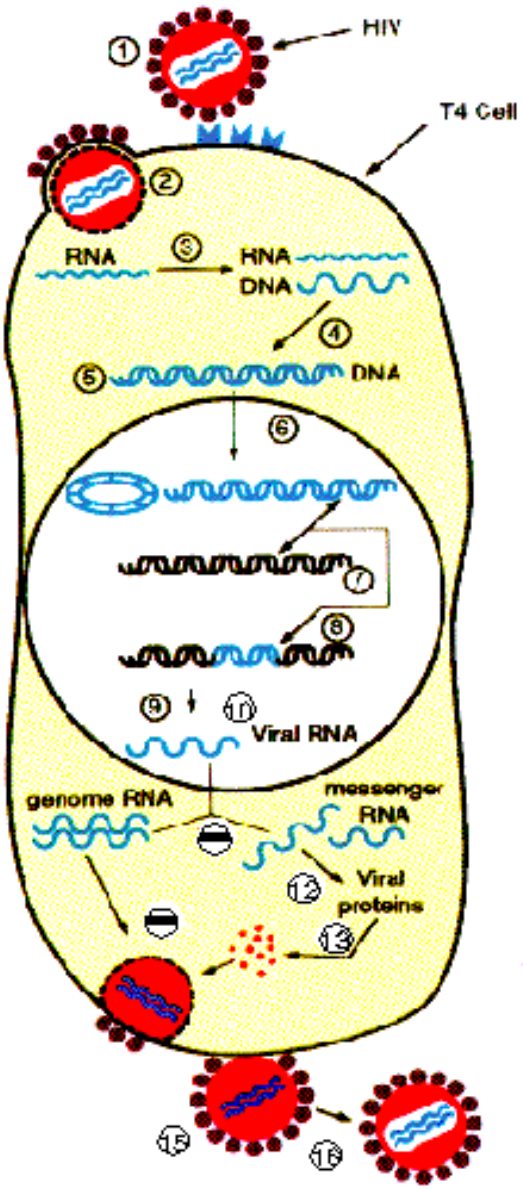
comparable to those of integrating vectors[66]. The significant expression of transgenes from lentiviral episomes was also demonstrated in a recent study using integrase-deficient lentivectors to deliver reprogramming factors to human fibroblasts, successfully inducing them to pluripotency [65].

Nondividing cells are an attractive target for nonintegrating vectors, as episomal vector genomes are not diluted out of the cell population. However, various groups have arrived at conflicting results on the efficacy of integrase-positive or integrase-defective lentiviral transduction of postmitotic cells. First, Kafri *et al*. transduced liver cells *in vivo* with an integrating lentiviral vector and demonstrated sustained gene expression [73]. However, Park *et al*. similarly used an integrase-positive lentiviral vector to transduce liver cells *in vivo* and found that partially hepatectomized livers, which undergo several rounds of cell division, were more than 25-fold more efficiently transduced than non-hepatectomized, postmitotic livers[74]. On the other hand, a number of groups have subsequently shown robust and stable expression in nondividing cells *in vivo* [31 38,75-78]. Finally, Yanez-Munoz indicated that nonintegrating vectors transduce postmitotic retinal and brain cells as efficiently as integrating vectors, demonstrating effective retinal transduction through the restoration of ocular function in mice deficient for *Rpe65*[79]. Taken together, these results suggest that nonintegrating lentiviral vectors can transduce nondividing cells as efficiently as integrating vectors, and while lentiviral transduction may be more efficient in dividing cells, transduction in nondividing cells can be efficient enough to achieve phenotype correction *in vivo*.

Research Presented in the Dissertation

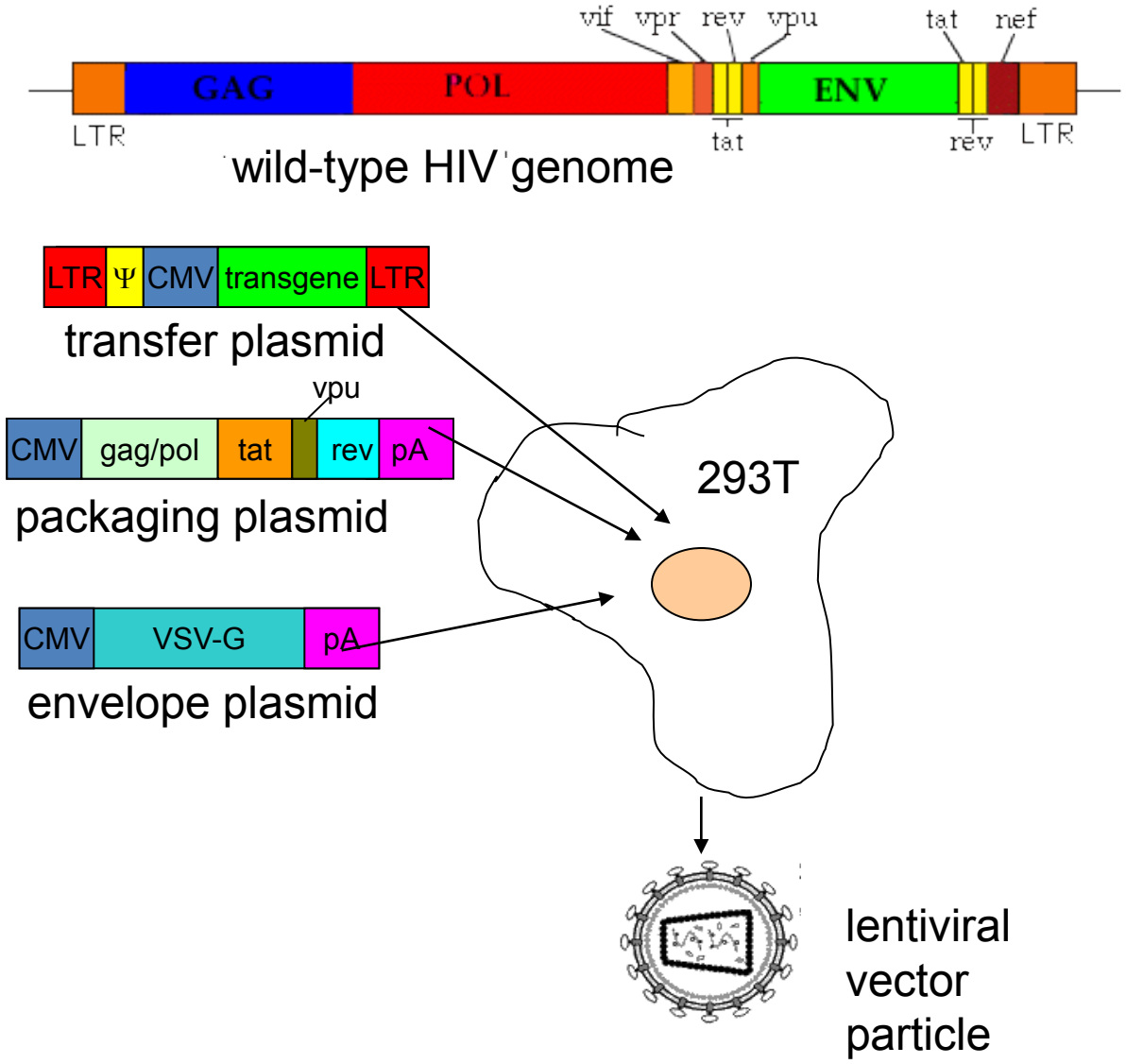
Integrated lentiviral genomes have been exhaustively characterized, but lentiviral

episomes are not thoroughly understood. As nonintegrating lentivectors continue to gain popularity as a substrate for the delivery of therapeutic transgenes, it becomes increasingly important to study the means by which lentiviral episomes form, persist, and express in transduced cells. Accordingly, the following studies examine the cellular, vector, and organismal factors affecting the formation, stability, and expression of unintegrated lentiviral vector genomes.



http://www.thebody.com/niaid/hiv_lifecycle/virpage.html

Fig. 1. Outline of the HIV life cycle. Briefly, an HIV particle binds to a CD4+ cell (1), enters and uncoats its RNA genome (2), and undergoes reverse transcription (3, 4). Reverse-transcribed, linear cDNA enters the nucleus (5), where it circularizes, is maintained as a linear episome, or integrates into the chromatin (7,8). Full-length, unspliced RNA (9), or spliced RNA (10) is expressed by viral cDNA and exported into the cytoplasm, where the spliced RNA is translated into viral proteins (12) which form the viral particle (13), while the full-length RNA is packaged into the viral particle. Once packaged, the viral particle buds out of the infected cell.



http://medstat.med.utah.edu/WebPath/TUTORIAL/AIDS/AIDS004.html

Fig. 2. Outline of the transient-transfection method of lentiviral vector production. The wild-type HIV genome is split into three plasmids, one encoding the HIV envelope gene or a heterologous substitute (envelope plasmid), one encoding the viral structural and enzymatic proteins (packaging plasmid), and one encoding the transgene of interest, along with the vector sequences necessary to package and express the transgene (transfer plasmid). Potentially pathogenic accessory genes are deleted.

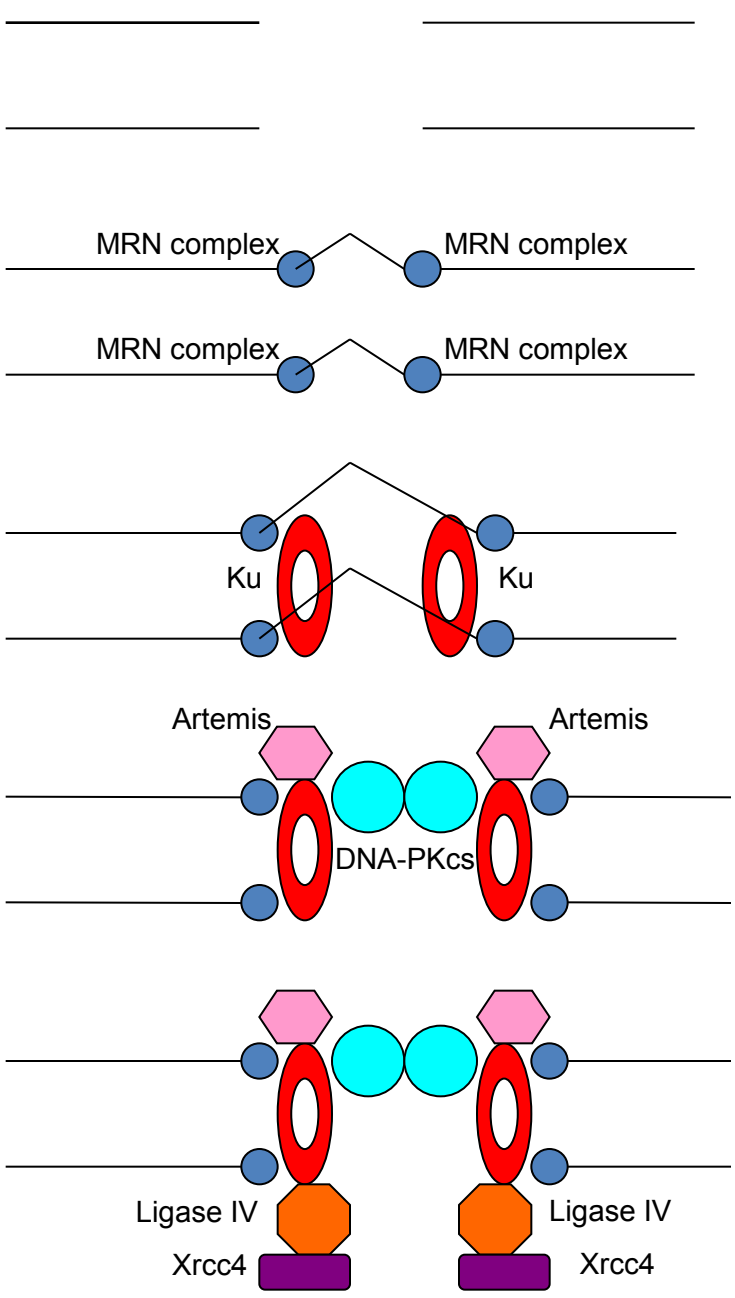


Fig. 3. Outline of the nonhomologous end-joining pathway of double-strand DNA break repair. In brief, a break is recognized by the MRN complex, which binds it and recruits the Ku proteins. The Ku proteins stabilize the break and recruit DNA-PKcs, which, if necessary, phosporylates Artemis, enabling it to process overhang or hairpin structures at the break. Finally, the Ligase IV/Xrcc4 complex brings together the broken DNA ends in a homology-independent manner.

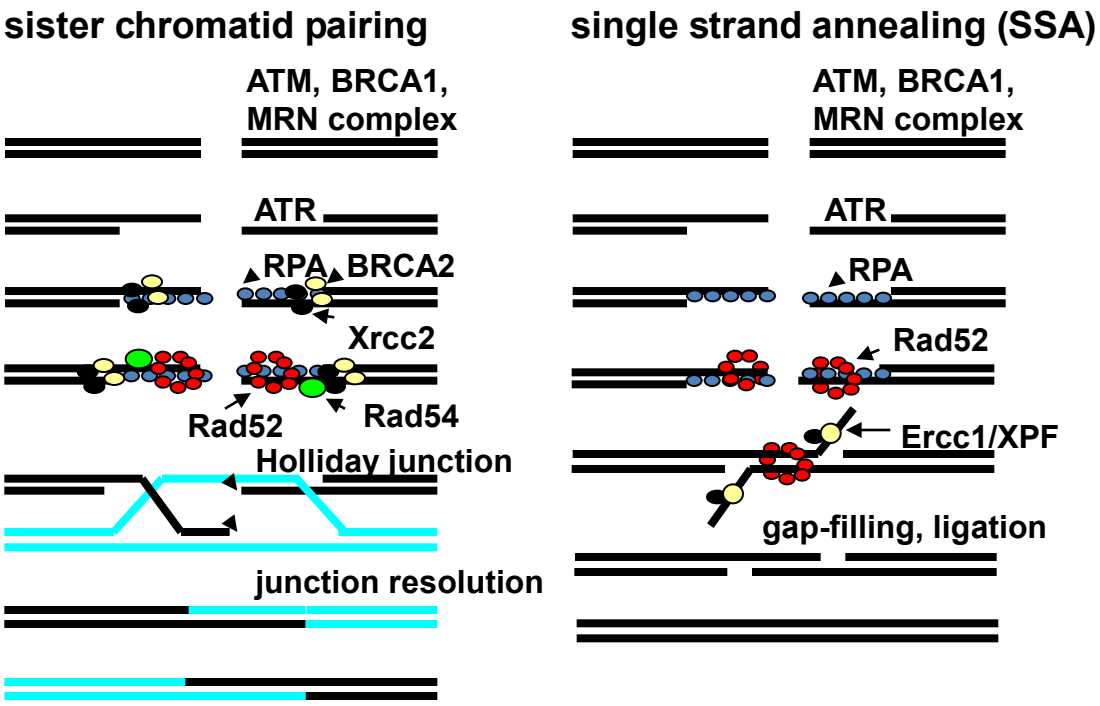
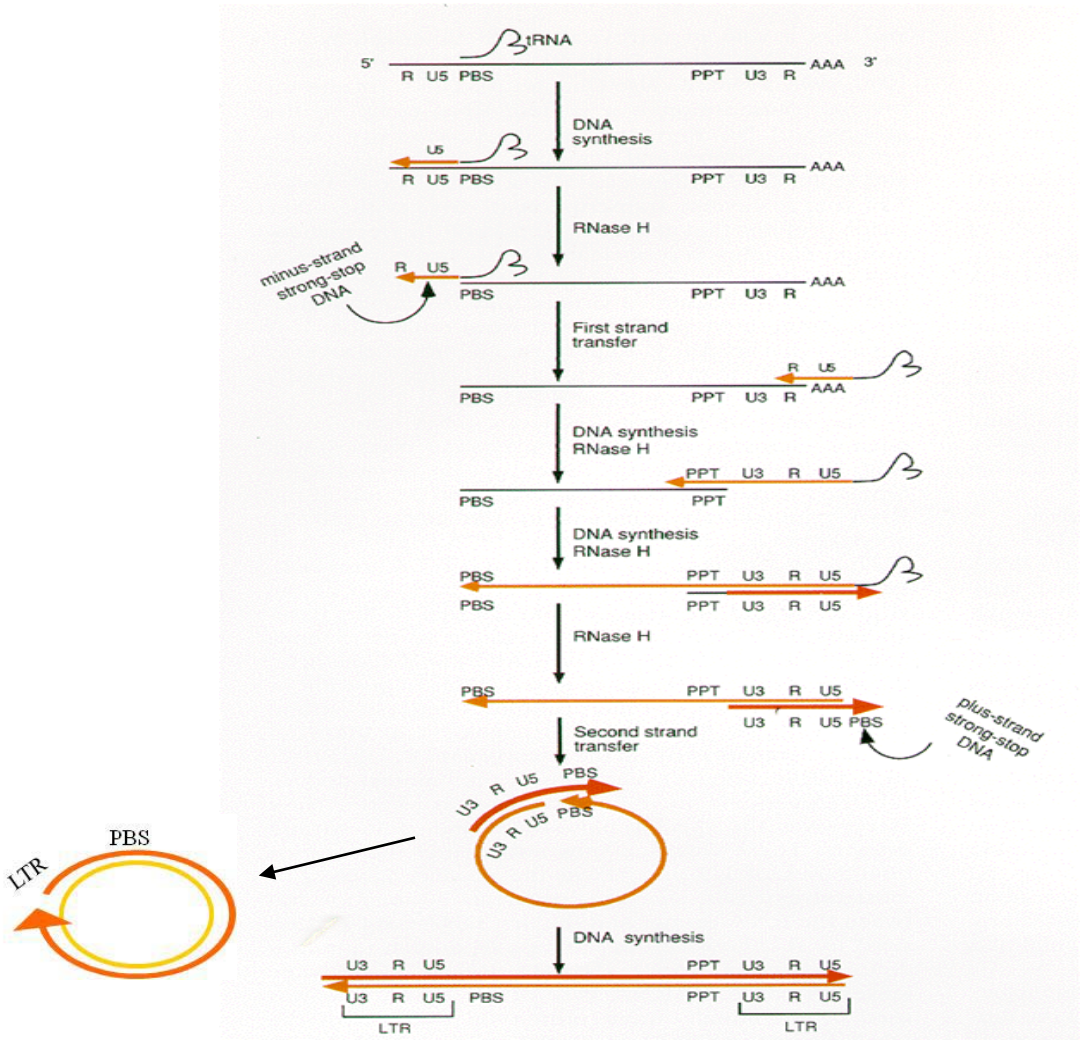


Fig. 4. Outline of the homologous-recombination pathway of double-strand DNA break repair. The DSB break is recognized by ATM, which recruits the MRN complex, which then mediates resection of the broken ends, creating single-stranded overhangs that are coated with RPA and protected by ATR. Then, if a sister chromatid is available, the ssDNA utilizes BRCA1, BRCA2, Rad51, Xrcc2, and Rad52 to effect strand invasion, extension, ligation and resolution of the broken sequence (left) . However, if no sister chromatid is available, the two resected ends are annealed at a region of homology by Rad52 and the intervening, noncomplementary sequences are excised by the XPF/Ercc1 complex (right).



Retroviruses
Coffin, John M.; Hughes, Stephen H.; Varmus, Harold E.
Plainview (NY): Cold Spring Harbor Laboratory Press; c1997

Fig. 5. Outline of 1-LTR circle formation as a product of reverse transcription. During reverse transcription, after plus-strand synthesis is initiated at the PPT and extends to the PBS, the plus-strand and minus-strand PBS elements associate, causing the vector to circularize. Typically, the LTR region then dissociates, enabling completion of the full-length, linear RT product. However, if the LTR failed to dissociate, then plus-strand synthesis could simply proceed around the circle, resulting in a 1-LTR circular RT product.

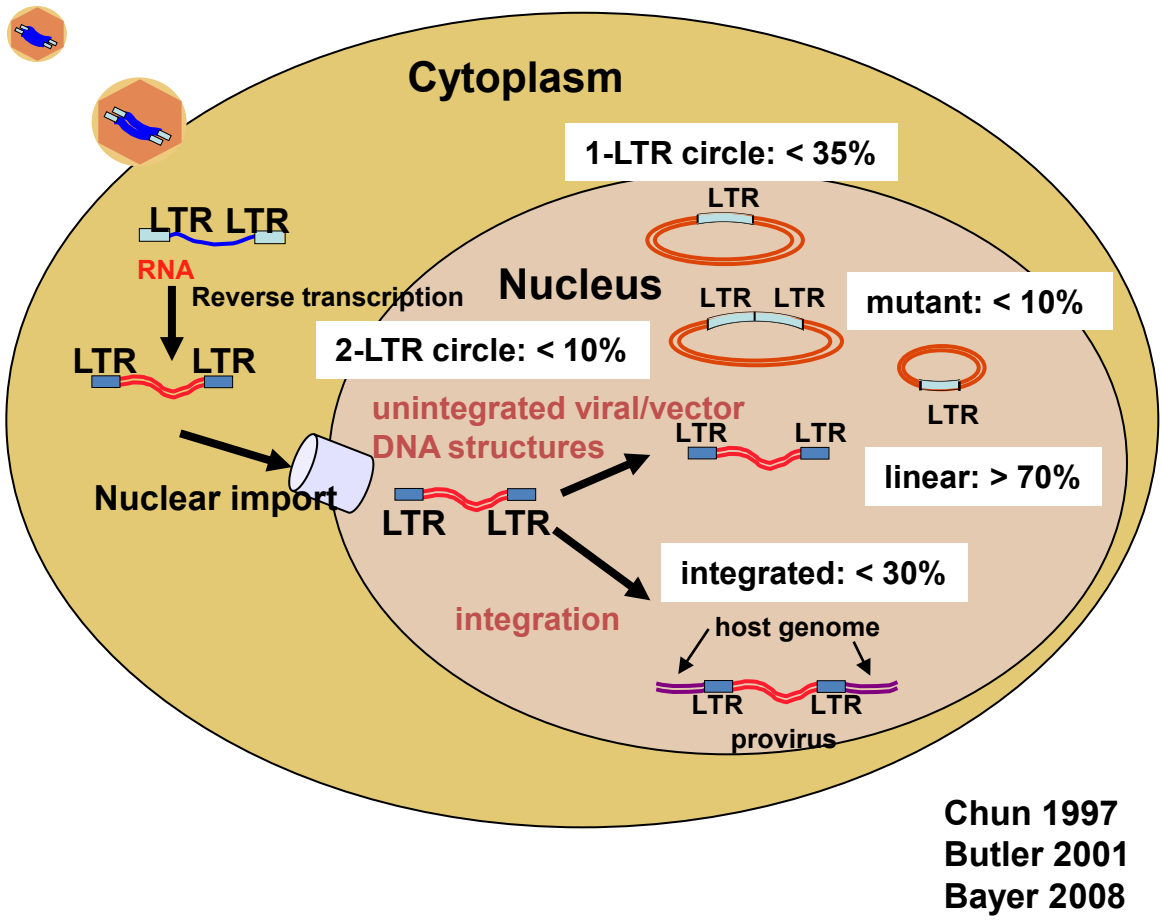


Fig. 6. Outline of the steps involved in lentiviral vector transduction. When a lentivector particle enters a target cell and uncoats its genome from the structural proteins, reverse transcriptase converts the genome from single-stranded RNA to a double-stranded DNA molecule, which then enters the nucleus, where the viral integrase protein may mediate the viral genome's integration into the host-cell chromatin. However, the majority of vector genomes are maintained as linear episomes, with a smaller percentage of genomes remaining as 1-LTR circular episomes and an even smaller percentage remaining as either 2-LTR circular or mutant circular episomes.

Chapter 2

CELLULAR FACTORS AFFECTING LENTIVECTOR EPISOME FORMATION

Introduction

In the course of lentiviral vector transduction, after the vector particle uncoats its RNA genome and reverse transcription converts the genome into a double-stranded linear DNA molecule, the vector genome presents a double-strand DNA break (DSB) to the cell [6] (Fig. 6), which may then interact with the cellular DSB repair machinery. However, the extent of the interaction has not been fully characterized. What is understood is that lentivector episomes appear in four forms: linear episomes, formed directly by reverse transcription (RT) [3]; 2-LTR circular episomes, generated by cellular nonhomologous end-joining (NHEJ) DNA double-strand break (DSB) repair machinery [39]; mutant circular episomes, formed, at least in part, by self-integration [41]; and 1-LTR circular episomes, formed either by the host cell's homologous recombination (HR) DSB repair pathway [42,44,46] or as abortive RT products [47-49].

Double-stranded DNA breaks (DSBs) are genetic lesions that can lead to apoptosis, cycle arrest, or genetic rearrangements resulting in oncogenesis [80]. Mammalian cells have NHEJ and HR pathways that process double-stranded DNA breaks. Because it does not require a homologous template, the NHEJ pathway is potentially active in every phase of the cell cycle. However, also due to its lack of a homologous-template requirement, NHEJ

is an error-prone mechanism [81]. In the NHEJ pathway, the MRN complex recognizes DSBs and recruits Ku, which binds the break and recruits the rest of the Ku/DNA-PK protein kinase complex, which processes any overhang or hairpin structures at the DSB [81]. Finally, the Lig. IV/Xrcc4 complex ligates the broken ends together in a homology-independent process (Fig. 3) [82].

The HR pathway, unlike the NHEJ pathway, is active only in late S and G2 phases of the cell cycle [51], and requires recognition of DSBs by ATM, which recruits the MRN (Mre11/Rad50/NBS1) complex. The MRN complex then mediates resection of the broken, double-stranded ends [81]. Resection creates single-stranded overhangs that are coated with RPA and protected by ATR [81]. Then, if a sister chromatid is available (i.e., in G2 phase), the ssDNA utilizes BRCA1, BRCA2, Rad51 and Rad52 to effect strand invasion at homologous sequences, followed by extension, ligation, and resolution of the broken sequence [80,81]. However, if no sister chromatid is available, the two resected ends are annealed at a region of homology by Rad52 and the intervening noncomplementary sequences are excised by the XPF/Ercc1 complex [83] (Fig. 4). This variant of HR, known as single-strand annealing (SSA), is the most likely pathway to circularize linear lentiviral cDNA and form 1-LTR circles, because lentiviral RT products would not present a sister chromatid to the cellular DNA-repair machinery,.

The relative contributions of the HR and RT pathways to 1-LTR circle formation have not been established. Accordingly, we attempted to establish further the contribution of HR to their formation. To this end, we characterized episome formation in cells transduced with lentivectors (Fig. 7) under inconducive conditions for homologous recombination, which were achieved either with cell lines mutant for the HR factors

BRCA1 [84], Xrcc2 [85], ATM [51], or Ercc1 [52,86], or with cells in G1 or G0 phase of the cell cycle, in which HR is not active [51,52]. To measure episome formation in these cells, we used two separate assays: Southern-blot analysis of total DNA from transduced cells, and restriction analysis of vector clones Hirt-extracted from cells transduced with a shuttle vector. The data presented here demonstrated that transduced HR-deficient cell lines, mutated for either BRCA1, Xrcc2, ATM, or Ercc1 did not exhibit a reduction in 1-LTR circles relative to linear episomes, nor did transduced G1-arrested cells show a decrease.

Materials and Methods

Viral vector production. All lentiviral vectors were prepared as previously described [87], transiently transfecting 10^7 293T cells with 15μg vector cassette, 10μg packaging cassette, and 5μg envelope cassette. Integrating vectors were made using the packaging cassette ΔNRF [87], which expresses functional integrase, while nonintegrating vectors were made using the packaging cassette pTK939, which was made by inserting the D64E-mutant integrase from pD64E into ΔNRF by standard cloning procedures. All vectors were pseudotyped with the VSV-G envelope cassette. For vectors constitutively expressing GFP, titers were assessed by serial dilution in 293T cells followed by visual analysis of GFP expression by fluorescence microscope. For other vectors, concentrations were determined by p24gag ELISA. The absence of replication-competent retroviruses was determined by three independent methods: tat transfer assay, vector rescue assay, and p24gag ELISA, as described previously [88].

Cell culture. 293T cells were grown in Dulbecco's modified Eagle medium (Mediatech,

Herndon, VA) supplemented with 9% fetal bovine serum and 1% penicillin/streptomycin solution. GM16147 (Coriell Institute for Medical Research, Camden, NJ) and CHO-K1 cells (American Type Culture Collection, Manassas, VA) E1KO7-5 and ATStg cells (kind gifts from Rodney Nairn, University of Texas MD Anderson Cancer Center, Smithville, TX), Irs1 and Irs1 wt. cells (kind gifts from John Thacker, Medical Research Council, Harwell, United Kingdom) and AA8 cells (ATCC) were cultured in minimum essential medium alpha medium (Invitrogen, Carlsbad, CA) supplemented with 9% fetal bovine serum and 1% penicillin/streptomycin solution. SUM149 cells (a kind gift from William Kaufmann, University of North Carolina, Chapel Hill, NC) were grown in HuMEC basal serum free medium (Invitrogen), supplemented with 5% fetal bovine serum, HuMEC supplement, 25mg bovine pituitary extract, and 1% penicillin/streptomycin solution. ME16C2 cells (a kind gift from William Kaufmann) were grown in HuMEC basal serum free medium (Invitrogen), supplemented with HuMEC supplement, 25mg bovine pituitary extract, and 1% penicillin/streptomycin solution. GM09607 cells (Coriell) and human embryonic fibroblasts were grown in minimum essential media with 9% fetal bovine serum and 1% penicillin/streptomycin solution (Invitrogen).

Shuttle-vector assay. 293T cells were transduced with integrating or nonintegrating vTK459 or vTK1054 (Fig. 8). Transduced cells were harvested 16 hours posttransduction using 0.85mL Hirt lysis buffer (0.6% sodium dodecyl sulfate, 0.01M Tris/HCl pH 7.4, and 0.1M ethylenediaminetetraacetate) and 0.25mL 5M NaCl per 10cm plate of transduced cells. Episomal DNA was subsequently subjected to phenol/chloroform extraction and D*pn*I digestion and electroporated into bacteria. Bacterial colonies were cultured

monoclonally, pelleted, and their episomes extracted by boiling in a hotprep buffer made with 2g sucrose, 0.4mL 0.5M Tris/EDTA solution, 40mL 10% Triton X-100 (Sigma, St. Louis, MO), 5mL 1M Tris/HCl pH 8.0 and water to 100mL. 125µL hotprep buffer and 2.5µg RNase A (Sigma) were added to each bacterial pellet before boiling. After boiling, samples were centrifuged at 14K for 10 minutes to dispose of cell debris. Episomes were digested with N*ot*I and S*ac*II and electrophoresed in a 1% agarose gel to be characterized as 1-LTR, 2-LTR, or mutant circular episomes, according to predicted sizes for backbone and 1-LTR or 2-LTR insert fragments. For every transduction, between 73 and 112 episome-bearing bacterial colonies were characterized, using recA-mutant *E. coli* strains.

Southern blot analysis. Cells were harvested three days after transduction with vTK945 and their total DNA was extracted as described previously [89]. 10µg total DNA was digested with D*pn*I, E*co*NI and P*fl*MI, electrophoresed in a 1% gel, transferred to a nylon membrane (GE Healthcare), UV-crosslinked, and probed with the 1.6kb A*fe*I/E*co*RV fragment of vTK945.

RecBCD treatment. Hirt-extracted, D*pn*I-treated DNA was treated with RecBCD (Epicentre Biotechnologies, Madison, WI), a bacterial exonuclease that degrades single-stranded and double-stranded linear DNA. Reactions were incubated at37°C in a volume of 300µL, with 6µL RecBCD, 30µL 10X RecBCD reaction buffer, and 12µL 25mM ATP.

Results

Shuttle-vector assay results are not skewed by bacterial recombination.

Retroviral shuttle-vector assays have been a means of measuring retroviral episome formation for over 25 years [90]. Because a shuttle vector includes a bacterial origin of replication and an antibiotic resistance gene in its transcribed region, circular episomes formed in vector-transduced mammalian cells can be recovered through Hirt extraction [91], used to transform bacterial cells, and analyzed as individual bacterial clones (Fig. 8). However, as linear episomes are transformed into bacteria along with circular episomes, the possibility exists that linear episomes may be circularized in bacteria by bacterial recombinases, distorting observations of relative circular episome abundance in transduced mammalian cells. To investigate the possibility that circularization of lentiviral shuttle-vector episomes occurs in bacteria, episomal DNA Hirt-extracted from shuttle vector-transduced cells was treated with the exonuclease RecBCD, which preferentially degrades linear DNA. To verify the activity of RecBCD, populations of treated and untreated episomal DNA were analyzed by Southern blotting, which demonstrates that RecBCD treatment degrades the linear form, but left intact the circular forms (Fig. 9). Importantly, following electroporation of treated and untreated episomal DNA into bacteria and analysis of bacterial clones, the relative abundances of 1-LTR, 2-LTR, and mutant circular episomes (as measured by N*ot*I/S*ac*II restriction at sites flanking the LTRs to produce insert bands characteristic of 1-LTR or 2-LTR circles, as well as irregularly sized bands, designated mutant) were not significantly altered by RecBCD treatment (Fig. 10). These findings indicate that linear episomes are not subject to significant circularization in bacteria.

The shuttle vector assay reproduces results of Southern-blotting analysis.

Previous findings indicate that lentiviral 2-LTR circular episomes are formed by the process of nonhomologous end-joining (NHEJ) DNA repair, which circularizes linear reverse transcription products by ligating their ends together [39]. To verify these results, cells mutant and wild-type for the NHEJ factor Xrcc4 were transduced with a lentiviral vector and analyzed for episome formation by Southern-blot analysis. As shown in Fig. 11, the relative abundance of 2-LTR circles was significantly reduced in Xrcc4-deficient cells, which was in keeping with prior studies on 2-LTR circle formation [39]. Next, to determine the accuracy of the shuttle-vector system in our hands, the same wild-type and Xrcc4-mutant cell lines were analyzed for episome formation with the shuttle-vector assay. As Fig. 12 shows, the relative abundance of 2-LTR circles was observed to be significantly reduced in Xrcc4-mutant cells, which was in line with the Southern-blotting analysis and the findings of Li *et al.* [39]. These results indicate that the shuttle-vector system is able to replicate results obtained by this lab and others.

1-LTR circle formation is unaffected by changes to the homologous-recombination status of lentivector-transduced cells.

As noted above, results obtained in this lab and others indicate that lentiviral 2-LTR circular episomes are formed by the process of nonhomologous end-joining (NHEJ) DNA double-strand break repair. Given that lentiviral LTRs are complementary to each other, the possibility arises that 1-LTR circles are formed by the homologous recombination (HR) pathway of DNA double-strand break repair, with a lentiviral genome's LTRs recombining with each other. Indeed, previous studies demonstrated that linear HIV genomes, purified from viral proteins, are able to form 1-LTR circles when incubated in cytoplasmic extracts along with nucleoside triphosphates [42], indicating that 1-LTR circle

formation can be mediated exclusively by cellular factors. Furthermore, a more recent study found that, when treated with siRNA against the HR factor Rad52, HIV-infected macrophages exhibited a specific reduction in 1-LTR circle formation, indicating that cellular HR factors play a role in forming 1-LTR circles [44]. To investigate the possibility that cellular DNA repair factors in the HR pathway generate 1-LTR circular episomes, we used a shuttle-vector assay to examine the formation of 1-LTR, 2-LTR, and mutant circular episomes in lentivector-transduced cells mutant for an array of homologous-recombination (HR) factors (Table 1).

BRCA1 is a genome surveillance protein involved in coordinating the activities of DNA damage-sensing proteins and DNA repair proteins in the canonical, sister-chromatid version of homologous recombination [84] (Fig. 4). To investigate the possibility that BRCA1-associated HR is involved in 1-LTR circle formation, we used the shuttle-vector assay to examine the episomes formed in the BRCA1-deficient cell line SUM149, along with the BRCA1-wild-type cell line ME16C2. We found that a cellular deficiency in BRCA1 does not have a significant effect on 1-LTR circle formation (Fig. 13).

Second, Xrcc2 is a Rad51C paralog, also involved in the sister-chromatid pathway of HR [85](Fig. 4). To determine if Xrcc2 plays a role in 1-LTR circle formation, we measured episome formation in the Xrcc2-mutant cell line Irs1, along with the Irs1 wt. cell line, which is complemented with a copy of wild-type Xrcc2. We found that a mutation to Xrcc2 did not notably reduce 1-LTR circle formation (Fig. 14).

Third, ATM is a member of the phosphatidyl inositol 3-kinase-like kinase (PIKK) family involved in initiating responses to DNA damage both through the sister-chromatid and single-strand annealing pathways of HR [51](Fig. 4). To examine the effect of ATM on

1-LTR circle formation, we measured episome formation in the ATM-deficient cell line GM09607, along with wild-type human embryonic fibroblasts (HEFs). We found that the ATM mutation did not significantly decrease 1-LTR circle formation (Fig. 15).

Fourth, Ercc1 is a protein that, in a complex with XPF, forms an endonuclease that specifically cleaves 3' single-stranded DNA flaps of double-stranded DNA as a later step in the single-strand annealing pathway of HR [52] (Fig. 4). To determine the role played by Ercc1 in 1-LTR circle formation, we examined the episomes formed in the Ercc1-deficient cell line E1KO7-5, along with the Ercc1-wild-type cell line ATStg. We found that the Ercc1 mutation does not have a significant effect on 1-LTR circle formation (Fig. 16).

Finally, previous findings indicate that HR does not occur in G1 phase of the cell cycle [52, 51]. To investigate the effect of G1 arrest on episome formation, we grew AA8 cells to confluency in 1% DMSO to achieve G1 arrest, as verified by PI staining (Fig. 17a) then transduced the cells, along with non-confluent, freely cycling cells, with a shuttle vector and measured relative episomal abundance with the shuttle-vector assay (Fig. 17b). No reduction in 1-LTR circular episome formation was observed (Fig. 17). Overall, we found that cellular deficiencies in the BRCA1, Xrcc2, ATM, and Ercc1 proteins and arrest in G1 phase had no notable effect in decreasing the relative abundances of 1-LTR circular episomes in transduced cells.

Discussion

Earlier studies suggest that 1-LTR circles might be made by one or both of two independent pathways: reverse transcription (RT) and HR [42,44,48,49]. Based on experiments performed on cells under conditions inconducive to HR, we provide here some indication

that HR is not required for 1-LTR circle formation. The data presented here demonstrate that transduced HR-deficient cell lines, mutated for either BRCA1, Xrcc2, ATM, or Ercc1, did not exhibit a reduction in the relative abundance of 1-LTR circle formation, nor did transduced G1-arrested cells show such a decrease. This finding represents a notable discrepancy with prior reports concerning lentiviral episome formation, which found that cellular factors, specifically homologous-recombination proteins, were the source of 1-LTR circle formation [42,44]. Differences in methodology may explain the discrepancy between previous findings and our own. Specifically, the study by Farnet and colleagues examined the formation of 1-LTR circles from linear HIV cDNA genomes incubated in cytoplasmic extracts, which may not fully mimic the episome formation observed in our study of intact cells' processing of vector-protein-associated linear lentiviral genomes [42]. Also, while our study determined episome formation in cells transduced with lentiviral vectors, as measured by shuttle-vector assay and Southern-blot analysis, the episome formation data presented by Jacque and colleagues were derived from cells infected with wild-type HIV and measured by qPCR [44]. Furthermore, wild-type HIV, employed by Jacque *et al*., expresses a number of accessory proteins that lentiviral vectors do not. Indeed, given that measurement of 1-LTR circles by PCR has been shown to be problematic [92], and that the study presented by Jacque *et al*. was substantially rebutted [93], the divergence of their data from our findings is not unexpected.

The novelty of the shuttle-vector system employed here underscores the importance of demonstrating that the results generated by it are reproducible and not artifactual. To verify that the shuttle-vector assay could replicate previously published results, we characterized 2-LTR circle formation in Xrcc4-deficient cells. We found that

2-LTR circle formation is significantly reduced in an Xrcc4-mutant cell line, which agreed with prior findings [39] and our own Southern-blot analysis, and confirmed the utility of the shuttle-vector system. Furthermore, the possibility that the relative abundances of episome in transduced cells is misrepresented by the shuttle-vector assay due to circularization of linear episomes in bacteria is ruled out by the current study, which showed that treatment of episomes with the exonuclease RecBCD prior to electroporation into bacteria did not influence the percentages of episomal forms reported by the assay. Taken together, these results reinforce the reproducibility and reliability of the shuttle-vector system.

The present study indicates that the formation of 1-LTR circular episomes in cells transduced with lentiviral vectors is not affected by mutations of a variety of HR factors, nor by arrest of transduced cells in G1 phase of the cell cycle. These results are, at first glance, not in line with earlier findings indicating that nonhomologous end-joining (NHEJ) factors are required for the formation of 2-LTR circles [39], as both double-strand break repair pathways could be expected to act on linear lentiviral episomes. However, a subsequent study by the same group found that several HR proteins did not appear to play a role in 1-LTR circle formation [94], which indicates that other factors may account for the formation of 1-LTR circles. Indeed, earlier studies on retroviral episome formation suggested that 1-LTR circles are formed as products of aberrant reverse transcription [48,49]. These results stress the importance of a thorough investigation of vector factors, such as sequences associated with RT, that may affect episome formation.

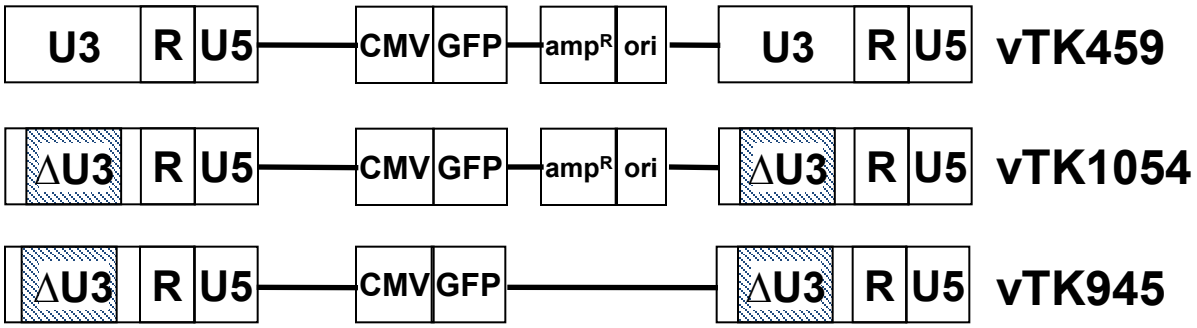


Fig. 7. Schematic of the vectors used in Chapter 2. vTK459 is a full-LTR shuttle vector, vTK1054 is a shuttle vector with a long U3 deletion, and vTK945 is a standard vector with a long U3 deletion.

cell line	mutation status	species	characteristics
293T	contains adeno and SV40 viral DNA	human	embryonic kidney-cell derived
CHO-K1	Xrcc4 wild-type	hamster	ovary-cell derived
GM16147	Xrcc4 mutant	hamster	ovary-cell derived
ME16C2	BRCA1 wild-type	human	breast tumor-derived
SUM149	BRCA1 mutant	human	breast tumor-derived
Irs1 wt.	Xrcc2 wild-type	hamster	ovary-cell derived
Irs1	Xrcc2 mutant	hamster	ovary-cell derived
HEF	ATM wild-type	human	fibroblast-derived
GM09607	ATM mutant	human	fibroblast-derived
ATStg	Ercc1 wild-type	hamster	ovary-cell derived
E1KO7-5	Ercc1 mutant	hamster	ovary-cell derived
AA8	wild-type	hamster	ovary-cell derived

Table 1. Cell lines used in Chapter 2.

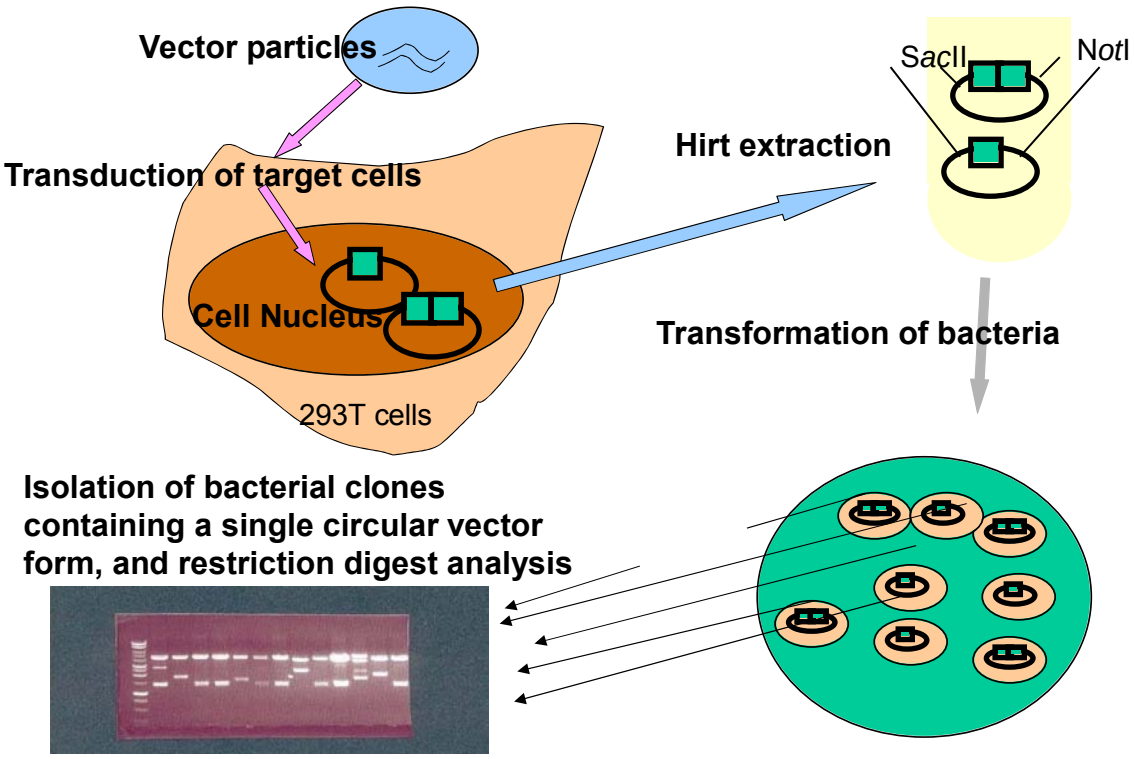


Fig. 8. Outline of the shuttle-vector assay for characterizing the relative abundance of circular episomes..

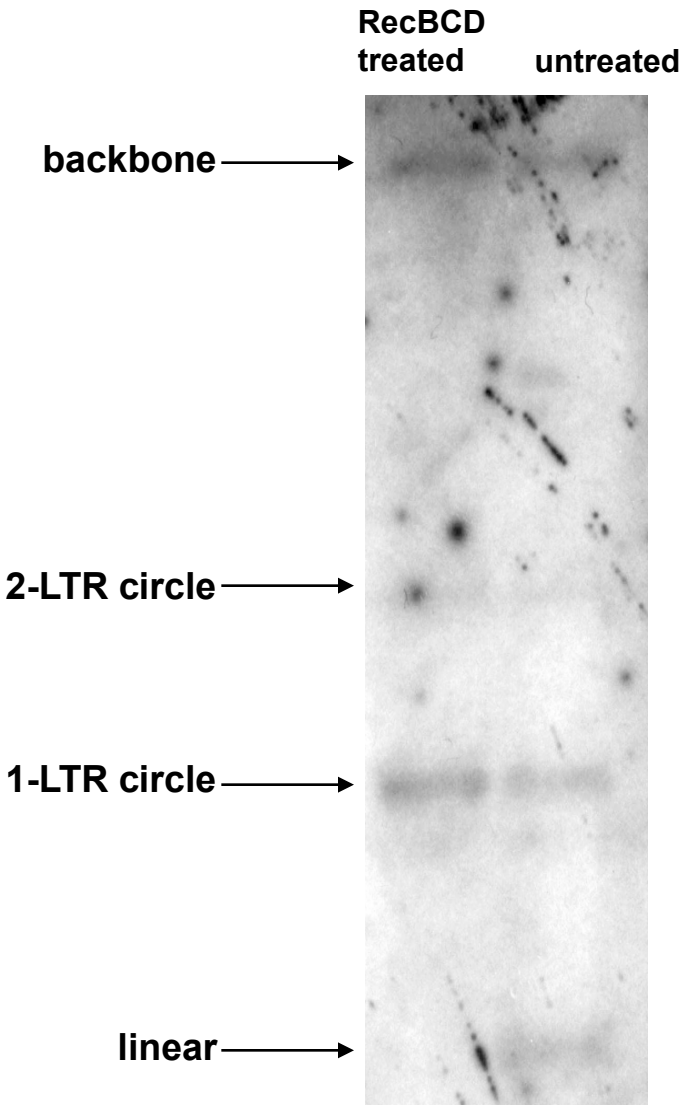


Fig. 9. Effect of RecBCD treatment on episome formation. DNA from 293T cells transduced with vTK459/IN+ was Hirt-extracted, treated with RecBCD (left lane) or left untreated (right lane), and analyzed by Southern-blot analysis.

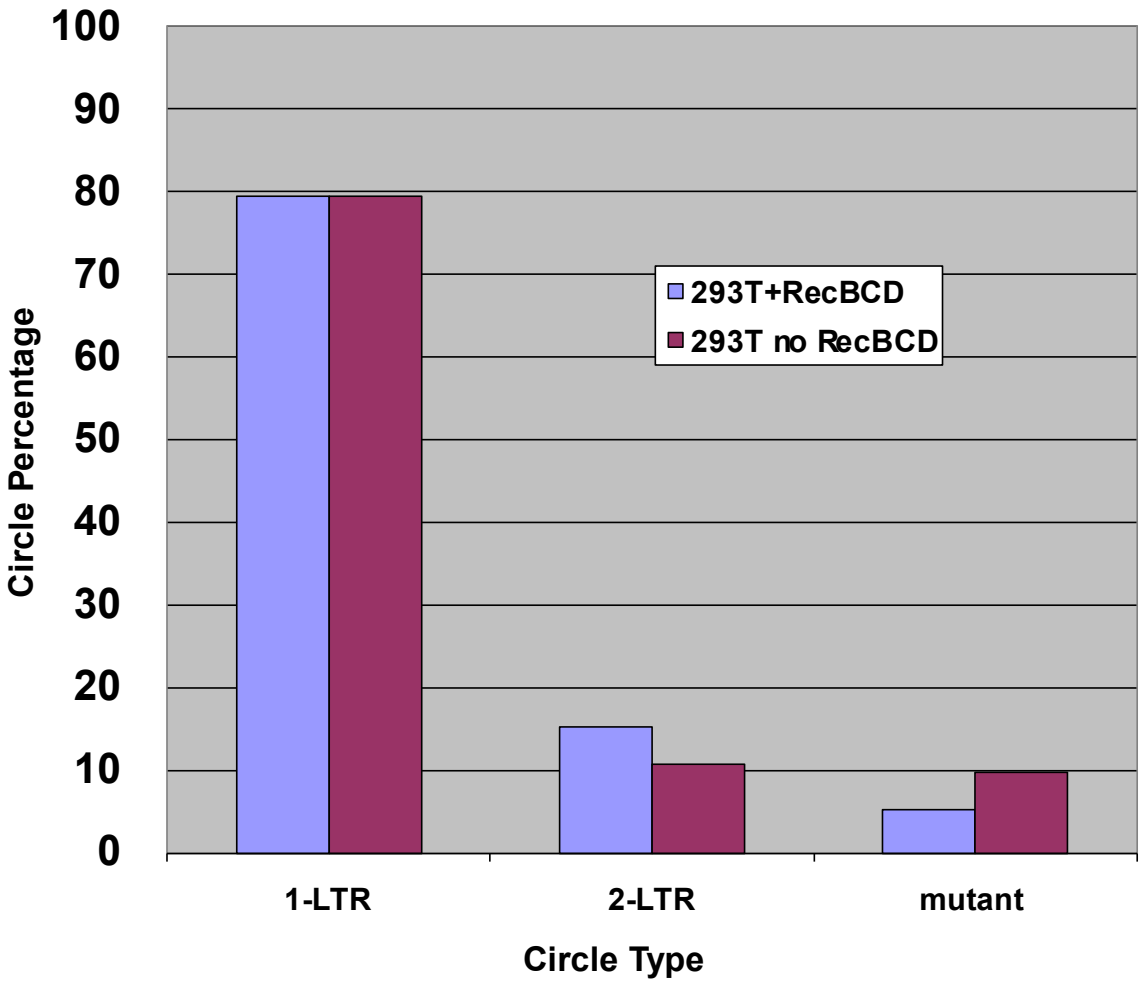


Fig. 10. Effect of RecBCD treatment on episome formation. DNA from 293T cells transduced with vTK459/IN+ was Hirt-extracted, treated with RecBCD (blue) or left untreated (red), and used to transform bacteria, which were clonally amplified and analyzed by restriction digest.

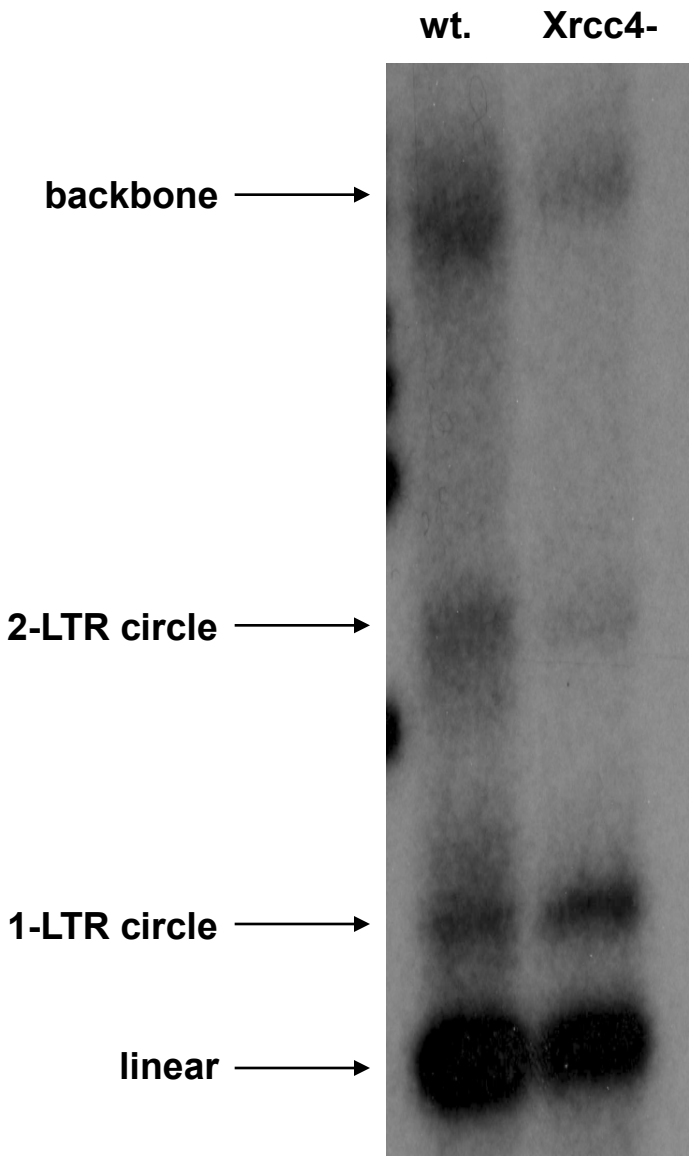


Fig. 11. Effect of Xrcc4 deficiency on episome formation. DNA from wild-type (left lane) or Xrcc4-mutant (right lane) cells transduced with vTK459/IN+ was extracted and analyzed by Southern-blot analysis.

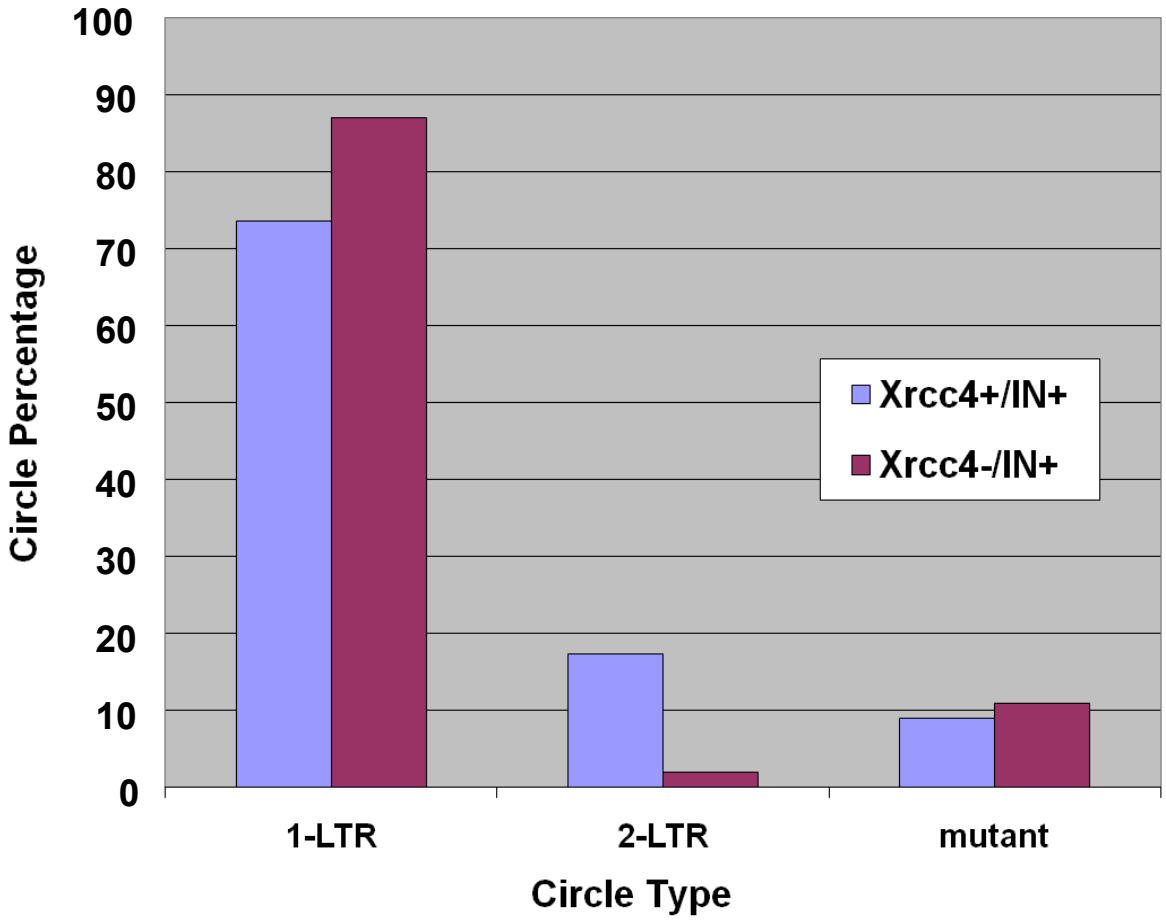


Fig. 12. Effect of Xrcc4 deficiency on episome formation. DNA from wild-type (blue) or Xrcc4-mutant (red) cells transduced with vTK459/IN+ was analyzed by shuttle-vector assay.

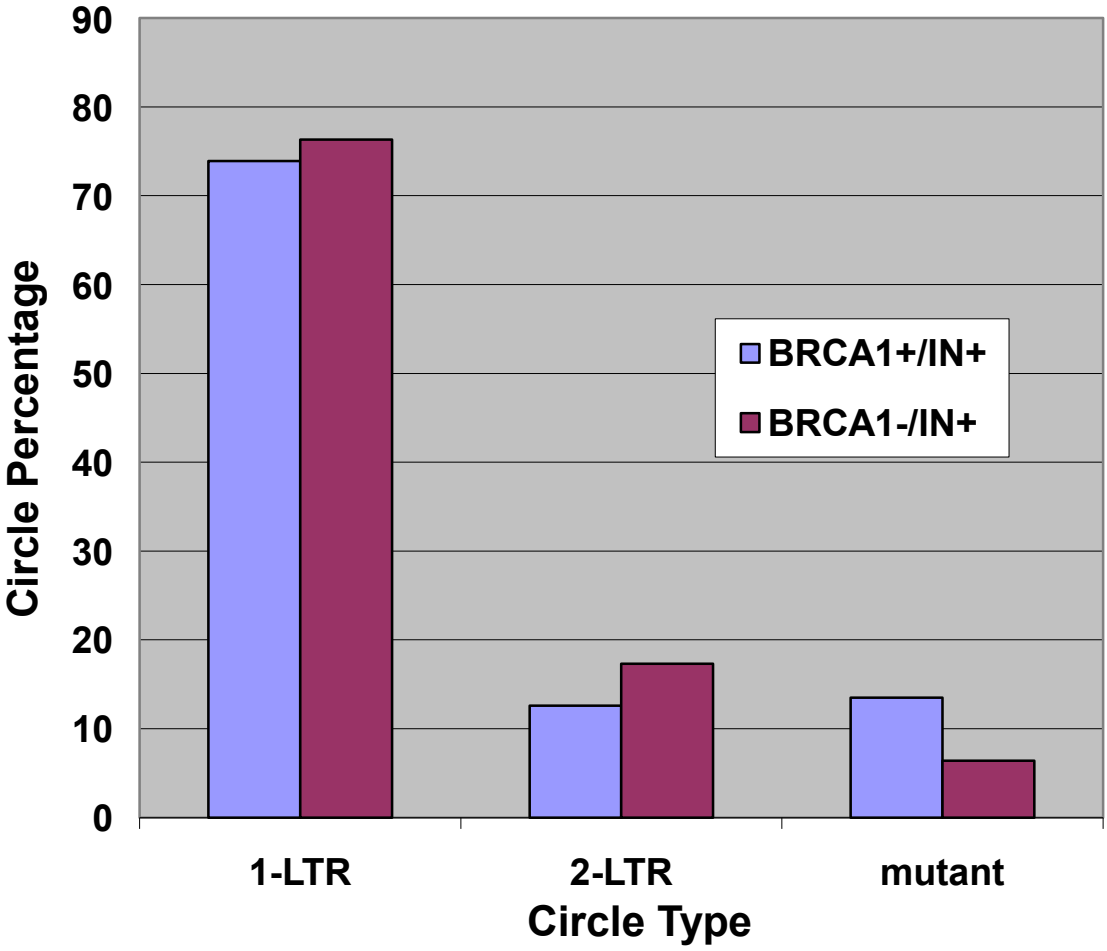


Fig. 13. Effect of BRCA1 deficiency on episome formation. DNA from wild-type (blue) or BRCA1-mutant (red) cells transduced with vTK459/IN+ were analyzed by shuttle-vector assay.

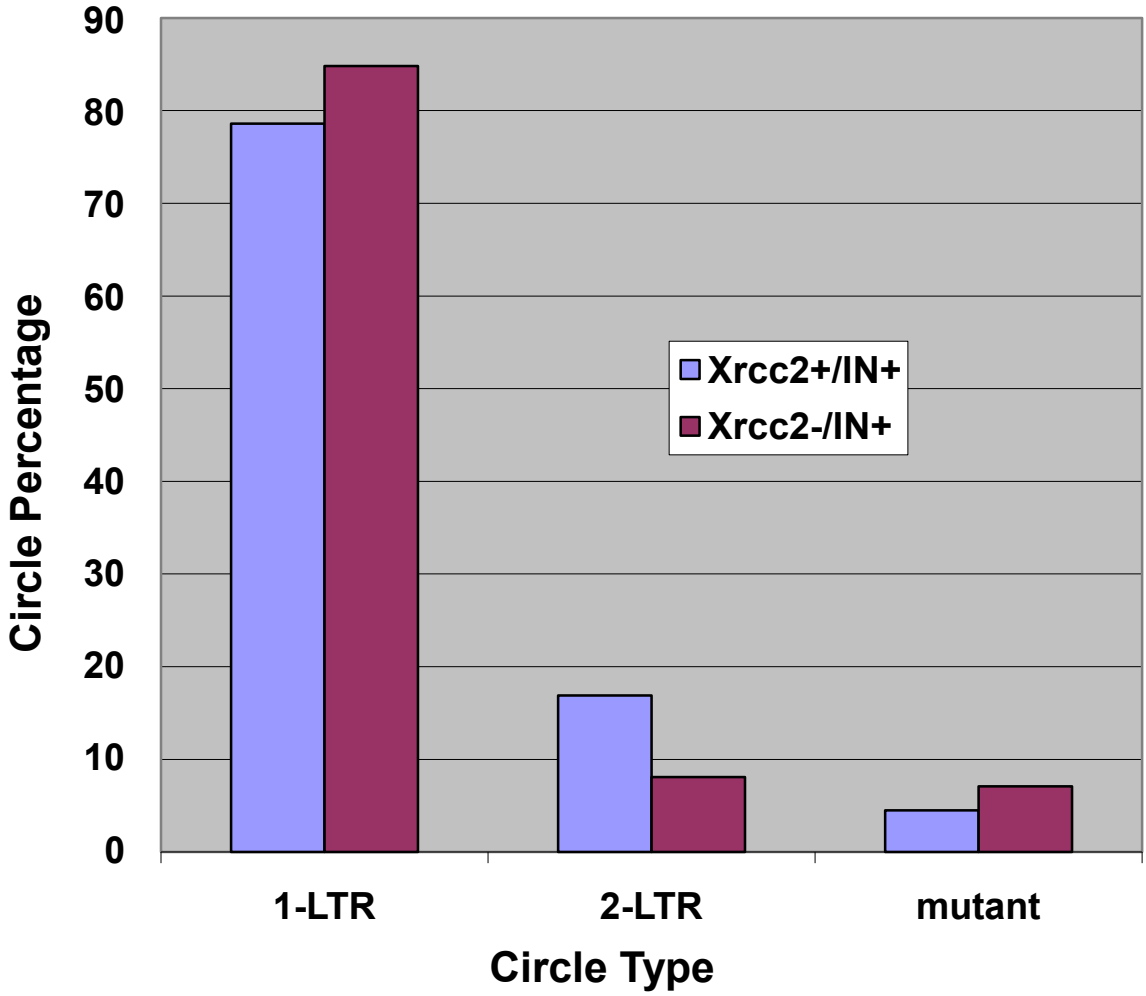


Fig. 14. Effect of Xrcc2 deficiency on episome formation. DNA from wild-type (blue) or Xrcc2-mutant (red) cells transduced with vTK459/IN+ were analyzed by shuttle-vector assay.

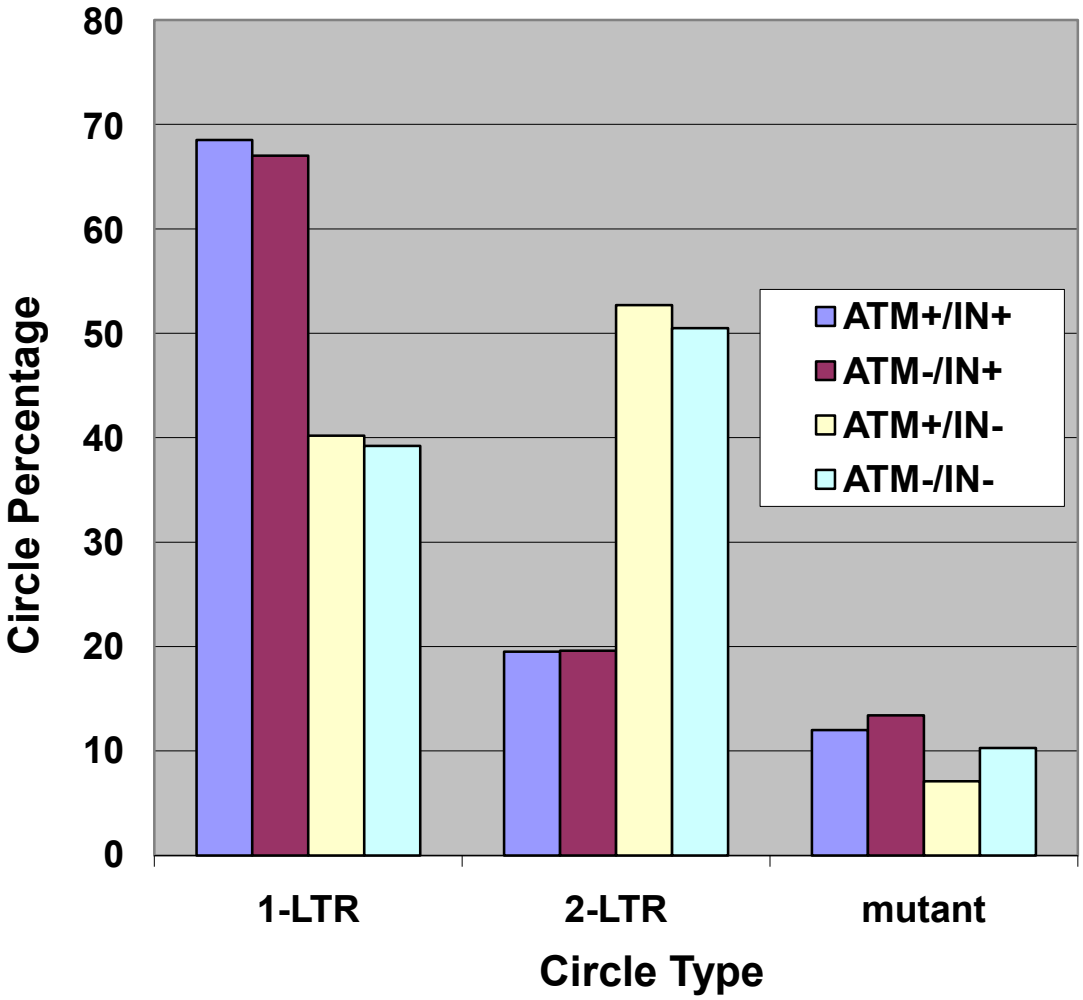


Fig. 15. Effect of ATM deficiency on episome formation. DNA from wild-type (blue and tan) or ATM-mutant (red and green) cells transduced with vTK459/IN+ were analyzed by shuttle-vector assay.

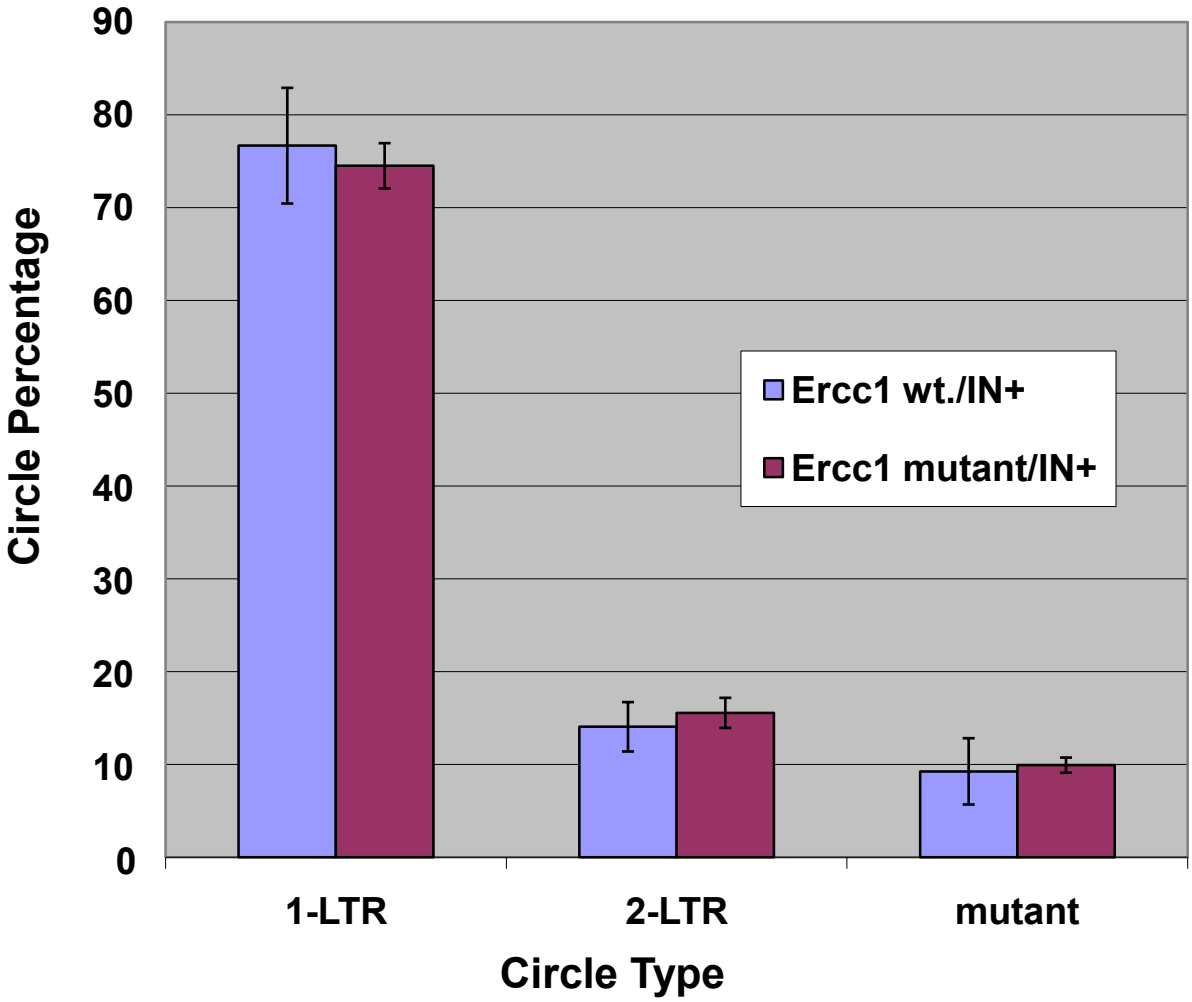


Fig. 16. Effect of Ercc1 deficiency on episome formation. DNA from wild-type (blue) or Ercc1-mutant (red) cells transduced with vTK459/IN+ were analyzed by shuttle-vector assay. Error = mean±SD, *n*=3.

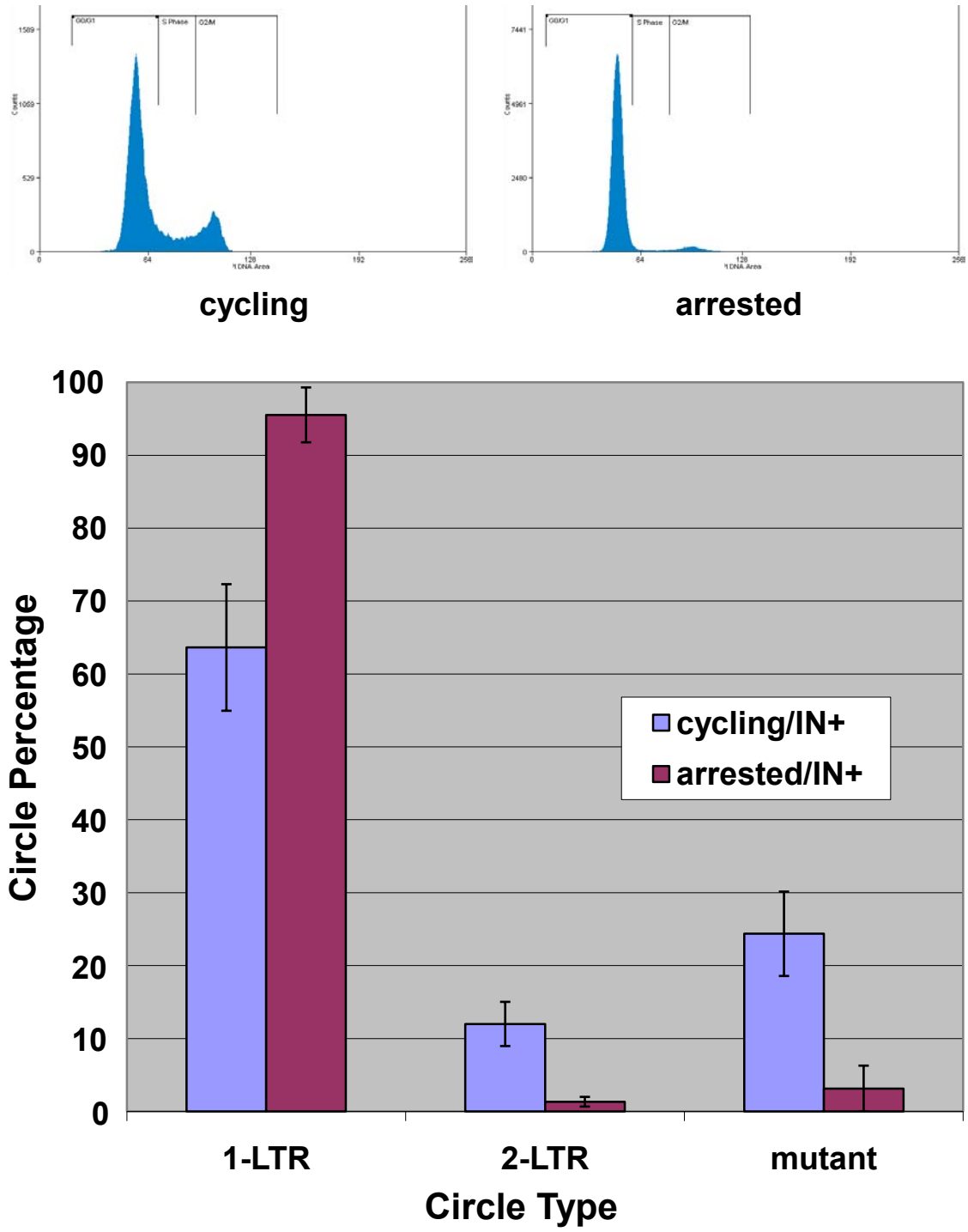


Fig. 17. Effect of cycle arrest on episome formation. DNA from cycling (blue) or G1-arrested (red) cells transduced with vTK1054/IN+ were analyzed by shuttle-vector assay. Error = mean±SD, *n*=3.

Chapter 3

VECTOR FACTORS AFFECTING EPISOME FORMATION AND EXPRESSION

A portion of the work in this chapter was published in *Molecular Therapy* 16(12): 1968-76.

Introduction

The large genetic payload, low immunogenicity, and ability to target nondividing cells of lentiviruses makes them an attractive vehicle for gene therapy. To that end, researchers have made a number of modifications to lentiviruses to make them nonpathogenic and replication-incompetent, as summarized in Chapter 1 (see Fig. 2) [6, 95].

Beyond the modifications necessary to make lentiviral gene-therapy vectors safe for researchers and patients, researchers have made other changes to the vectors. For instance, they have replaced the wild-type lentiviral envelope protein with the G protein of the vesicular stomatitis virus (VSV-G), which imparts broader tropism and the ability to withstand ultracentrifugation [96] (Fig. 2). Another change was packaging lentiviral vectors using an integrase mutated at the D64, D116, or E152 residues of its catalytic-core domain[4,67,97], resulting in a vector that cannot integrate into target-cell chromosomes, but can still import its cDNA genome into the nucleus. A notable outcome of integrase-defective transductions is that the number of 2-LTR circles increases relative to the total number of episomes in target cells [98], which may be due to the extra vector

genomes that would otherwise undergo integration, or may be because the mutant integrase does not cleave the episomes' LTRs, leaving them blunt and more amenable to NHEJ. Regardless of the mechanism for the change in relative abundance of episome forms, the possibility exists that other vector modifications could also cause changes in episome formation. Furthermore, the significantly downregulated transgene expression generated by integrase-deficient lentivectors has been observed in a number of studies [26,66], indicating that vector modifications may influence episomal transgene expression levels. For instance, previous studies indicate that the U3 region of HIV may harbor a transcriptionally repressive element [15-17]. Furthermore, several studies suggest that RT mediates the formation of 1-LTR circular retroviral episomes[48,49], suggesting that vector modifications may also influence episome formation.. Accordingly, expression and episomal formation by lentiviral vectors with largely truncated U3 sequences and with a complete deletion of the RT-associated PPT region were analyzed. We saw that the U3 deletion increases episomal expression without significantly changing episome formation, while the PPT deletion does not notably modify episomal expression, but leads to the formation of predominantly 1-LTR circular episomes in lentivector-transduced cells, which has the effect of reducing both integrase-mediated and integrase-independent integration.

Materials and Methods

Lentiviral vector constructs. The vector cassette pTK113 has been described previously [87] and contains a U3 deletion extending from –7 to –141. The vector cassette pTK945 bears a U3 deletion spanning –48 to –396, and is otherwise identical to pTK113. The vector cassette pTK1125 bears a U3 deletion extending from –18 to –418, and is otherwise

identical to pTK113. Shuttle vectors were derived from the vector plasmid pTK459 [99], which includes a bacterial origin of replication and ampicillin-resistance gene in the transcribed region of the plasmid, as well as a full-length U3 (in pTK459), short-deletion U3 (pTK1055), or long-deletion U3 (pTK1054). The vector cassettes pTK647 and pTK979 were constructed by inserting the U3 sequence from pTK113 and pTK945, respectively, into pTK646, which bears the liver-specific promoter hAAT and the firefly luciferase gene, by standard cloning procedures. The Flp9 cell line was prepared as follows: pTK113 was cloned into Flp-In expression vector (Invitrogen, Carlsbad, CA) and the resulting plasmid was cotransfected with pOG44 (Flp recombinase) into the Flp-In™ host cell line, such that pTK113 integrated into the genomic FRT site (Invitrogen). The single-copy incorporation of the expression cassette per diploid genome was verified by Southern blot, following digestion of DNA isolated from Flp9 cells with A*fl*II (New England Biolabs, Ipswich, MA, and all restriction enzymes to follow), which recognizes 2 sites in the LTR of the expression vector, or X*ba*I, which recognizes a single site in each FRT sequence. pTK1023 was generated by deleting an N*he*I fragment from vTK945, eliminating the PPT, then inserting an N*he*I fragment from vTK978, which replaced the U3 sequence's *att* site. pTK1179 was constructed by deleting a P*fl*MI/E*co*RV fragment from pTK945 and replacing it with a P*fl*MI/E*co*RV fragment from pTK1074, which contains a precise PPT deletion. pTK1074 was generated by performing two separate PCR experiments with two sets of primers, both using pTK459 as template:
CCTGGTTGCTGTCTCTTTATGAGG (Fw) and
GAATTAGCCCTTCCAGTAAAAAGTGGCTAAG (Rev), and
CTTAGCCACTTTTTACTGGAAGGGCTAATTC (Fw) and

CAATGTCAACGCGTATATCTGGCCCG (Rev). The resulting amplicons served as templates in a second PCR experiment, in which CCTGGTTGCTGTCTCTTTATGAGG (Fw) and CAATGTCAACGCGTATATCTGGCCCG (Rev) were the primers. The resulting single amplicon was cloned into pTK459 with S*ac*II/A*pa*I to create pTK1074. pTK1187 was created by cloning pTK529, a BSD-GFP-containing vector, into pTK945 with A*fe*I/X*ho*I, while pTK1188 was made by cloning pTK529 into pTK1179 with A*fe*I/X*ho*I. pTK1034 was generated by cloning pTK1023 into pTK464, a luciferase-containing vector, with K*pn*I/P*me*I.

Viral vector production. All lentiviral vectors were prepared as previously described [87], transiently transfecting 10^7 293T cells with 15μg vector cassette, 10μg packaging cassette, and 5μg envelope cassette. Integrating vectors were made using the packaging cassette ΔNRF [87], which expresses functional integrase, while nonintegrating vectors were made using the packaging cassette pTK939, which was made by inserting the D64E-mutant integrase from pD64E into ΔNRF by standard cloning procedures. All vectors were pseudotyped with the VSV-G envelope cassette. For vectors constitutively expressing GFP, titers were assessed by serial dilution in 293T cells followed by visual analysis of GFP expression by fluorescence microscope. For other vectors, concentrations were determined by p24gag ELISA. The absence of replication-competent retroviruses was determined by three independent methods: tat transfer assay, vector rescue assay, and p24gag ELISA, as described previously [100].

Cell culture. 293T cells were grown in Dulbecco's modified Eagle medium (Mediatech,

Herndon, VA) supplemented with 9% fetal bovine serum and 1% penicillin/streptomycin solution. Jurkat cells were cultured in Roswell Park Memorial Institute medium (Mediatech) supplemented with 9% fetal bovine serum and 1% penicillin/streptomycin solution.

FACScan analysis. 293T cells were transduced at an m.o.i. of 1. At 3-5 days (p0) and four passages (p4) posttransduction, cells were harvested, fixed in 1X phosphate-buffered saline (PBS)(HyClone, Waltham, MA) containing 2% formaldehyde/0.2% glutaraldehyde, and analyzed by FACscan as previously described [88]. For cell-cycle analysis, cycling and arrested cells were harvested, fixed in 70% ethanol (Pharmco-AAPER, Brookfield, CT), incubated in a solution containing 50μg/mL propidium iodide (Molecular Probes, Eugene, OR), 100 units/mL RNase A (Sigma), 100mg bovine serum albumin fraction V (Roche, Mannheim, Germany), and 10mL PBS.

Northern blot analysis. 293T cells were transduced with the indicated vectors at an m.o.i. of 1. Total cell lysates were prepared 3-5 days posttransduction for RNA isolation using the PARIS™ kit (Ambion, Austin, TX) or Rneasy® Plus Mini kit (Qiagen, Hilden, Germany). 3μg of total RNA was denatured at 70°C and resolved on a 1.2% denaturing formaldehyde/agarose gel. RNA was transferred to a Zeta-Probe GT® membrane (BioRad, Hercules, CA). Hybridization was executed at 68°C for ~18 hours with a ^{32}P-labeled probe comprising a 595-base region spanning the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) that was cut from pTK113 with C*la*I. The Northern blot was imaged with BioMax MR film (Kodak, Rochester, NY). Relative quantification of RNA

species was obtained via a Storm phosphorimager (GE Healthcare, Chalfont St. Giles, UK) using ImageQuant 5.2 software (GE Healthcare).

Southern blot analysis. Cells were harvested three days and three passages after transduction and their total DNA was extracted using the Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen) or as described previously (Kantor 2009)[89]. 10μg total DNA was digested with D*pn*I and either E*co*NI and P*fl*MI (for 293T cells) or N*ot*I and P*fl*MI (Jurkat cells), electrophoresed in a 1% gel, transferred to a nylon membrane (GE Healthcare), UV-crosslinked, and probed with the 1.4kb K*as*I/*Bam*HI fragment of vTK945 or the 1.6kb A*fe*I/E*co*RV fragment of vTK945. Some membranes were also probed with a 731bp region of the endogenous gene BDNF, produced with the PCR primers 5'CGTTTGACCAATCGAAGC3' (forward) and 5'TCCCCTCAGTCAGGACCCTCG3' (reverse). Quantification of DNA density was achieved either using ImageJ software or on a Storm phosphorimager using ImageQuant 5.2 software.

Shuttle-vector assay. 293T cells were transduced with integrating or nonintegrating vTK459, vTK1055, vTK1054, or vTK1074 (see above for construction details), and mouse livers were transduced with integrating or nonintegrating vTK1054. Transduced cells were harvested 16 hours posttransduction using 0.85mL Hirt lysis buffer and 0.25mL 5M NaCl per 10cm plate of transduced cells, and livers were harvested one day or 21 days posttransduction using 14.56mL SDS-free Hirt lysis buffer, 0.92mL 10% SDS, and 4.36mL 5M NaCl per liver. Episomal DNA was subsequently subjected to phenol/chloroform extraction and D*pn*I digestion and electroporated into *E. coli*. Bacterial

colonies were cultured monoclonally, pelleted, and their episomes extracted by boiling in a hotprep buffer made with 5g sucrose, 4mL 0.5M Tris/EDTA solution, 40mL 10% Triton X-100 (Sigma, St. Louis, MO), 5mL 1M Tris/HCl pH 8.0 and water to 100mL. 700μL hotprep buffer and 25μL lysozyme solution (10mg/mL lysozyme (Sigma) in 0.25M Tris/HCl pH 8.0) were added to each bacterial pellet, which was resuspended and placed on ice for 5-10 minutes before boiling for 1 minute. After boiling, samples were centrifuged at 14K for 10 minutes to dispose of cell debris. The supernatant was then mixed with 700μL isopropanol and centrifuged at 14K for 10 minutes, then the DNA pellet was washed with 70% ethanol, dried, and resuspended in 50-200μL water containing 10μg/mL RNase A. Episomal DNA clones were digested with N*ot*I and S*ac*II and electrophoresed in a 1% agarose gel to be characterized as 1-LTR, 2-LTR, or mutant circular episomes. For every transduction, between 73 and 112 episome-bearing bacterial colonies were characterized.

Luciferase assay. 48 hours posttransduction, target cells were lysed with cell culture lysis reagent (Promega, Madison, WI), harvested, and centrifuged to remove debris. Lysates were combined with luciferin reagent and luciferase expression was measured as relative light units (RLU), using a Victor^3 multilabel counter (PerkinElmer, Waltham, MA). Each experiment was performed in quadruplicate.

qPCR analysis. 48 hours posttransduction, total genomic DNA was extracted from transduced 293T cells and treated with D*pn*I (New England Biolabs, Ipswich, MA). The cell-equivalent content of all genomic DNA preparation was calculated through PCR

amplification of the human β-globin gene, and the DNA equivalent of 330 cells was used for viral quantification. To target the loading-control gene β-globin, the following primers were used: 5'-CAGAGCCATCTATTGCTTAC-3'(forward), 5'-GCCTCACCACCAACTTCATC-3'(reverse). To target the vector woodchuck hepatititis virus posttranscriptional regulatory element (WPRE), the following primers were used: 5'-ACGTCCTTCTGCTACGTCC-3' (forward), 5' AAAGGGAGATCCGACCGACTCGTC-3' (reverse). A range of 1.5x 10^4 to 1 cell-equivalent units was used in duplicate to establish the standard curve. Reactions were labeled with SYBRÆ Green I (Cambrex, East Rutherford, NJ) and performed in triplicate on an iCycler iQ-Multicolor real time PCR detection system (BioRad, Hercules, CA). Genomic DNA from Flp9 cells was used as a standard. Genomic DNA isolated from RT deficient-transduced cells, Flp-In™ cells, and uninfected 293T cells, and template-free reaction mix served as negative controls. Reactions were analyzed using the BioRad Optical System software (version 3.1). 10μL of each PCR sample was subjected to gel electrophoresis and visualized by staining with ethidium bromide.

Results

Increased *in vitro* episomal expression from a vector with a large U3 deletion

The first vector modification to be examined for its effect on episomal expression and episome formation was the U3 region of the LTR. Several groups recently demonstrated notable *in vitro* and *in vivo* expression from nonintegrating, U3-truncated, self-inactivating (SIN) lentiviral vectors [31,38,66,77,79, 101]. These results are not in line with previous studies on integrase-deficient lentiviral vectors, which exhibited low to negligible

viral gene expression from genomes with full-length U3 regions [4,74,102]. The improved efficiency of episomal expression exhibited by SIN vectors suggests that *cis*-acting sequences in the U3 may negatively regulate expression. Indeed, a sequence element in the 5' U3 region has been shown to reduce both LTR-driven and internally promoted expression [16,17,19]. However, the level and efficiency of episomal lentiviral expression has not been rigorously characterized. To investigate the possibility that a larger U3 deletion on a SIN vector could enhance its expression, two SIN vectors were made, one bearing a short U3 deletion (vTK113, deleted from –7 to –141, as in the vector described by Miyoshi *et al.* [14], and one containing a larger U3 deletion (vTK945, deleted from –48 to –396, thus retaining the TATA box)(Fig. 19a)(see Fig. 18 for a full list of vectors used for this study); both vectors were packaged with functional or deficient integrase and used to transduce 293T cells (Fig. 19b). To minimize the risk of expressing saturating quantities of GFP, thereby confounding subsequent GFP expression analysis, cells were transduced with decreasing amounts of vector, and target-cell populations exhibiting <60% transduction were analyzed by FACS analysis at five days posttransduction for GFP expression. As shown in Fig. 19b, a larger U3 deletion increased GFP fluorescence nearly threefold, from an MFI of 26.47 for the nonintegrating short-deletion vector vTK113/IN- to an MFI of 69.96 for the nonintegrating long-deletion vector vTK945/IN-. However, regardless of the length of the U3 deletion, integrating vectors expressed more GFP than either nonintegrating vector, and the difference between the levels of transgene expression exhibited by vTK113/IN+ and vTK945/IN+ was not significant. Not surprisingly, after four passages GFP was not detected by FACS analysis in cells transduced with

nonintegrating vectors (Fig. 19b), and less than 0.1% of cells displayed GFP measurable by fluorescence microscopy (data not shown).

To support further the possibility that U3 sequences inhibit transcription, a Northern-blot assay was employed to measure the effect of U3 deletion length on transcription in 293T cells. Cells were transduced as for FACS (see above paragraph) and RNA was harvested from nuclei, hybridized to a radiolabeled woodchuck hepatitis virus posttranscriptional regulatory element (WPRE)-specific probe, which recognizes transcripts both originating from the internal CMV promoter and LTR-derived transcripts, and quantified via a phosphorimager. In keeping with FACS analysis, the Northern assay indicated that the larger U3 deletion enhanced episomal transcription nearly fourfold, that both integrating vectors produced significantly more RNA than either nonintegrating vector, and that short-deletion and long-deletion integrating vectors generated comparable levels of RNA (Fig. 19c, Table 2). Furthermore, full-length transcripts were not detected, indicating that vTK113 and vTK945 are SIN vectors, despite the presence of a TATA box in vTK945's U3. These findings are in line with a prior study that also failed to detect full-length transcription from SIN vectors with TATA-containing U3 sequences [12]. However, mindful of the possibility that the TATA box in vTK945 may contribute to its increased episomal expression, we constructed a new vector (vTK1125, Figs. 17 and 18) containing a U3 deletion (from –18 to –418, as described in Zufferey *et al.*) that eliminates both the TATA box and 5' U3 sequences, rendering it "state-of-the-art." Interestingly, extending the deletion in the U3 to include the TATA box did not improve episomal transgene expression beyond the levels obtained by vTK945 (Fig. 19). Finally, the notion that the larger U3 deletion mediates increased episomal expression was further confirmed

by a qPCR-normalized luciferase assay comparing short-deletion (vTK464) and long-deletion (vTK993) vectors (Fig. 20).

Relative abundances of episomal forms are unaffected by U3 deletion length

These results were in line with earlier studies suggesting that the U3 sequence contains an expression-repressing element in its 5' region [15-17,19]. However, we could not rule out the possibility that other factors account for the increase in expression associated with the long U3 deletion in vTK945. To date, there is no evidence either suggesting or contradicting the notion that the various episomal forms (2-LTR circular, 1-LTR circular, mutant, and linear) express transgenes with differing efficiencies. Thus, the question remains of whether the increased episomal expression generated by nonintegrating long U3-deletion vectors is associated with a concomitant change in the relative abundance of episomal forms. Similar to earlier studies characterizing the relative abundance of vector episomes and integrated provirus [32,39,94], Southern analysis was employed on DNA extracted from transduced Jurkat and 293T cells at three days posttransduction and following three passages (Fig. 21a). As shown in Fig. 21b and quantified in Table 3, the relative abundance of episomal forms in integrating and nonintegrating vectors was not changed significantly by the longer U3 deletion. However, and in keeping with a prior study [98], differences in relative episomal abundance were caused by integrase status, with nonintegrating vectors producing significantly more 2-LTR circles than integrating vectors, and by cell type, with 293T cells exhibiting a notably greater proportion of linear episomes than Jurkat cells. Interestingly, and differing from a recent report [32], the relative abundance of linear episomes was not dramatically increased by integrase deficiency (Fig. 21b). Under the condition of harvesting DNA three days posttransduction, linear episomes

were comparable to, and in some cases exceeded by, 1-LTR circles in terms of relative abundance (Table 3). Furthermore, integrated provirus accounted for between 21 and 33% of all vector genomes, regardless of U3 deletion size (Fig. 21b). While Southern-blot analysis did not show significant changes in episome formation mediated by U3 length, cell line-specific changes were noted, with all vectors producing a greater share of linear episomes and a smaller proportion of 1-LTR circles in 293T cells than in Jurkat cells (Fig. 21b). Prior studies on simple retroviruses demonstrated the existence of autointegrated lentiviral episomes [41]; however, the relative contribution of this unique episomal form to the total population of episomes in the context of lentiviral/retroviral vectors has not been elucidated, largely because autointegrated episomes, being variably sized, cannot be adequately detected by Southern blot. To overcome this technical difficulty, we developed an HIV-1-derived shuttle vector containing a bacterial origin of replication and a drug-resistance gene within the vector's transcribed region. This vector configuration enables circular episomes produced in vector-transduced cells to be extracted by the Hirt protocol [91], and monoclonally isolated from bacteria (Fig. 8). Simple restriction analysis allows individual episomal clones to be characterized either as 1-LTR, 2-LTR, or mutant (autointegrated or self-recombinant) circles, according to predicted backbone and insert sizes. As shown in Fig. 21c (and in keeping with previous results [98,103], results of the shuttle-vector assay indicate that, in integrating-vector-transduced cells, 1-LTR circular episomes, 2-LTR circular episomes, and autointegrated circular episomes account for roughly 75%, 15%, and 10% of circular episomes, respectively, while in nonintegrating vector-transduced-cells, their relative amounts were 60%, 35%, and 5%, respectively. Shuttle vectors bearing a full-length LTR (vTK459, described in detail in Ma *et al.* [99]), a

short U3 deletion (vTK1055), or a long U3 deletion (vTK1054) (Fig. 18c) were assayed, leading to the finding that U3 length did not affect the relative abundances of circular episomes, with the notable exception that vTK1054 demonstrated significantly more autointegrated episomes than vTK459 (p-value of 0.0005 by chi^2 test)(Fig. 21c, Table 4).

PPT-deleted vectors exhibit expression levels comparable to integrase-deficient lentivectors.

Having determined the effect of one vector modification (the large U3 deletion) on episome formation and expression, we turned our attention to another vector modification with the potential to affect those vector functions. Specifically, we investigated the effect of the polypurine tract (PPT) on episome formation and episomal expression. Previous studies indicating that 1-LTR circular lentiviral episomes may be formed through reverse transcription [47-49], coupled with our finding that cellular homologous –recombination factors do not appear to mediate 1-LTR circle formation, raise the possibility that reverse-transcription-associated factors and sequences may affect 1-LTR circle formation. One such associated sequence is the PPT, which is involved in the plus-strand initiation step of reverse transcription and may influence the efficiency of strand displacement synthesis, which, when aberrant, may lead to 1-LTR circle formation [3]. We hypothesized that initiating plus-strand synthesis at a cryptic site distant from the viral 3' LTR may reduce integration by increasing 1-LTR circular episome formation and thereby reducing the formation of linear episomes, which are the natural substrate for retroviral integrase [3]. Accordingly, we deleted the 3' polypurine tract (PPT), which borders the 3'LTR and serves as a primer for plus-strand synthesis (Fig. 22). To examine the effect of deleting the PPT on vector expression and integration, we employed FACS analysis and quantitative

PCR to measure vector-derived GFP expression and vector-genome persistence, respectively. As shown in Fig. 23a, the integrase-proficient, PPT-deleted (PPT-/IN+, vTK1188 in Fig. 22) vector produced a notably low level of GFP expression, with a mean fluorescence intensity (MFI) of 40, which was typical of nonintegrating lentivectors and comparable to the GFP expression generated by the integrase-mutant vectors, which produced MFIs of 30 (PPT+/IN-) and 39 (PPT-IN-); conversely, the conventional, PPT-positive, integrase-proficient vector (vTK1187 in Fig. 22) exhibited a high level of GFP expression (MFI of 280)(Fig. 23a). The low level of GFP expression, which is a hallmark of nonintegrating vectors, produced by the PPT-deleted vectors indicates indirectly that they fail to integrate efficiently. The idea that PPT-deleted vectors integrate poorly was supported by the finding that only 2.4% of cells transduced with the PPT-/IN+ vector (out of 70% three days after transduction) still expressed GFP after four passages (p4), while more than a third of cells transduced with the conventional PPT+/IN+ (vTK1187) vector continued to express GFP at p4 (Fig. 23b), which is in line with previously measured levels of integrase-mediated integration [103]. Similar to earlier studies demonstrating minimal vector integration in the absence of integrase activity [25,32], 0.5% of cells transduced with integrase-deficient vectors, either with or without the PPT, were GFP-positive after four passages (compared to 50% GFP-positive cells at three days posttransduction). This low level of GFP-positive cells, generated by illegitimate integration, which is near the level of background signal detected by FACS, constituted a technical limitation necessitating the use of a more sensitive, qPCR-based assay in order to characterize illegitimate integration more accurately. Accordingly, qPCR analysis of integration efficiency was determined as the ratio of vector genome copies per cell

measured three days posttransduction to those detected after four passages. Indeed, as shown in Fig. 23c, employing the qPCR assay showed that the integration efficiency of the PPT-deleted, integrase-proficient vector was 2.7%, in comparison to 27% for the PPT-positive, integrase-proficient vector, indicating that the PPT deletion alone causes a tenfold reduction in integration efficiency. Indeed, these results are consistent with the results of the FACS analysis (Fig. 23a), which also suggest a roughly tenfold difference in integration between PPT-positive and PPT-deleted, integrase-competent vectors. Most importantly, the qPCR assay provides direct evidence that the PPT deletion reduces illegitimate integration from integrase-deficient lentivectors. As shown in Fig. 23c, the PPT-/IN- vector exhibited an integration level of 0.08%. In comparison, the PPT+/IN- vector's integration level was 0.2%, indicating that the PPT deletion reduces illegitimate integration 2.5-fold. Clearly, these data indicate that the reduced levels of illegitimate integration generated by PPT-deleted, integrase-defective lentivectors render them more applicable to clinical settings, in which inadvertent integration events must be minimized.

Deleting the PPT reduces lentivector integration and leads to the nearly exclusive formation of 1-LTR circular episomes.

To determine the mechanism of the PPT deletion's effect of reduced integrase-mediated and integrase-independent integration, we sought to elucidate the effect of the PPT deletion on episome formation. To this end, 293T cells were transduced with either PPT-positive (vTK945) or PPT-deleted (vTK1179) lentivectors, packaged with or without functional integrase. Employing Southern-blot analysis (Fig. 24) three days after transduction, we observed that the PPT deletion increased 1-LTR circle formation and reduced or eliminated linear episomal DNA formation (Fig. 25a). This finding supports

the possibility that altering the process of reverse transcription can increase the formation of 1-LTR circles, reducing linear episomes and, consequently, decreasing the downstream products of linear episomes: namely, 2-LTR circles and integrated proviruses. Indeed, PPT-deleted vectors displayed minimal 2-LTR circle formation, consistent with the previously established notion that 2-LTR circles are formed from linear episomes by the process of NHEJ [39]. A novel PPT-deleted shuttle vector, containing a bacterial origin of replication and an ampicillin resistance gene, was developed to confirm these interesting results, using restriction digestion of individual vector clones rescued by Hirt extraction (Fig. 25c). This assay corroborates the results of the Southern-blot analysis, demonstrating that the PPT deletion mediates a significant increase in 1-LTR circle formation and decrease in 2-LTR circle formation.

Encouraged by these results, we sought to explore the possibility that, by deleting the PPT, we could decrease linear-spisome formation and thereby inhibit integrase-mediated integration. To test this hypothesis, 293T cells were transduced with either wild-type or PPT-deleted vectors, as shown above (Fig. 25a), and, after four passages, were analyzed by Southern blotting to determine the relative abundances of episomal and integrated vector genomes. As shown in Fig. 25a, lanes 5-8, the PPT-deleted vectors generated an integrated-provirus signal that, even in the presence of functional integrase, was either nonexistent or below the threshold of detection by Southern-blot analysis, using a radiolabeled probe complementary to a sequence in the vector genome's 5' region. The nearly undetectable level of linear episomes in cells transduced with PPT-deleted vectors, as measured by Southern-blot analysis (Fig. 25a, lanes 1-4) were not fully in line with earlier studies characterizing illegitimate integration of plasmid DNA,

which indicated that linear DNA is 40-fold more efficient than circular DNA as a substrate for illegitimate integration [104]. This finding raised the possibility that not all linear episomes were accounted for by the Southern-blot analysis shown in Fig. 25a. In that vein, we conjectured that the aberrant reverse transcription induced by the PPT deletion mediates plus-strand synthesis from several cryptic sites in the vector genome, producing linear episomes with a uniform 3' end, but with sequence variation at the 5' end (Fig. 26). This precludes the use of a molecular probe directed to the 5' end of the vector genome as an efficient means of detecting linear episomes of various lengths. To overcome this obstacle, we designed a probe complementary to the 3' end of the vector genome as a means to detect all episomal forms (Fig. 24). Indeed, Southern-blot analysis with a 3' probe indicated that PPT-deleted vectors produce detectable levels of linear episomes, though at roughly a threefold lower level than PPT-positive vectors (Fig. 25b) (Table 5).

Discussion

We present an examination of expression and episomal formation by lentiviral vectors with largely truncated U3 sequences and with the RT-associated PPT region deleted. We observed that the U3 deletion increases episomal expression, but does not notably affect episome formation. Conversely, the PPT deletion does not markedly modify episomal expression, but leads to the formation of predominantly 1-LTR circular episomes in lentivector-transduced cells, which has the effect of reducing both integrase-mediated and integrase-independent integration.

Effect of U3 deletion length on episome formation and expression

We characterized episomally derived transgene expression and episome formation from SIN lentiviral vectors *in vitro*. In general, while all vectors demonstrated measurable levels of transgene expression regardless of the SIN vector used, nonintegrating vectors exhibited significantly less transgene expression than their integrating counterparts. This finding represents a notable discrepancy with recent reports concerning nonintegrating vectors, which do not indicate any major difficulty in obtaining robust expression from nonintegrating vectors [38,66]. Differences of methodology in quantifying expression may explain the discrepancy. Specifically, in the present study efforts were made to evaluate the efficiency of expression by Northern analysis and direct measurement of transgene abundance, either by mean fluorescence intensity (MFI) or by relative light units (RLU); furthermore, to avoid saturation, transduction was carried out at a low m.o.i. and expression was normalized to vector copy number. Conversely, previous reports measured the percentage of cells expressing a given transgene; this method does not measure the degree of expression in a transgene-positive cell. Another methodological concern is that expression from integrating vectors, if measured shortly after transduction or from nondividing cells, is generated primarily from episomes, which account for 70-95% of vector genomes [18,40,103]. Therefore, early expression seen from cells transduced with integrating vectors may still be largely episome-derived and not as efficient, on average, as expression from exclusively integrated genomes, possibly leading to the undervaluation of expression generated from integrated provirus. However, the mechanism by which expression from unintegrated lentiviral genomes is repressed is not clear. The fact that the first (non-SIN) lentiviral vectors to be developed exhibited the lowest level of episomal expression raised the possibility that a *cis* element in the U3 region may inhibit

transcription from lentiviral vectors, and, in fact, early studies on HIV-1 suggest the existence of such an element [15-17]. While the effect of the long U3 deletion on episomal expression implies that *cis*–acting elements in the lentiviral vector genome influence extrachromosomal transcriptional activity, the possibility exists that cell-specific *trans*-acting factors are also involved in the mechanism of downregulated transgene expression from lentiviral episomes. In keeping with the concept of cell-specific factors influencing episomal expression, previous findings indicate varying levels of gene expression across a number of cell lines infected with nonintegrating lentivirus [58]. Interestingly, transduction of murine dorsal root ganglia in culture by wild-type U3 or long U3-deletion integrating lentiviral vectors resulted in strong expression exclusively in neurons, or both neurons and stromal cells, respectively, further suggesting that the silencing mechanisms affecting integrating and nonintegrating retroviral vector expression bear cell-specific characteristics [19].

The possibility that increased efficiency of expression from long U3-deletion vectors is due to differences in episome formation is ruled out by the present study, which indicates that no significant change in the relative abundances of episomal forms is associated with the large U3 deletion. However, analysis of episome formation revealed discrepancies with published results. In contrast to a prior report, the relative abundance of linear episomes did not increase in the absence of functional integrase [32]. In keeping with the findings of Svarovskaia *et al*. [98], the relative abundance of 2-LTR circular episomes increased in the absence of integrase activity. Two putative mechanisms proposed earlier by Engelman *et al*. [28] to explain this phenomenon suggest either that blunt-ended, linear DNA, unprocessed by mutant integrase, is more amenable to circularization by

end-joining, or that more linear DNA is available for circularization in the absence of integrase activity. Interestingly, the efficiency of integration in the present study, as measured by Southern blot, ranged from 23% to 35% of all integrase-functional vector genomes, which is higher than the 5-16% measured previously by PCR [103]. The present study employed a novel shuttle-vector assay to quantify the mutant circular episomal form produced by lentivectors, and observed that integrase-deficient vectors produced a measurable number of mutant circles, suggesting that they may be formed partly through integrase-independent self-recombination instead of self-integration. Furthermore, integrating long U3-deletion vectors were found to produce notably more autointegrated circular episomes than did other vectors, implying that U3 sequences help prevent the formation of autointegrated circles.

This study showed that a nonintegrating lentiviral vector with an extensively truncated U3 region can be an efficacious means of delivering transgenes to target cells. However, the fact that nonintegrating long-deletion lentiviral vectors still do not express transgenes as efficiently as their integrating counterparts indicates that an additional mechanism of episomal silencing may be involved, possibly one that is inherent to unintegrated retroviral genomes. The transcriptional downregulation of lentiviral episomes, combined with the reduced probability that they are influenced by the chromosomal transcriptional regulatory environment, indicate that the characteristics of integrated lentiviral genomes cannot be extrapolated to lentiviral episomes, and that further characterization of nonintegrating lentiviral vectors is required to optimize their use in basic research and gene therapy applications.

Effect of PPT deletion on episome formation and expression

An investigation of the episomal expression and episome formation exhibited by PPT-deleted vectors demonstrated an intriguing reduction in illegitimate and integrase-mediated integration. To date, efforts to reduce lentivector integration have focused on the integration step of the viral life cycle. A number of well-characterized mutations to the integrase's catalytic-core domain, at residues D64, D116, and E152, have demonstrated the ability to inhibit vector integration without affecting reverse transcription or nuclear import [29]. These mutations have reduced integration to a baseline level of illegitimate integration, which was determined by our group to occur once per 5×10^2 to 2×10^3 vector genomes (Fig. 23), and by others to occur at a rate of once per 4.59×10^3 to 7.5×10^6 vector particles [105, 58, 38]. However, attempts to reduce illegtitimate integration by further targeting the integration pathway through mutating the vector's *att* sites in combination with integrase mutations have not been successful [31,32]. In light of these results, minimizing illegitimate integration for safety purposes is an imperative for nonintegrating lentivectors, especially in applications that require the expression of potentially genotoxic genes, such as zinc-finger nucleases, which mediate site-specific double-strand breaks in genomic loci to stimulate homologous recombination with vector-borne sequences or nonhomologus end-joining within the genome, allowing the replacement, addition, editing, or disruption of host-cell genes [106,107]. Importantly, as zinc-finger nucleases have exhibited off-target activity, their expression must be transient to minimize the inadvertent disruption of untargeted genes [108]. Furthermore, the novel technology of inducing pluripotent stem cells from somatic cells [109,110] has employed lentiviral vectors as a vehicle for delivering reprogramming factors. However, some reprogramming factors have exhibited oncogenic potential, which renders their integration

into host genomes highly undesirable [22,111,112]. In an attempt to alleviate this biohazard, Mali *et al*. explored the possibility of using nonintegrating lentivectors to deliver reprogramming factors, including SV40 large T antigen [65]. Although iPS colonies were generated via integrase-deficient HIV vectors, all tested colonies demonstrated unintended integration of the large T coding sequence, underscoringing the importance of minimizing illegitimate vector integration [65]. In a similar vein, applications in which transient transgene expression is desired, such as vector-mediated expression of antigens to elicit an immune response, could also benefit from minimized illegitimate integration [76,113]. To identify a novel, complementary approach to reduce the illegitimate integration exhibited by the currently used integrase-deficient vectors, we investigated the mechanisms underlying lentivector episome formation.

Since linear genomes are the preferred substrate for integration, it was not surprising that 1-LTR circles, the putative result of an unproductive detour in the process of RT, were termed "dead-end byproducts of aborted infection" [3]. Intrigued by a previous study demonstrating that, when injected into mammalian nuclei, linear DNA is 40-fold more likely to integrate than supercoiled circular DNA [104], we sought to reduce illegitimate integration by making this natural detour in the process of reverse transcription (1-LTR circle formation) more prominent. Our experiments were premised on the notion that the PPT deletion could alter the reverse transcription process, causing plus-strand synthesis initiation to a more 5' location or location, lengthening the plus-strand DNA and possibly inhibiting the strand-dissociation synthesis step required to form full-length linear episomes, thereby resulting in an increase in the quantity of 1-LTR circles being made, with a concomitant decrease in linear episomes, the putative substrate for illegitimate

integration (Fig. 26). Indeed, we determined that the PPT deletion caused a notable (threefold) decrease in linear episomes (Table 5), leading to a proportional reduction in illegitimate integration (Fig. 23c). Importantly, the various PPT-deleted vectors exhibited titers comparable to those of PPT-positive vectors (Table 6), indicating that the PPT deletion did not hinder their ability to transduce target cells. Interestingly, other studies employing lentiviral vectors, in which the native PPT was either mutated or swapped for a heterologous PPT (rather than completely deleted, as in our study), have reported on reduced viral titer and increased irregularities at the LTR-LTR junction of 2-LTR circles [114,115].

Overall, we show here that 1-LTR circles are the predominant episomal form generated by a novel, PPT-deleted lentivector. This vector, packaged without functional integrase, exhibits a 2.5-fold lower level of illegitimate integration than currently used nonintegrating lentivectors. Mechanistically, the findings presented here suggest that linear episomes are the main substrate for illegitimate integration. Evidently, these novel PPT-deleted vector may offer a safer gene-therapy platform for clinical and research applications, especially ones requiring large doses of vector particles in vivo or transient expression of hazardous genes *ex vivo*.

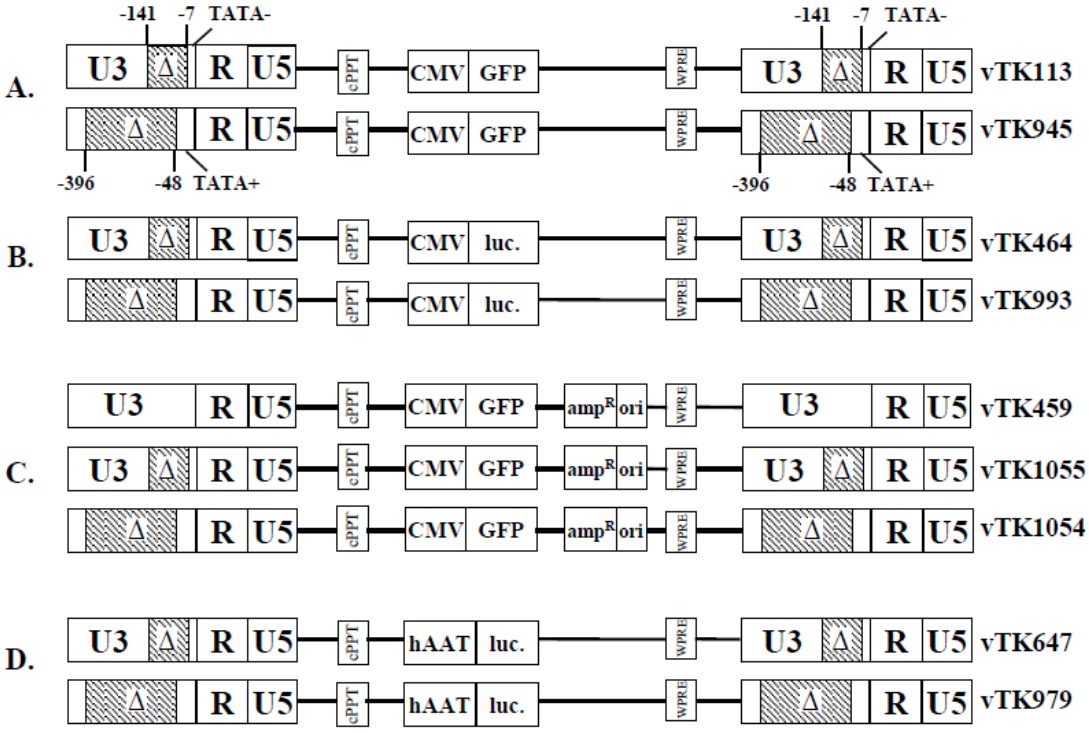


Fig. 18. Schematic of vectors with short or long deletions in the U3 region of the LTR.

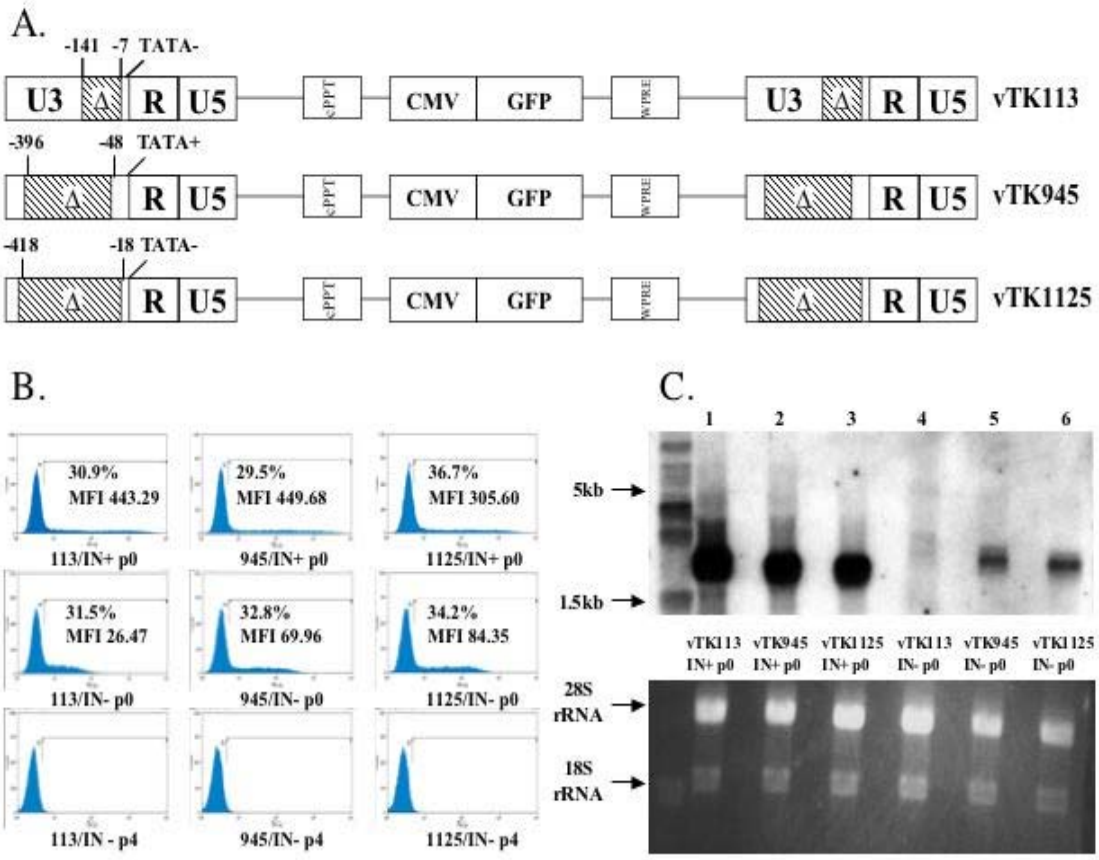


Fig. 19. Comparative analysis of *in vitro* expression from lentiviral vectors with various deletions in the U3 sequence. (**a)** Schematic of vectors with a short (vTK113), longer (vTK945), or nearly full-length (vTK1125) U3 deletions; deleted sequences are indicated by crosshatching. (**b)** FACS analysis of GFP expression generated by vTK113 (left), vTK945 (center), or vTK1125 (right), with (upper) or without (middle and lower) functional integrase, in 293T cells. Mean fluorescence intensity (MFI) was measured 5 days posttransduction (p0) and after four passages (p4) (lower). **(c)** Northern-blot analysis of transcription mediated by vTK113 (lanes 1 and 4), vTK945 (lanes 2 and 5) or vTK1125 (lanes 3 and 6), with (lanes 1, 2, and 3) or without (lanes 4, 5, and 6) functional integrase, in 293T cells. RNA was harvested 5 days posttransduction. Equal loading of RNA was verified through ethidium bromide staining of ribosomal RNA (lower panel).

Vector	RNA band density
vTK113/IN+	121
vTK945/IN+	125
vTK113/IN-	7.7
vTK945/IN-	31

Table 2. Quantification of Northern blot characterizing RNA produced by integrating and nonintegrating lentivectors in 293T cells.

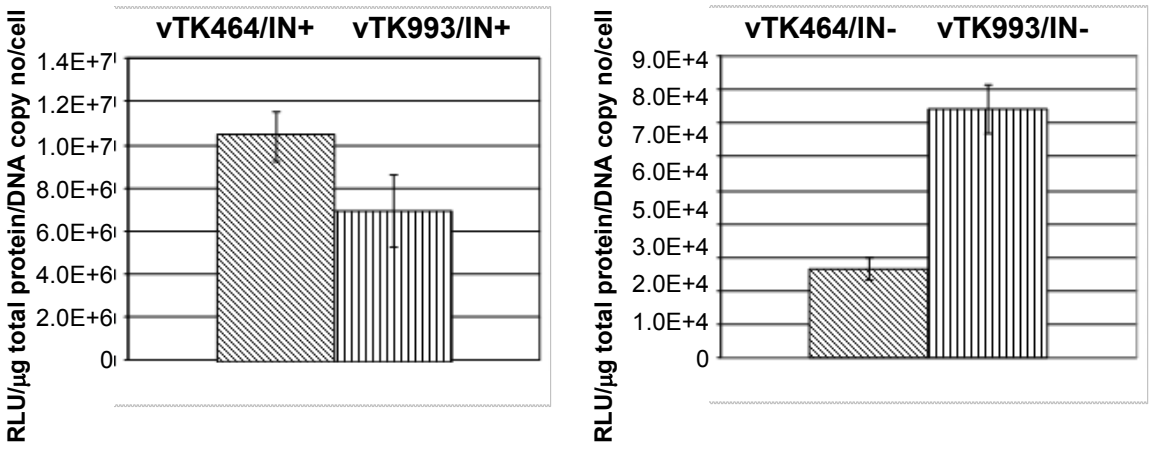


Fig. 20. Comparative analysis of *in vitro* expression efficiency generated by vectors with long or short U3 deletions, as normalized to vector genome copy number by qPCR. Error = mean±SD, *n*=4.

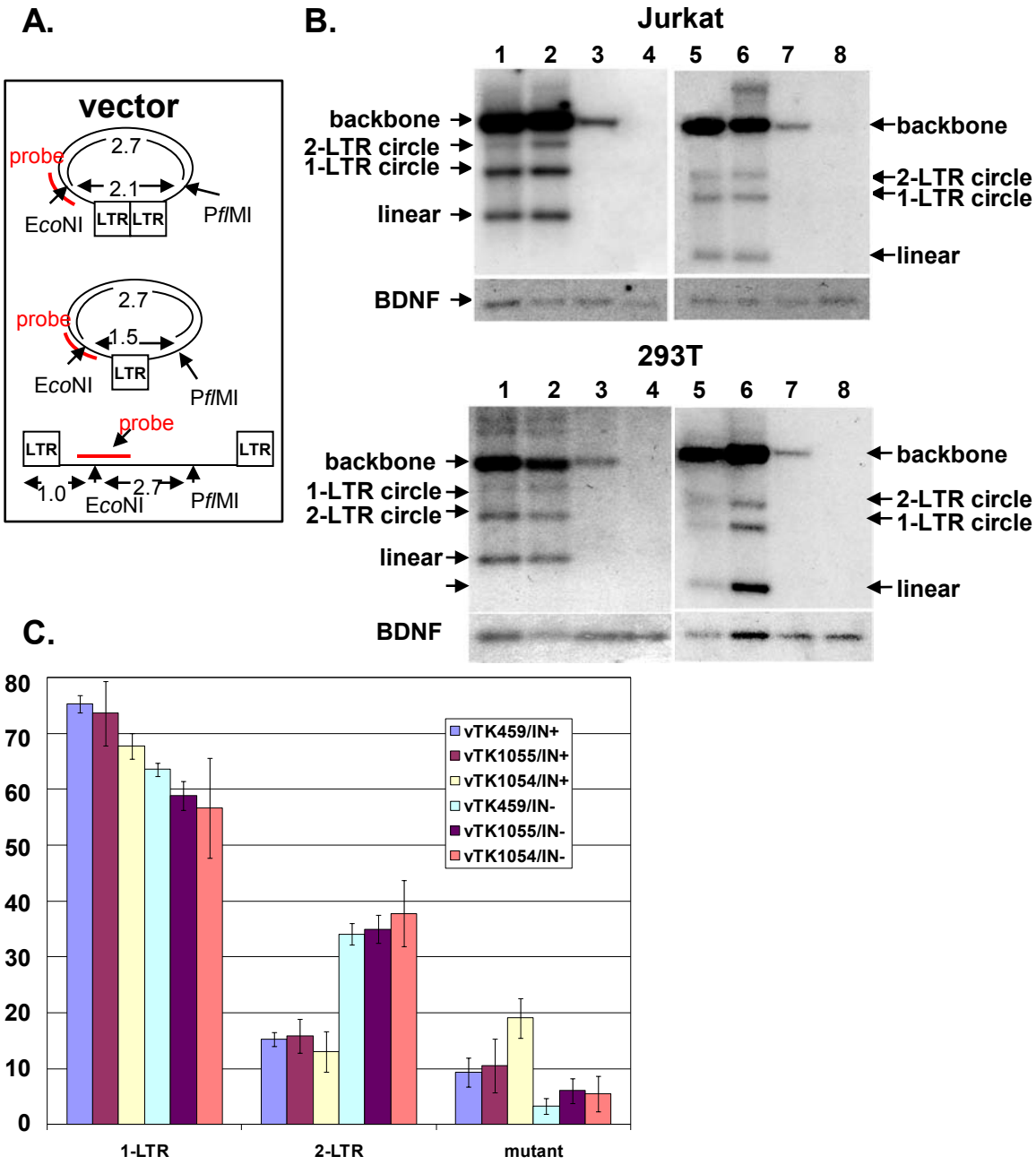


Fig. 21. Comparative analysis of episome formation in 293T cells and Jurkat cells. (a) To simultaneously analyze integrated and nonintegrated vector genomes, total DNA from transduced cells was cut at sites (*Eco*NI and *Pfl*MI) flanking the LTR(s), and a radiolabeled probe complementary to a region spanning the *Eco*NI site was employed on vTK113 (left) and vTK945 (right) vector genomes. **(b)** Episome formation and integration efficiency was examined by Southern blot in Jurkat (upper) and 293T (lower) cells. Jurkat cells were transduced with vTK113, integrating (lanes 1 and 3) or nonintegrating (lanes 2 and 4), or with vTK945, integrating (lanes 5 and 7) or nonintegrating (lanes 6 and 8) and total DNA was extracted from transduced cells 3 days (no passages)(lanes 1–2 and 5–6) or ~14 days (3 passages)(lanes 3–4 and 7–8) posttransduction, digested as shown in **(a),** and analyzed by Southern blot, and the same procedure was followed using 293T cells (lanes 9–16). Size markers are shown in red. **(c)** Episomes were harvested from 293T cells 16 hours posttransduction. For **(c)**, error = mean±SD, *n*=3.

	Vector and Cell Type											
DNA form	Jurkat 113 IN+[a]	Jurkat 113 IN-[b]	Jurkat 113 IN+ integ.[c]	Jurkat 945 IN+	Jurkat 945 IN-	Jurkat 945 IN+ integ.	293T 113 IN+	293T 113 IN-	293T 113 IN+ integ.	293T 945 IN+	293T 945 IN-	945 IN+ integ.
2-LTR	8.97%	16.29%		8.48%	17.69%		5.42%	19.58%		4.78%	15.76%	
1-LTR	46.21%	43.50%		41.16%	41.26%		40.16%	26.77%		26.64%	30.42%	
linear	44.82%	40.21%		50.36%	41.05%		54.41%	53.65%		68.58%	53.82%	
integrated			35.04%			30.51			23.03%			29.76%

Table 3. Quantification of relative amounts of integrated lentiviral genomes and linear, 1-LTR, and 2-LTR episomal genomes produced by integrating and nonintegrating lentivectors in 293T and Jurkat cells.

a. IN+ = vector packaged with wild-type integrase
b. IN- = vector packaged with D64E-mutant integrase
c. integ. = proportion of lentivector genomes remaining after three passages of transduced cells

	Circular Episomal DNA Type		
Vector	1-LTR	2-LTR	mutant
vTK459/IN+[a]	75.7+/-2.3%[b]	14.3+/-1.9%	10+/-2.5%
vTK1055/IN+	73.6+/-5.8%	15.9+/-3.0%	10.5+/-4.8%
vTK1054/IN+	61.5+/-2.3%	13+/-3.7%	24.5+/-3.5%
vTK459/IN-[c]	63.6+/-1.2%	34.1+/-2.6%	3.3+/-1.4%
vTK1055/IN-	58.9+/-2.6%	35+/-2.5%	6.1+/-2.2%
vTK1054/IN-	53.5+/-9.0%	39.8+/-5.9%	6.6+/-3.2%

Table 4. Quantification of 1-LTR, 2-LTR, and autointegrated episomal genomes produced by lentiviral shuttle vectors in 293T cells.

a. IN+ = vector packaged with wild-type integrase
b. error = mean±SD, *n*=3
c. IN- = vector packaged with D64E-mutant integrase

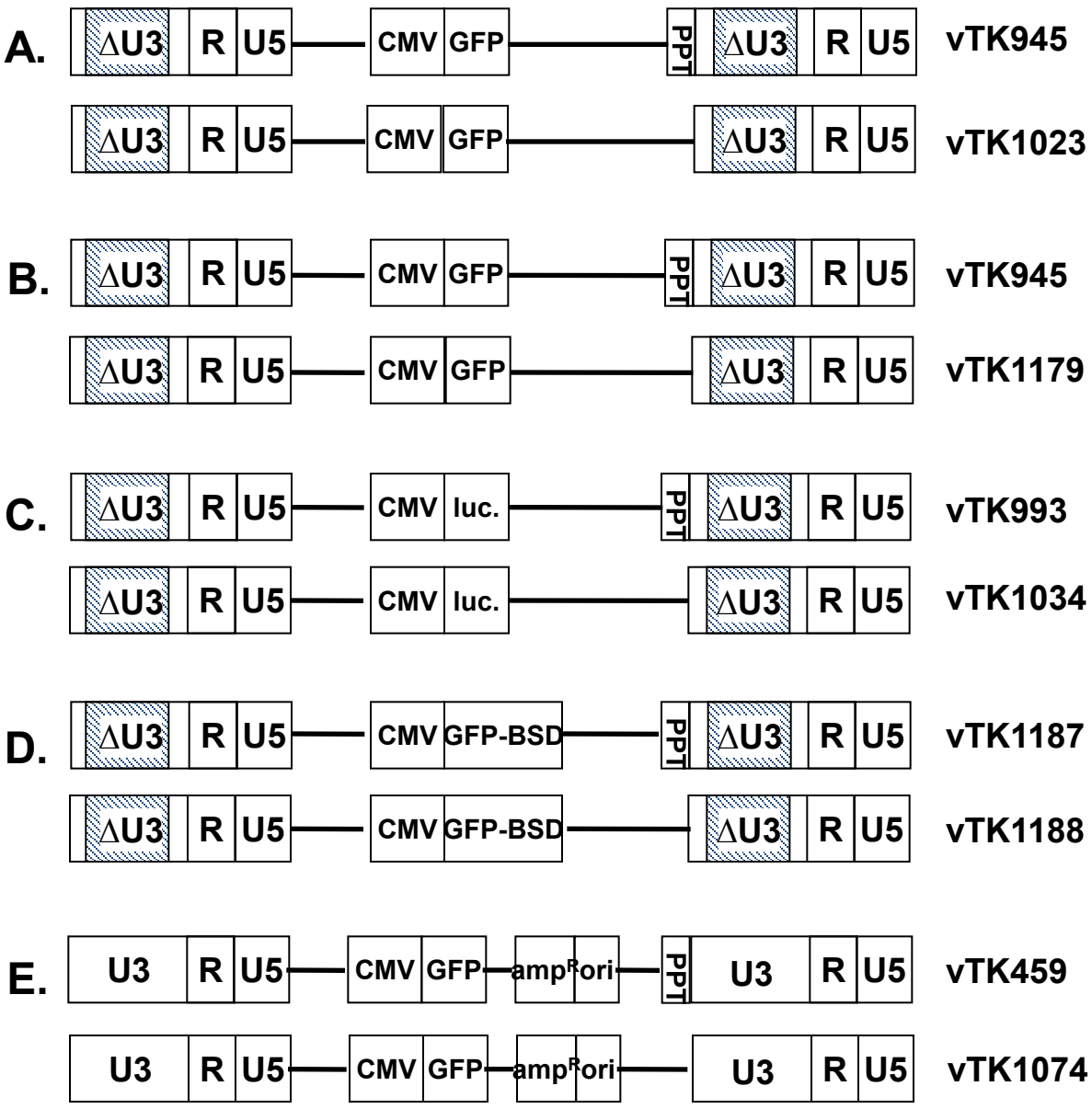


Fig. 22. Schematic of vectors with or without the PPT deletion.

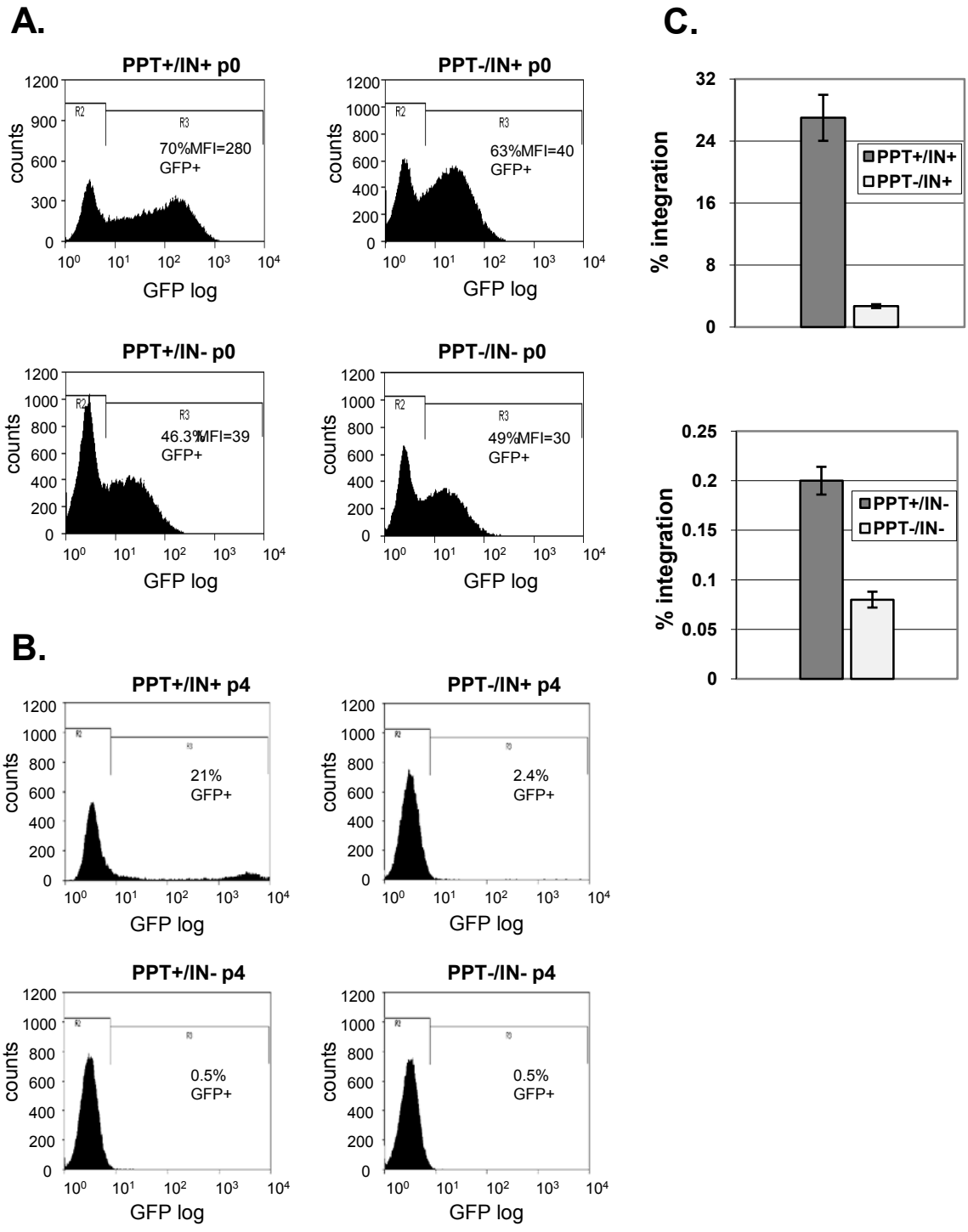


Fig. 23. Comparative analysis of *in vitro* expression generated by PPT-positive and PPT-deleted vectors. Expression was measured by FACS analysis (**a, b**) and luciferase assay, normalized by qPCR (**c**). For (**c**), error = mean±SD, *n*=3.

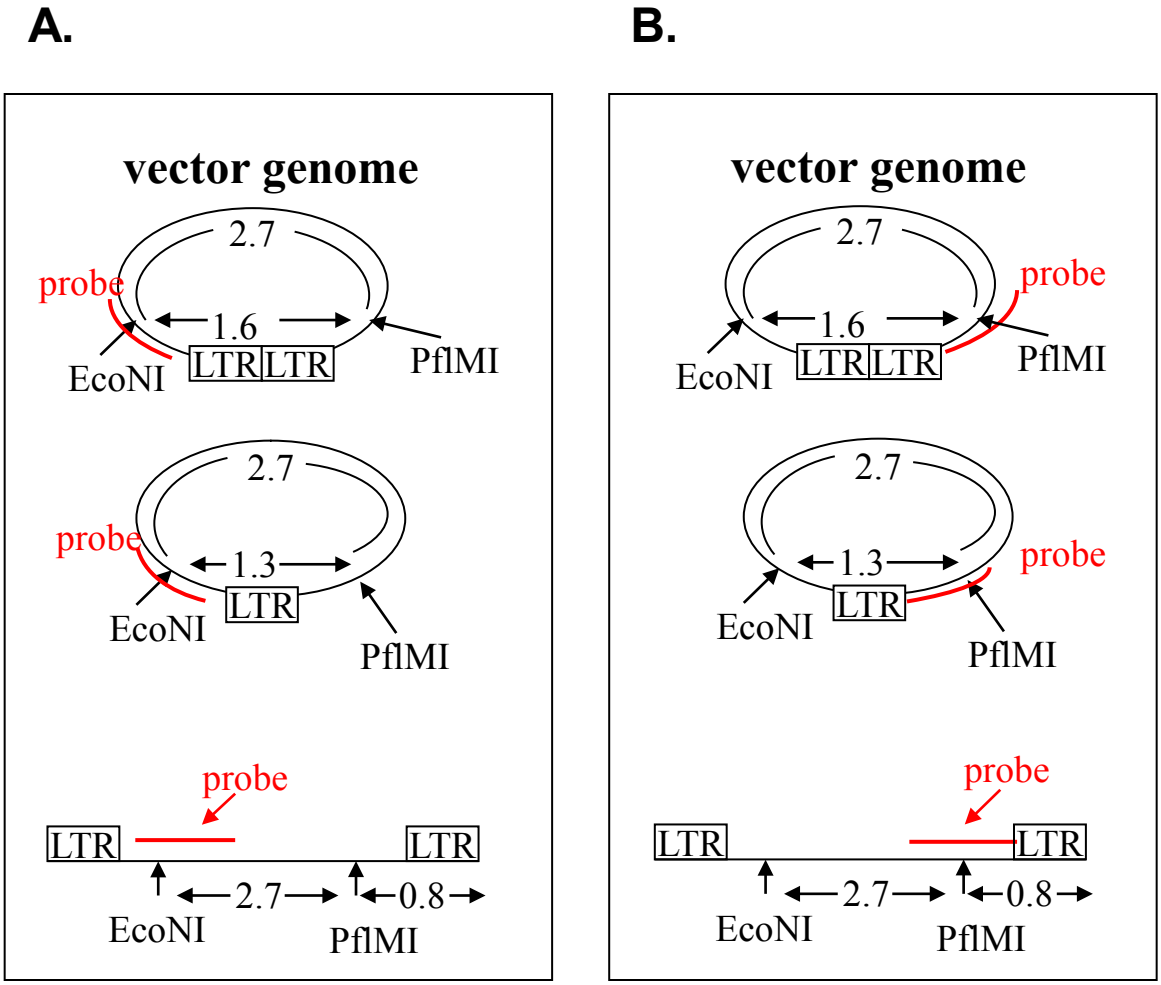


Fig. 24. Outline of the digest and probing scheme employed for the Southern-blot analysis of PPT-deleted vectors.

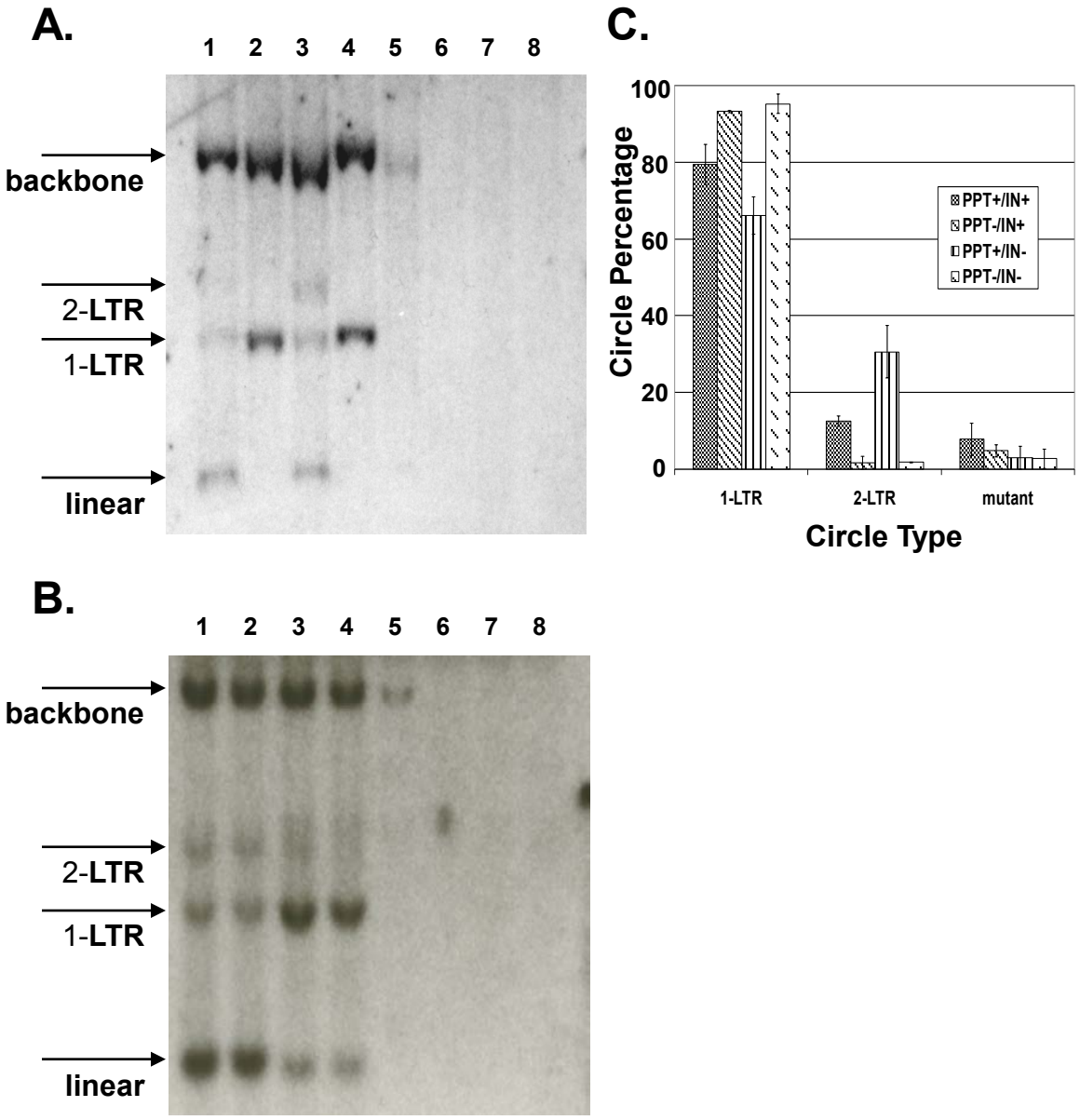


Fig. 25. Comparative analysis of episome formation and integration in 293T cell stransduced with PPT-positive or PPT-deleted vectors. Episome formation and integration was assayed by Southern-blot analysis (**a**,**b**), and episome formation was analyzed by shuttle-vector assay (**c**). **(a)** Cells were transduced with vTK945, integrating (lanes 1 and 5) or nonintegrating (lanes 3 and 7), or with vTK1179, integrating (lanes 2 and 6) or nonintegrating (lanes 4 and 8) and total DNA was extracted from transduced cells 3 days (no passages)(lanes 1–4) or ~14 days (4 passages)(lanes 5–8) posttransduction and analyzed by Southern blot, as shown in Fig. 24a. **(b)** Cells were transduced with vTK945, integrating (lanes 1 and 5) or nonintegrating (lanes 2 and 6), or with vTK1179, integrating (lanes 3 and 7) or nonintegrating (lanes 4 and 8) and total DNA was extracted from transduced cells 3 days (no passages)(lanes 1–4) or ~14 days (4 passages)(lanes 5–8) posttransduction and analyzed by Southern blot, as shown in Fig. 24b. For (**c**), error = mean$\pm$SD, *n*=3.

	Vector and Passage Number				
	p0 PPT[a]+/IN+[b]	p0 PPT+/IN-[c]	p0 PPT-[d]/IN+	p0 PPT-/IN-	p4 PPT+/IN+ integ.[e]
2-LTR circle	10.7%	8.5%	2.8%	2.8%	
1-LTR circle	33.2%	36.6%	78.2%	79.2%	
linear	56.1%	54.9%	19.0%	18.1%	
integrated					22.8%

Table 5. Quantification of relative amounts of integrated lentiviral genomes and linear, 1-LTR, and 2-LTR episomal genomes produced by PPT-positive, PPT-deleted, integrating, and nonintegrating lentivectors in 293T cells.

a. PPT+ = vector with an intact polypurine tract
b. IN+ = vector packaged with wild-type integrase
c. IN- = vector packaged with D64E-mutant integrase
d. PPT- = vector with a deleted polypurine tract
e. integ. = proportion of lentivector genomes stably integrated after three passages of transduced cells

Vector	PPT[a]	vector titer (transducing units/mL)	
		IN+[b]	IN-[c]
vTK945	+	$6.70 \pm 0.46 \times 10^7$	$4.83 \pm 0.31 \times 10^7$
vTK1179	-	$7.83 \pm 0.80 \times 10^7$	$5.23 \pm 0.31 \times 10^7$
vTK945	+	$6.77 \pm 1.08 \times 10^7$	$7.40 \pm 1.20 \times 10^7$
vTK1023	-	$8.53 \pm 0.94 \times 10^7$	$8.33 \pm 1.04 \times 10^7$

Table 6. The effect of the PPT deletion on titers of lentivectors packaged with wild-type or mutant integrase.

a. PPT = vector polypurine tract
b. IN+ = vector packaged with wild-type integrase
c. IN- = vector packaged with D64E-mutant integrase

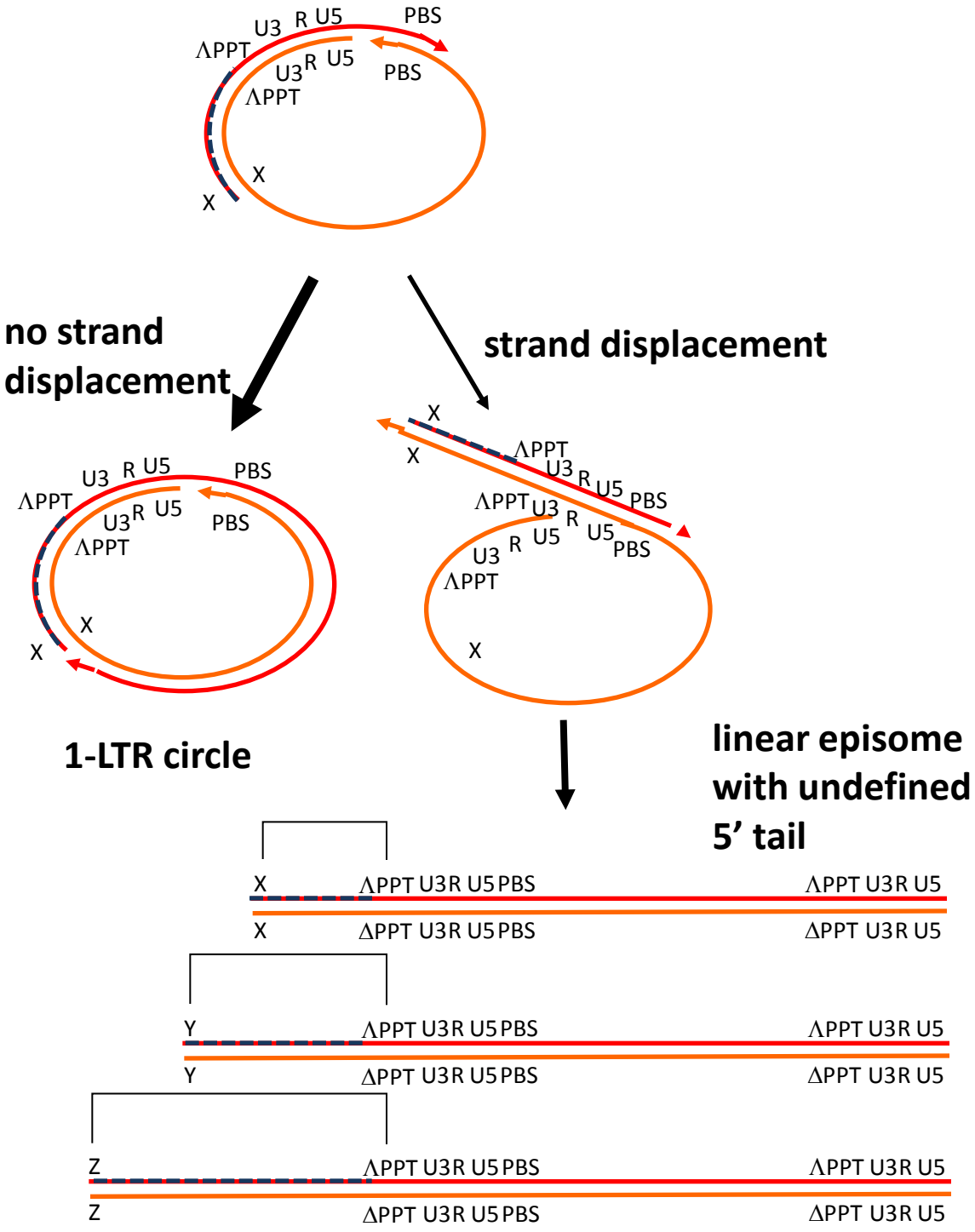


Fig. 26. Proposed model for the formation of linear episomes by PPT-deleted vectors.

Chapter 4

ORGANISMAL FACTORS AFFECTING LENTIVECTOR EPISOME FORMATION, STABILITY, AND EXPRESSION

A portion of the work in this chapter was published in *Molecular Therapy* 16(12): 1968-76.

Introduction

Nondividing cells are a potentially advantageous target for nonintegrating vectors, as episomal vector genomes are not diluted out of them, potentially enabling the long-term expression of transgenes in postmitotic cells, which account for the majority of adult somatic tissue. However, various groups arrived at conflicting results in early studies on the efficacy of integrase-positive or integrase-defective lentiviral transduction of nondividing cells. Studies conducted with first-generation lentiviral vectors indicated that integrase deficiency nearly eliminated expression [4, 102]. Furthermore, Park *et al*. used an integrase-positive lentiviral vector to transduce liver cells *in vivo* and found that partially hepatectomized livers, which undergo several rounds of cell division, were more than 25-fold more efficiently transduced than non-hepatectomized, postmitotic livers [74], indicating that lentiviral vectors could not efficaciously transduce nondividing cells *in vivo*. However, Kafri *et al*. similarly transduced liver cells *in vivo* with an integrating lentiviral vector and demonstrated sustained gene expression [73]. In consonance with these

results, another study showed that nonintegrating vectors transduce postmitotic retinal and brain cells as efficiently as integrating vectors, demonstrating effective retinal transduction through the restoration of ocular function in mice deficient for *Rpe65* [79]. Nonintegrating lentivectors have also been used to impart significant transgene expression in muscle and lymph nodes [31,101]. The discrepancy between early, negative findings from nonintegrating lentivectors and recent, positive results may be due to differences in promoter or transgene choice, which have been shown to affect *in vivo* transgene expression and stability [116,117]. Taken together, these results suggest that nonintegrating lentiviral vectors can transduce nondividing cells efficiently, and while lentiviral transduction may be more efficient in dividing cells, transduction in nondividing cells can be efficient enough to achieve phenotype correction *in vivo*.

Though lentivectors have generated robust transgene expression in certain nondividing cells *in vivo*, not all *in vivo* lentivector transductions produce identical results. Previous studies have demonstrated that HIV permissivity varies across cell lines [58], and several studies have arrived at diverging results on the ability of lentivectors to transduce cardiomyocytes in adult or neonatal animals [118, 119, 120]. Furthermore, one study found that different target organs may express unintegrated vector transgenes with varying degrees of efficiency [78]. These findings, along with the varying results observed in the mouse liver as outlined above, indicate that *in vivo* nonintegrating lentivector transductions may be subject to organismal factors that may affect episome formation, as well as episomal expression and stability. However, none of the studies mentioned above thoroughly examines the types of episomes formed by nonintegrating lentiviral vectors *in vivo*. The goal of this study was to elucidate the organismal factors affecting lentivector episome

formation, stability, and expression.

Materials and Methods

Vector construction. The shuttle vector pTK1054 includes a bacterial origin of replication and ampicillin-resistance gene in the transcribed region of the plasmid. The vector cassettes pTK1238 and pTK1239 were constructed by inserting the GFP-Cre sequence from pTK577 into pTK402 and pTK646, which bear the liver-specific human alpha-1 antitrypsin (hAAT) promoter and the ubiquitous human elongation factor 1alpha (EF1α) promoter, respectively, by standard cloning procedures. pTK113, pTK945, and pTK1023 were described in Chapter 3.

Cell culture. 293/lacZ cells (kind gift of Norman Sharpless, University of North Carolina) were grown in Dulbecco's modified Eagle medium (Mediatech, Herndon, VA) supplemented with 9% fetal bovine serum and 1% penicillin/streptomycin solution.

Viral vector production. All lentiviral vectors were prepared as previously described [87], transiently transfecting 10^7 293T cells with 15μg vector cassette, 10μg packaging cassette, and 5μg envelope cassette. Integrating vectors were made using the packaging cassette ΔNRF [87], which expresses functional integrase, while nonintegrating vectors were made using the packaging cassette pTK939. All vectors were pseudotyped with the VSV-G envelope cassette. For vectors constitutively expressing GFP, titers were assessed by serial dilution in 293T cells followed by visual analysis of GFP expression by fluorescence microscope. For other vectors, concentrations were determined by p24gag ELISA. The

absence of replication-competent retroviruses was determined by three independent methods: tat transfer assay, vector rescue assay, and p24gag ELISA, as described previously [100].

Tissue-specific expression assay. 293/lacZ (non-hepatic) cells were transduced with integrase-proficient and integrase-deficient vTK1238 (containing the ubiquitous EF1α promoter) and vTK1239 (containing the liver-specific hAAT promoter) at an m.o.i. of 0.5. Five days posttransduction, cells were rinsed twice with 100 mM sodium phosphate buffered saline (PBS), fixed in lacZ FIX buffer (2% formaldehyde and 0.2% gluteraldehyde in PBS) for 5 minutes, then washed twice more with PBS. LacZ substrate buffer (5mM potassium ferricyanide, 5mM potassium ferrocyanide and 2mM magnesium chloride in PBS), mixed with X-Gal at a concentration of 0.3mg X-Gal/mL lacZ substrate buffer, was then incubated with the cells overnight at 37°C. LacZ-positive cells were counted under direct light.

In vivo *experiments in rat brain.* All of the animals were pathogen-free male Sprague-Dawley rats obtained from Charles Rivers. All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH]85-23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee. Virus vector infusions were performed as previously described [69]. Briefly, rats first were anesthetized with 50 mg/kg pentobarbital and then placed into a stereotaxic frame. Using a 32 gauge stainless steel injector and a Sage infusion pump, the rats received 1 μl (at 6 x 10^9 GFP transducing

units/mL) of the integrating and nonintegrating vTK113 and vTK945 vectors over a 10 minute period into the striatum (1.0mm anterior to bregma, 3.0mm lateral, 5.5mm vertical) according to the atlas of Paxinos and Watson [121]. In all cases, the injector was left in place 3 minutes postinfusion to allow diffusion from the injector.

Immunohistochemistry. Two weeks or 3 months after the vector infusion, rats received an overdose of pentobarbital (100 mg/kg pentobarbital, i.p.) and subsequently were perfused transcardially with ice-cold 100 mM sodium phosphate buffered saline (PBS) (pH=7.4), followed by 4% paraformaldehyde in 100 mM phosphate buffer (pH=7.4). After overnight fixation in paraformaldehyde-phosphate buffer, vibratome sections (40 µm thick) were taken through the striatum and rinsed in PBS. For immunohistochemistry, tissue sections were incubated in 10% normal goat serum and 0.1% Triton X-100 in PBS for 45 minutes. Next, sections were incubated with a primary antibody to NeuN (1:500, Chemicon, Temecula, CA) or glial fibrillary acidic protein (GFAP)(1:4,000, DAKO A/S, Denmark) overnight in 3% normal goat serum, 0.2 % Triton X-100 and PBS. Tissue sections were then rinsed in PBS, incubated in blocking serum (10% normal goat serum, 0.1% Triton X-100, PBS) for 1hr. and then incubated with a secondary fluorescent antibody (Alexa-fluor 594 goat anti-mouse (NeuN), goat anti-rabbit (GFAP), (Molecular Probes, Eugene, OR) for 1 hour at 4^0C. Following 3 rinses in PBS, the sections were mounted on slides and coverslipped with fluorescent mounting media. eGFP fluorescence initially was visualized on an Olympus IX 71 fluorescence microscope (Olympus, Center Valley, PA), and digital pictures were taken. In the cases where co-localization was to be determined, both the GFP and the Alexa 594 fluorescence was visualized with a Zeiss 510 Meta laser

scanning confocal microscope (Zeiss, Oberkochen, Germany). Co-localization was determined by multiple scans through the Z axis of the sample.

In vivo *experiments in mouse liver*. All procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee. C57BL/6NHsd mice (Harlan Sprague Dawley, Indianapolis, IA) were injected intraperitoneally at eight weeks of age with 100μg p24 of lentivector. At 10 days, 45 days, and 6 months postinjection mice were assayed for luciferase expression following luciferin (NanoLight, Pinetop, AZ) injection and using the Xenogen IVIS imaging system (Xenogen, Hopkinton, MA). Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally at eight weeks of age with 40μg p24 of lentivector. At one day and 21 days postinjection, livers were harvested, strained into single-cell suspensions, and subjected to shuttle-vector analysis.

Results

Sustained episomal expression in the brain and liver from a integrase-deficient vectors

In accordance with the notion that lentiviral episome formation may vary across cell types, previous findings indicate that the level of episomal lentiviral gene expression may be cell type-specific [58]. These findings spurred an investigation into the ability of nonintegrating lentiviral vectors to transduce effectively a variety of cell types *in vivo*, including brain and liver cells. Accordingly, rats were injected intracranially with 6×10^6 transducing units of integrating and nonintegrating small-deletion (vTK113) and

long-deletion (vTK945) vectors. Two weeks and three months after striatal infusion, brain tissues were harvested and imaged for GFP, as well as the neuron-specific marker NeuN and the astrocyte-specific marker GFAP. As shown in Fig. 27a, the U3 deletion notably increased episomal GFP fluorescence in striatal tissue three months postinfusion. However, while nonintegrating vTK113 expressed GFP in both neurons and astrocytes, nonintegrating vTK945 appeared to transduce astrocytes to a significantly lesser degree (Fig. 27b). Furthermore, while vTK945 expressed GFP strongly in the striatum, episomal expression of vTK945 in the corpus callosum was relatively weak (Fig. 27a).

Intrigued by the long U3 deletion's effect on episomal expression in the rat brain, we sought to replicate the vector's efficacy in the slowly dividing tissue of the liver, which prior findings had found resistant to sustained episomal lentiviral transduction [74]. To that end, mice were injected intraperitoneally with 100 μg p24 of small-deletion (vTK647) or large-deletion (vTK979) vector expressing luciferase from the liver-specific promoter hAAT. After imaging for luciferase activity at 10 days, 45 days, and 6 months postinjection, nonintegrating vTK979 was found to generate roughly fourfold more luciferase activity than nonintegrating vTK647, but still yielded approximately 40-fold less luciferase activity than integrating vTK647 (Fig. 27c and d). Furthermore, in disagreement with a previous report, we found that transgene expression in the liver was largely stable over a six-month time course [122] (Fig. 27c and d). Discrepancies may be due to differences in promoters and mouse strains employed. Taken together, the results in Fig. 27 indicate that the U3 deletion improves episomal expression in several tissues *in vivo*, albeit with variation across tissues, and that the expression is sustained over time.

PPT-deleted vectors exhibit sustained *in vivo* expression

The *in vitro* findings outlined in Chapter 3 suggest that PPT-deleted vectors exhibit reduced transgene expression, comparable to that of integrase-deficient vectors. To investigate the possibility that the expression capacities of the vectors might be different *in vivo*, rats were injected intracranially with 1 x 10^6 transducing units of integrating and nonintegrating PPT-positive (vTK945) and PPT-deleted (vTK1023) vectors. Two months after striatal infusion, brain tissues were harvested and imaged for GFP. We found that both PPT-negative vectors expressed at approximately the same level as the PPT+/IN- vector, but at a considerably lower level than the PPT+/IN+ vector (Fig. 28).

1-LTR vector circles increase over time in the mouse liver

Previous findings have demonstrated that lentiviral vectors express transgenes stably in the mouse liver and other nondividing cell types [31,79]. However, the stability and relative abundances of episomes formed in transduced livers has not been thoroughly characterized. To investigate the stability and formation of lentivector episomes in the mouse liver, we injected mice intraperitoneally with 40 μg p24 of integrase-proficient shuttle vectors and measured the relative abundances of 1-LTR, 2-LTR, and mutant circular episomal vector genomes Hirt-extracted from the liver at one day and 21 days posttransduction. As shown in Fig. 29, day1 hepatocytes exhibited roughly 60% 1-LTR circles, 30% 2-LTR circles, and 10% mutant circles, while day 21 hepatocytes displayed approximately 90% 1-LTR circles, 5% 2-LTR circles, and 5% heterogenous circles. Importantly, these results indicate that circular lentivector episomes are stable enough in the liver to be recovered via bacterial rescue after 21 days. Interestingly, the change in relative episomal abundance between day 1 and day 21 could indicate that delayed reverse transcription kinetics in slowly dividing heptocytes may slowly generate primarily 1-LTR

circular RT products after day 1, leading to an increase in relative abundance of 1-LTR circles at day 21. Indeed, previous studies have indicated that the kinetics of reverse transcription are slowed in nondividing cells, taking 3 to 5 days to reach completion [123,124]. On the other hand, the data could suggest that linear episomes are subject to degradation after day 1, leading to a decrease in relative abundance of linear episomes by day 21, which would cause a corresponding decrease in the relative abundance of 2-LTR circles. Of course, the increase in relative abundance of 1-LTR circles may be due to a combination of both phenomena.

Episomal expression is more tissue-specific than expression from integrated provirus

The use of tissue-specific promoters in lentiviral vectors is a logical development, as tissue-specific promoters are less likely to be silenced in target cells or to induce an immune response in non-target cells [122,125]. However, given that lentiviral vectors preferentially integrate into actively transcribing genes [126,127], the question arises of whether active endogenous genes may have an enhancer effect on integrated vector cassettes. To investigate the notion that integrated, tissue-specific promoters may be subject to activation by adjacent genes, we examined the expression, in non-hepatic cells, of liver-specific promoters in integrase-proficient and integrase-deficient vectors. Specifically, we used integrating and nonintegrating lentivectors, expressing Cre recombinase from liver-specific and ubiquitous promoters, to transducer the 293/lacZ cell line, which consists of 293 cells containing a β–galactosidase gene bearing a stop codon flanked by loxP sequences (Fig. 30). Given that only vectors expressing Cre could excise the stop codon and activate lacZ expression, we investigated whether putatively liver-specific promoters expressed Cre and induced lacZ expression in non-hepatic cells.

We found that integrating, liver-specific vectors mediated lacZ expression in approximately as many cells as either ubiquitous-promoter vector, but that the nonintegrating, liver-specific vector induced notably fewer lacZ-positive cells, indicating that liver specificity was significantly improved by expression from integrase-deficient vectors (Fig. 31).

Discussion

The goal of this study was to elucidate the organismal factors affecting lentivector episome formation, stability, and expression. Specifically, we investigated episomal expression over time in the brain and liver, episome formation over time in the liver, and episomal expression as a means of improving tissue-specific expression. We found that episomal expression was stable over time *in vivo*, that 1-LTR circular lentivector episomes increased in relative abundance over time in the liver, and that the tissue specificity of integrase-deficient vectors was superior to that of integrase-proficient vectors.

The potentially variable nature of episomal expression and U3-mediated transcriptional repression across cell types, coupled with the clinical relevance of *in vivo* transduction, underscored the importance of measuring gene expression in a variety of cell types *in vivo*. Here we show that the large U3 deletion had a pronounced effect on episomal expression in rat-brain striatal cells; however, this improvement in SIN vector design did not confer robust episomal expression in corpus callosum cells, suggesting that additional mechanistic elements contribute to the silencing of episomal expression.

Prior studies administered nonintegrating vectors *in vivo* by direct injection into nondividing target organs, including eye, brain or muscle [31,38,79], thereby delivering

potentially saturating quantities of vector particles; however, the present study, for the first time, administered nonintegrating lentiviral vectors systemically to slowly dividing liver cells, which allowed testing of the vector's efficacy in a condition of low concentration and, by extension, relatively low multiplicity of infection in the target organ. Intriguingly, a long U3-deletion vector expressed four times more liver-specific luciferase than a short U3-deletion vector, in keeping with *in vitro* results and indicating that cells in the liver may express transcriptionally repressive factors binding to the 5' U3 region eliminated from the large U3-deletion vector. The significant, hepatocyte-specific luciferase expression noted by the present study contrasts with previous reports characterizing lentiviral expression in the liver as insignificant [74]; discrepancies with the prior study may be due to differences in transgene and promoter employed, as well as LTR length. Furthermore, in divergence from prior findings suggesting that lentiviral vector transgene expression in the liver induces an effective immune response [122], the present study found no significant loss of transgene product activity in immunocompetent mice. In fact, transgene expression from every vector assayed was sustained for at least six months after administration, in agreement with prior studies stably expressing transgenes episomally in the liver from adeno-associated viral vectors and plasmid DNA [70,71]. A slight (~15%) reduction over time of episomal expression in mouse livers observed in this study may be due to the slow division of liver cells, and could eventually necessitate readministration of alternately pseudotyped vector.

In keeping with the results obtained with vTK113 and vTK945, the PPT-deleted vector vTK1023 was found to stably express GFP in the rat brain for up to two months, whether packaged with functional or defective integrase. This finding indicates that, while the PPT deletion leads to increased 1-LTR circle formation and reduced linear-episome

and 2-LTR circle formation, the deletion does not appear to present deleterious long-term effects on episomal stability or expression *in vivo*, and that PPT-deleted vectors, therefore, may present a viable alternative to traditional nonintegrating lentivectors for the long-term expression of transgenes in nondividing cells and the short-term expression of transgenes in dividing cells.

In keeping with the results obtained from vTK647 and vTK979, the shuttle vector vTK1054 formed episomes that were detectable in the mouse liver up to 21 days posttransduction, confirming that lentivector episomes are relatively stable *in vivo*. Importantly, shuttle-vector analysis of episomes harvested one day and 21 days posttransduction measured, for the first time, the relative abundances of episomal forms over time *in vivo*. This study demonstrates that the relative abundance of 1-LTR circles increases between the two time points, implying that linear episomes are subject to degradation over time, and/or that 1-LTR circles are the preferential products of reverse transcription between day 1 and day 21. These results are in line with previous results indicating that lentiviral episomes are stable in nondividing cells [56,59], as well as prior studies showing that reverse transcription takes up to five days to reach completion in nondividing cells [123,124].

We used a non-hepatic reporter cell line to compare expression from liver-specfic and ubiquitous promoters in integrating and nonintegrating lentivectors. We found that a liver-specific promoter in an integrating vector effects detectable transgene expression in as many reporter cells as a ubiquitous promoter in an integrating vector, though the reporter cells are not permissive for liver-specfic promoters. Although the reporter cell line requires only a small amount of vector transgene expression to activate an internal transgene with a

readily visible product, the leakiness of the putatively liver-specific promoter cannot be overlooked. One possible explanation for the nonspecific expression is lentiviruses' demonstrated preference for integrating in the coding regions of active genes [126,127], where the endogenous promoter and associated active chromatin may have an activating effect on the integrated liver-specfic promoter. Interestingly, an integrase-deficient vector bearing a liver-specific promoter mediated transgen expression in considerably fewer reporter cells than its integrating counterpart, indicating that episomal expression cassettes are not subject to the same transcriptional-regulatory environment as integrated provirus.

The data presented here indicate that lentivector episomes are stable and capable of long-term expression in the mouse liver and rat brain, although their expression does not match that of integrated vector genomes. Furthermore, this study indicates that episomal lentiviral genomes may be less prone than integrated vector genomes to "leaky," non-tissue-specific expression from tissue-specific promoters. These findings suggest that nonintegrating lentivectors are a potential source of stable therapeutic-transgene expression in nondividing cells, and while their expression levels are below those of integrating vectors, their expression (in a reporter cell line requiring only a low level of vector-mediated transgene expression to activate an integrated reporter gene) appears to be more tissue-specific, possibly because integrated provirus may associate with the transcription factors and chromatin-remodeling factors present near preferred lentiviral integration sites [126, 127]. Therefore, the improved tissue specificity of integrase-deficient vectors may make them less likely to express in non-targeted antigen-presenting cells and elicit an immune response to targeted cells.

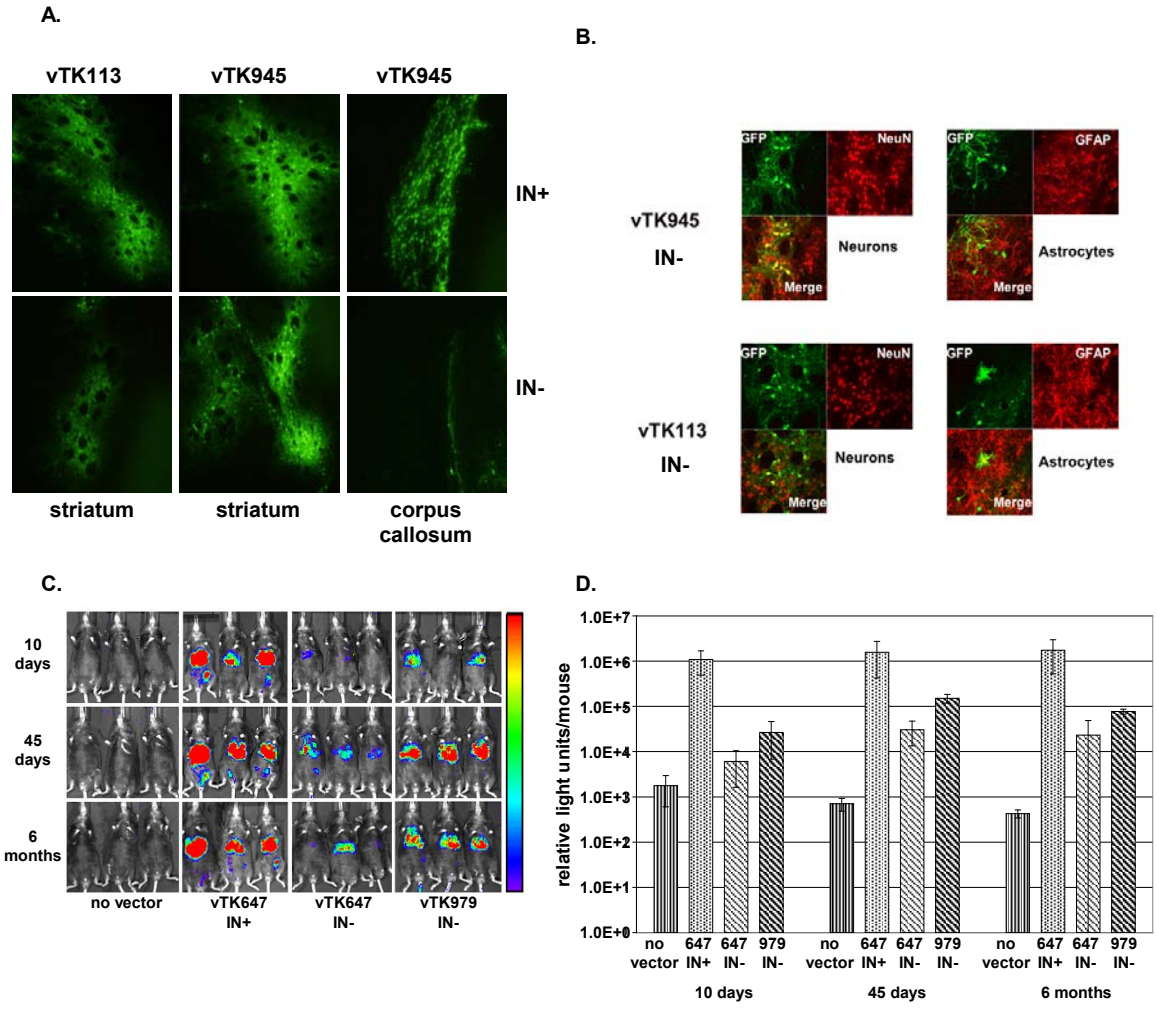


Fig. 27. Analysis of *in vivo* episomal expression from lentiviral vectors with short or large deletions in the U3 sequence. (a) Tissue compartment-specific GFP expression mediated by short-deletion or long-deletion vectors, with (top) or without (bottom) functional integrase, in the striatum and corpus callosum of the rat brain, measured three months after vector infusion. **(b)** Cell type-specific GFP expression mediated by short-deletion (lower) and long-deletion (upper) nonintegrating vectors in neural (fluorescing with NeuN, left side) and glial (fluorescing with GFAP, right side) cells of the rat brain, measured three months after vector infusion. A 20-micron size bar appears in the 113/IN- GFAP/GFP merge panel. **(c)** Long-term luciferase expression produced by short-deletion or long-deletion vectors, with or without functional integrase, in the mouse liver. Animals were imaged 10 days, 45 days, and 6 months after intraperitoneal injection of vector. **(d)** Quantification of luciferase expression described in **(c)**. Error = mean±SD, n=3.

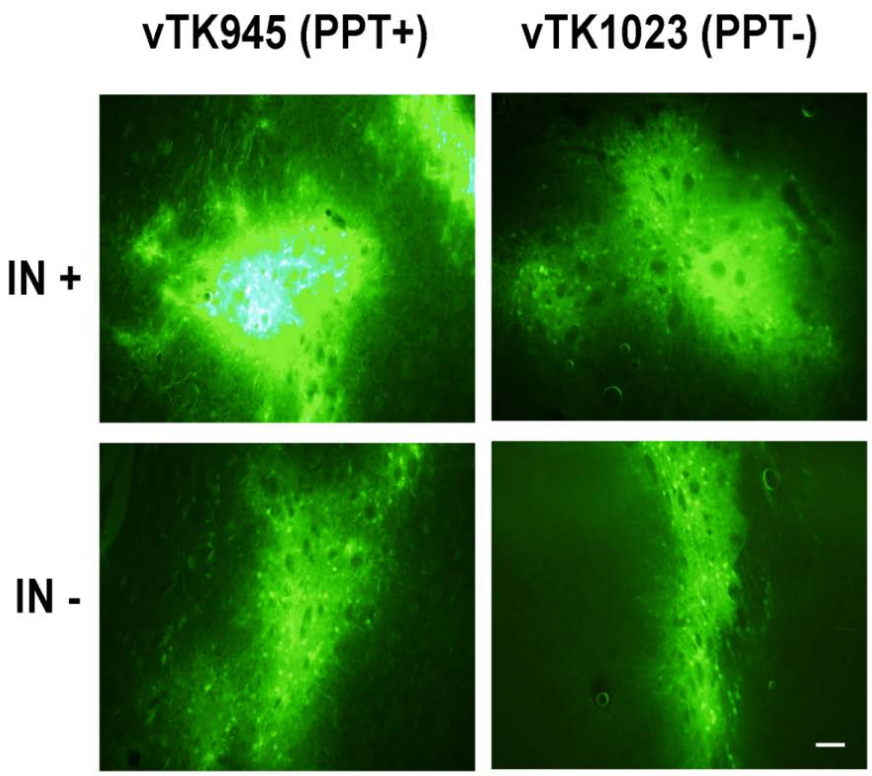


Fig. 28. Comparative analysis of *in vivo* expression generated by PPT-positive and PPT-deleted vectors, packaged with and without functional integrase.

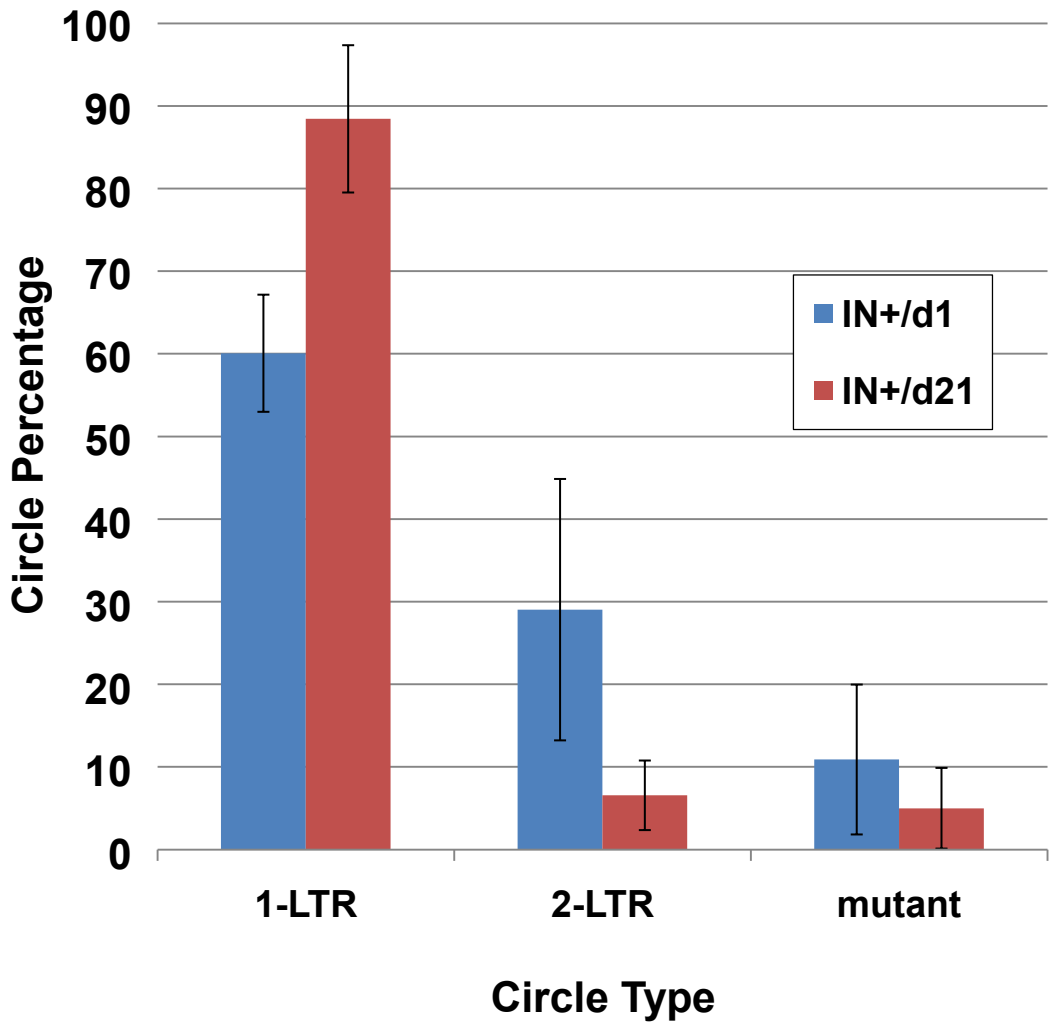


Fig. 29. Comparative analysis of *in vivo* episome formation generated by a shuttle vector. Mouse livers were harvested one day and 21 days postinjection and analyzed by shuttle-vector assay. Error = mean±SD, *n*=3.

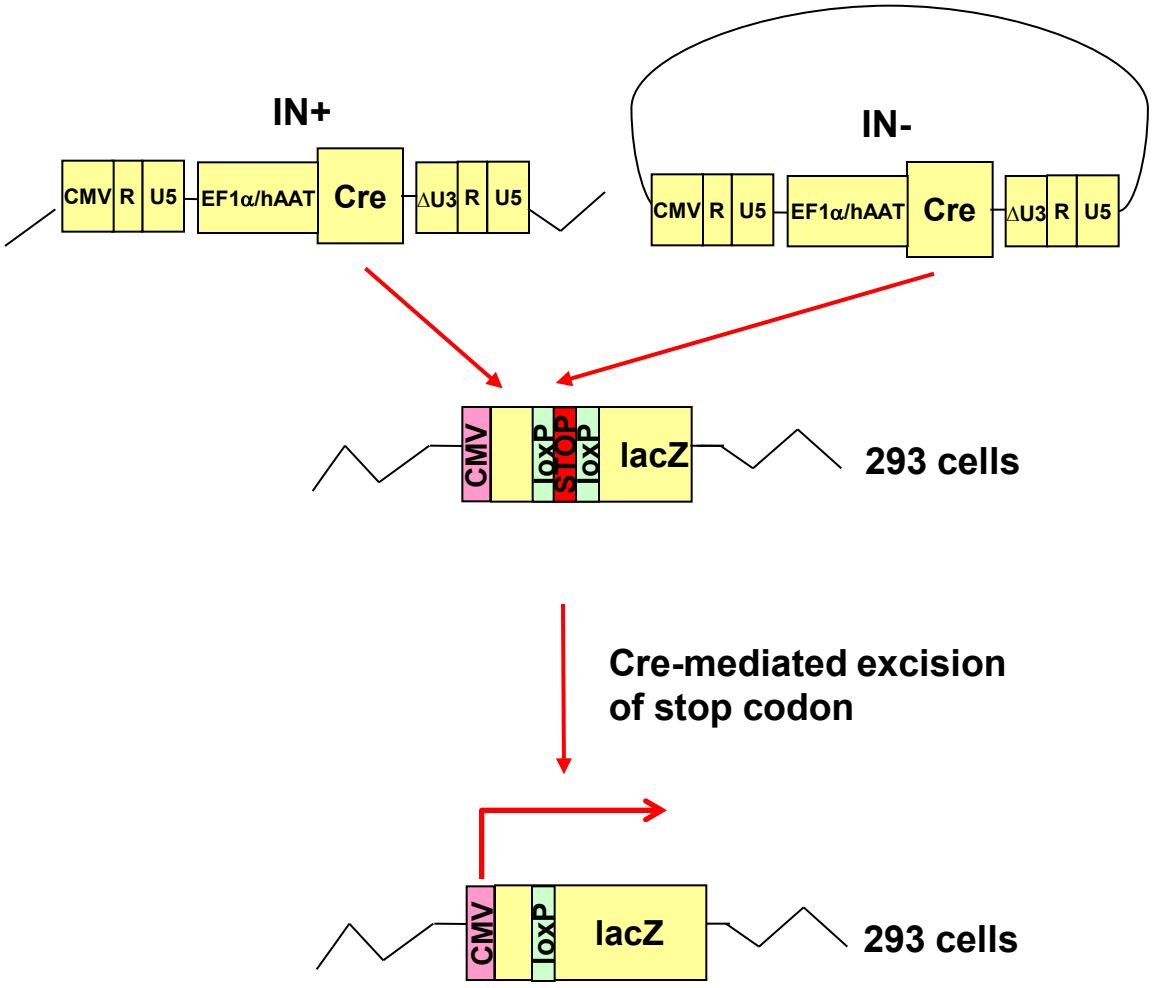


Fig. 30. Outline of the 293/lacZ cell line as a means of measuring expression of Cre recombinase.

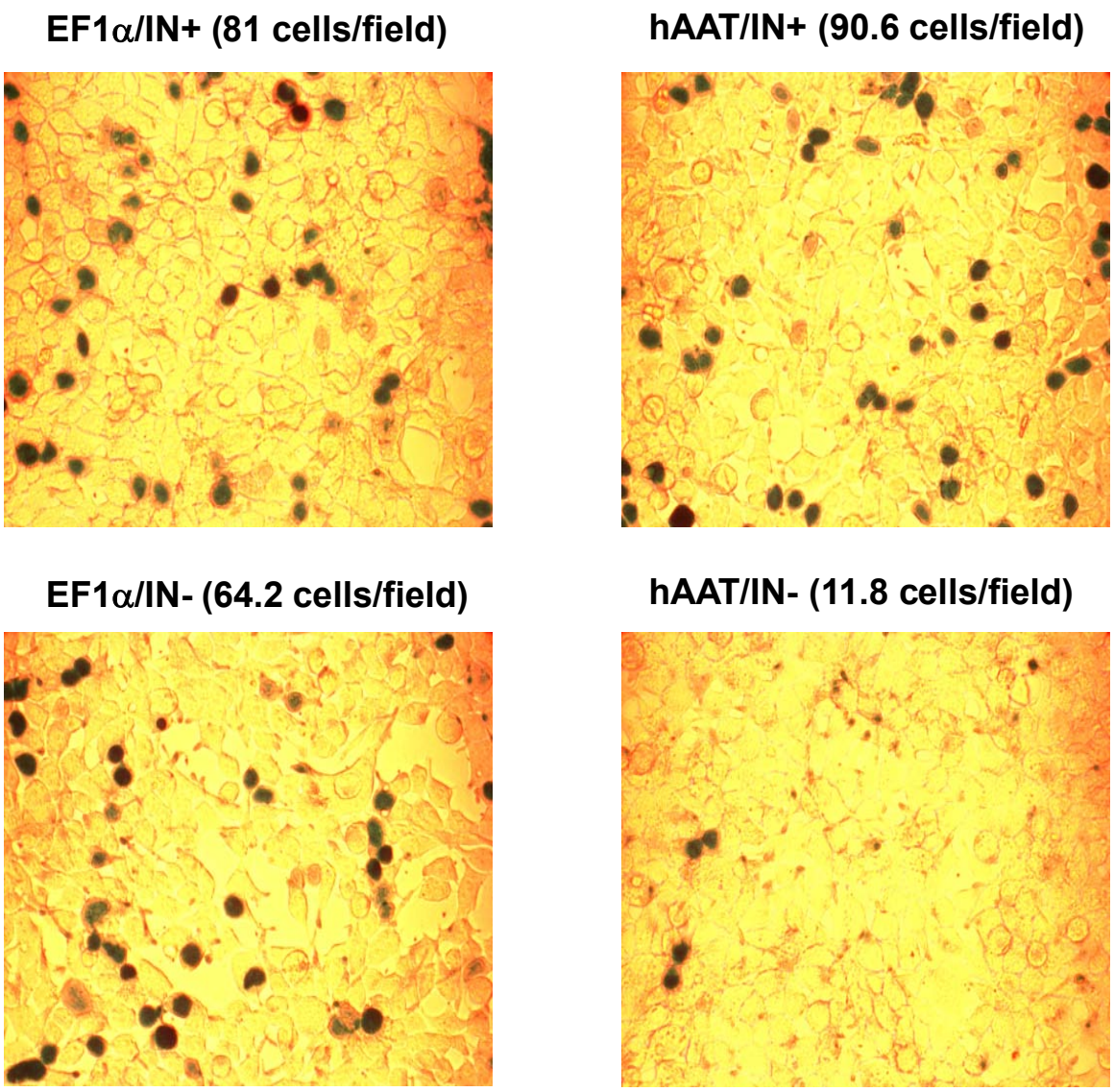


Fig. 31. Comparative analysis of expression exhibited by integrase-proficient and integrase-deficient vectors bearing ubiquitous (EF1α) and liver-specific (hAAT) promoters.

Chapter 5

CONCLUSIONS

The results of the recent X-SCID gene-therapy trials in Europe have illustrated both the benefits and risks of retroviral vectors [20, 21]. Clearly, a different strategy for reducing the genotoxicity of retroviral and lentiviral vectors would make them more suitable for use in human trials. One such method is to mutate the vector genome or packaging cassette to render the vector integration-incompetent, but still capable of nuclear import and transgene expression from episomal vector genomes. In fact, preliminary studies have shown that such vectors can deliver genetic payloads efficiently and stably to a variety of nondividing cell types, both *in vitro* and *in vivo* [31,38,79,101]. However, the fact that lentivector episomes have not been examined as thoroughly as integrated lentivector genomes underscores the importance of understanding the factors mediating the formation, stability and expression of unintegrated lentivector genomes, especially if they are to be the sole substrate for the expression of therapeutic transgenes in potential gene-therapy applications. Accordingly, these studies examined the cellular, vector, and organismal factors affecting the formation, stability, and expression of unintegrated lentiviral vector genomes, showing that HR deficiencies and U3 deletions did not affect episome formation, but that a PPT deletion significantly increased 1-LTR circle formation, and that 1-LTR circles (which correlate with reduced illegitimate integration) also became more relatively abundant over time in

the mouse liver. Furthermore, these experiments demonstrated that the U3 deletion increased episomal expression, while the PPT deletion had no effect on episomal expression, and *in vivo* transductions demonstrated robust and sustained episomal expression over time in the rat brain and mouse liver.

Cellular Factors

The origin of 1-LTR circular episomes is one of the oldest questions facing the field retroviral and lentiviral vectors. The most widely accepted explanation, and perhaps the most intuitive, is that 1-LTR circles are the products of homologous recombination (HR) between the LTRs at either end of linear, unintegrated vector genomes. However, the evidence supporting this hypothesis is less than conclusive, based on the observation of linear lentiviral genomes circularizing into 1-LTR episomes in a virus-free cellular extract, and HIV infection yielding fewer 1-LTR circles in cells knocked down for the HR protein Rad52 [42,44]; furthermore, transduction of cells mutant for other HR factors did not result in reduced 1-LTR circle formation [94]. Another possible explanation for 1-LTR circle formation, one dating back to the early days of retrovirology, is that they may form as byproducts of incomplete reverse transcription, as suggested by the findings that 1-LTR circles can form in the course of *in vitro* vector transduction, in the absence of cellular proteins, and that 1-LTR circles appear both in the nucleus of transduced cells, where HR proteins are active, and in the cytoplasm, where HR proteins are not active, but reverse transcriptase is [48,49].

To determine which mechanism is responsible to 1-LTR circle formation, a number of HR-deficient cell lines were transduced with lentivectors. Surprisingly, none of the

mutant cell lines assayed had a significant effect on 1-LTR circle formation (Figs. 12-15). Furthermore, as HR has been shown to occur only in cells cycling through late S and G2 phases of the cell cycle, cultured cells arrested in G1 phase were transduced with lentivectors, and also failed to show measurable reduction of 1-LTR circle formation (Fig. 17). These findings are at odds with previous results, and while the possibility exists that the HR genes analyzed were not needed for 1-LTR circle formation, or that they retained enough residual activity to form the circles, the fact that the G1-arrested cells showed undiminished 1-LTR circle formation indicates that HR does not mediate their formation. The possibility that vector factors, such as those involved in reverse transcription, catalyze the formation of 1-LTR circles was investigated in later experiments. However, given that several proteins important to HR, including Rad52 and RPA, were not fully characterized, an intriguing future experiment would be to use shRNA-encoding lentiviral vectors to target RPA and Rad52 in wild-type cells, then analyze lentivector episome formation in the knocked-down cells.

Vector Factors

Perhaps the most pressing issue facing the field of nonintegrating lentiviral vectors is their relatively low level of expression, compared to that of integrating vectors. In fact, early nonintegrating lentiviruses and lentivectors were considered transcriptionally inert [4,30,102], and only in recent years have advances in vector development led to significant episomal expression [31,38,79,101]. Given that, in the course of an integrase-proficient vector transduction, only 1-30% of vector genomes integrate and the rest remain episomal, the relative silence of this unintegrated majority suggests that cellular factors may act on

episomes to inhibit their expression. In fact, previous studies in wild-type lentivirus suggest that a sequence in the viral LTR may be negatively regulated by cellular factors [15,17]. Furthermore, recent study suggested that an LTR sequence may repress expression from an internal promoter [19]. Therefore, in the interest of increasing lentiviral episomal expression, a vector with a long U3 deletion was constructed and analyzed *in vitro*.

Transduction of cultured cells with the long U3-deletion vector revealed that, while the deletion had no significant effect on expression from an integrating vector, the long deletion increased vector expression from an integrase-mutated vector roughly threefold (Fig. 19), indicating that the U3 may contain negative regulatory sequences that, in turn, affect expression from the vector's internal promoter. An intriguing future experiment would be to perform a series of small (20-50bp) deletions to determine the location of the negative regulatory element, analyze the sequence to identify several candidate binding proteins, and performs ChIP analysis on the vector sequence in transduced cells to determine its enrichment for the proteins in question.

To determine if the increase in transgene expression generated by the long U3-deletion vector was associated with a change in the relative abundances of episomal forms present in transduced cells, episomes were analyzed by Southern blot and shuttle-vector assay. No significant change in the relative abundances of episomes was observed between the short and long U3-deletion vectors, indicating that the increase in expression did not have a structural explanation (Fig. 21). However, one exception was that the mutant-circular form was significantly upregulated in the case of the integrating long U3-deletion vector (Fig. 21d), suggesting that the deleted U3 region contains not only a negative regulatory element, but, possibly, a sequence capable of preventing the

autointegration that leads to the formation of some mutant episomes. As mentioned above, an interesting experiment would be to perform a series to deletions to locate the sequence responsible for this phenomenon.

To investigate the possiblity that reverse transcription is the source of 1-LTR circles, and mindful of the role of the polypurine tract (PPT) in delineating the site of plus-strand synthesis initiation in the course of reverse transcription, a number of PPT-deleted vectors were constructed. Transducing cultured cells with PPT-deleted vectors resulted in significant increases in 1-LTR circles, as well strong reductions in 2-LTR circles, linear episomes, and integrated provirus (Fig. 25), indicating that the PPT, and, by extension, reverse transcription, affects 1-LTR circle formation, causing 1-LTR circles to be the primary product of reverse transcription instead of linear episomes, with a resulting reduction in linear episomes and the 2-LTR circles and integrated genomes derived from them. A possible mechanism for the PPT-deletion-induced upregulation of 1-LTR circles is that the absence of the PPT may force the RT to search for a cryptic plus-strand synthesis initiation site upstream of the LTR, and that the resulting plus strand, abnormally long, may be difficult to dissociate prior to the end of reverse transcription, thereby favoring an undissociated, incomplete reverse-transcription product in the form of a 1-LTR circle (Fig. 26). To further illustrate this mechanism, it would be interesting to move the PPT various distances upstream from the LTR and possibly modulate the prevalence of 1-LTR circles as RT products, as well as to perform a series of upstream deletion in PPT-deleted vectors to attempt to locate the cryptic plus-strand synthesis initiation site.

To determine if the PPT-deleted vectors could transduce cells efficienctly, their

transgene expression was assayed on cultured cells as a function of both mean fluorescence intensity and relative light units per genome. PPT-deleted vectors expressed at a considerably lower level than integrase-competent, PPT-bearing vectors, and, in fact, their expression was comparable to that of integrase-defective vectors (Fig. 23a), in keeping with the reduced levels of integration exhibited by PPT-deleted vectors (Fig. 23b).

The ability of the PPT deletion to singlehandedly reduce vector integration levels to those of integrase-mutant vectors, but still allow significant transgene expression, implicated it as a novel means of reducing vector genotoxicity by inhibiting integration. To determine if, when used in combination with a conventional integrase mutation, the PPT deletion could drive illegitimate integration levels below those seen with the integrase mutation alone, integration levels of wild-type, PPT-deleted, integrase-mutated, and PPT-deleted/integrase-mutated vectors were measured by qPCR. In fact, as well as reducing integration from an integrase-competent vector tenfold, the PPT-deleted vector reduced illegitimate integration from an integrase-defective vector 2.5-fold (Fig. 23b). This finding is significant, because, in previous studies, the combination of *att*-site mutations and integrase mutations had failed to reduce the level of illegitimate integration below that produced by either mutation alone [31,32], indicating that the PPT deletion is an advance, albeit incremental, in the field of vector safety. A possible future experiment in that vein, accordingly, would be to compare the genotoxicity of integrase-deficient, PPT-deleted vectors to those of conventional integrase-deficient vectors through an *in vitro* genotoxicity assay.

Organismal Factors

Determining the formation, stability, and expression of lentivector episomes *in vivo* is a necessary undertaking for gene-therapy researchers, because, regardless of how robustly and stably a vector expresses transgene in culture, organismal factors such as circulation, tissue-specific transduction and expression, and immune response may hinder *in vivo* vector efficacy [74,117,122]. Accordingly, we measured episome expression and formation mediated by several vectors over time in the rat brain and mouse liver.

To verify that the U3 deletion's effect on episomal expression was conserved *in vivo*, rat brains and mouse livers were transduced with short and long U3-deletion vectors. As seen in cultured cells, the long U3 deletion imparted threefold higher expression on nonintegrating vectors (Fig. 27c and d). However, the effect in rat brain was cell-type- and tissue-compartment-specific, suggesting cell- and tissue-specific variability in the proteins interacting with lentivector episomes and, presumbably, mediating their chromatinization (Fig. 27a and b). More importantly, every vector assayed imparted significant expression in mouse liver for up to six months, in contradiction to a number of studies suggesting that lentiviral vectors could not transduce the liver effectively [74,122] (Fig. 27c). The discrepancy may be due to differences in promoter type and transgene used. Similarly, analysis of *in vivo* expression generated by PPT-deleted vectors in the rat brain two months posttransduction also showed transgene expression comparable to that of integrase-mutated vectors (Fig. 28), reinforcing that nonintegrating lentivectors are capable of long-term expression, as has been demonstrated by other nonintegrating viral vectors [68,70].

To investigate the relative abundance of and stability of circular lentivector episomes in the mouse liver over a three-week period, mice were injected with lentiviral

vectors and episome formation in the liver was analyzed one day and 21 days after transduction. 1-LTR circles were found to increase in relative abundance between one and 21 days, indicating that reverse transcription preferentially generates 1-LTR circles in the period between 1 and 21 days posttransduction, or that linear episomes or 2-LTR circles are subject to degradation over the three-week period in question (Fig. 29). An intriguing future experiment on this subject would be to transduce mice with PPT-deleted vectors as well as PPT-positive vectors and harvest episomes after two and five days as well as one day and 21 days, measuring absolute episomal DNA levels by qPCR. This experiment could determine the stability of PPT-deleted vector genomes *in vivo*, measure the relative stability (or continued production) of 1-LTR circles *in vivo*, and, with more time points, elucidate the kinetics of episome formation or degradation *in vivo*.

Some groups, when using an integrating, liver-specific vector, have reported problems with non-tissue-specific transduction of antigen-presenting cells, leading to the mounting of an immune response to the transgene and the liver cells that express it [117,122]. Therefore, as tissue-specific promoters in unintegrated vector genomes may be less likely to be activated by chromosomal enhancer elements, we set out to test the tissue specificity of an integrase-deficient vector. Transducing a non-hepatic reporter cell line (Fig. 30) with integrating and nonintegrating vectors bearing ubiquitous and liver-specific promoters, we found that nonintegrating, liver-specific promoters mediated a low level of expression, as expected, but that integrating vectors with liver-specific promoters were surprisingly leaky, imparting measurable expression in as many cells as did the ubiquitous-promoter vectors (Fig. 31). These data indicate that episomal vector transgenes are less prone to activation from active endogenous genes, which is not surprising, given that lentiviruses

have been shown to preferentially integrate into the coding regions of active genes [126,127]. An interesting experiment suggested by these results would be to inject animals with the set of vectors outlined above to determine if the improved tissue specificity of nonintegrating lentivectors is replicated *in vivo*.

Significance

In summary, this work has contributed to understanding the cellular, vector, and organismal factors affecting the formation, stability, and expression of unintegrated lentiviral vectors. Specifically, the findings presented here indicate that 1-LTR circles are unaffected by HR deficiency and a long U3 deletion, but are significantly increased by a PPT deletion, and that, over a 21-day period, 1-LTR circles become more relatively abundant in the mouse liver. Furthermore, these data suggest that nonintegrating lentivectors are capable of greater tissue specificity than integrating lentivectors. These results provide a more detailed picture of lentiviral episome formation and maintenance in transduced cells. Two of the primary challenges facing researchers employing nonintegrating lentiviral vectors are the vectors' relatively low levels of trangene expression relative to those exhibited by integrating lentiviral vectors, and the phenomenon of illegitimate expression, which could lead to the deleteriously sustained expression of potentially genotoxic transgenes intended to be only transiently expressed in dividing cells; for example, in targeted-integration applications, the expression of zinc-finger nucleases would necessarily be short-term to avoid the accumulation of double-strand DNA breaks. The results presented here suggest methods for improving episomal expression (in the case of the U3 deletion) and reducing illegitimate integration (in the instance of the PPT deletion), which may benefit future vector applications.

REFERENCES

1 Lever, A. M. *et al.* Lentivirus vectors for gene therapy. *Biochem Soc Trans* **27**, 841-847 (1999).

2 Berger, E. A., Murphy, P. M. & Farber, J. M. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* **17**, 657-700 (1999).

3 Coffin, J. M., Hughes, S. M. & Varmus, H. E. *Retroviruses*. (Cold Spring Harbor Laboratory Press, 1997).

4 Naldini, L., Blomer, U., Gage, F. H., Trono, D. & Verma, I. H. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci USA* **93**, 11382-11388 (1996).

5 Shimada, T., Fujii, H., Mitsuya, H. & Nienhuis, A. W. Targeted and highly efficient gene transfer into CD4+ cells by a recombinant human immunodeficiency virus retroviral vector. *J Clin Invest* **88**, 1043-1047 (1991).

6 Wanisch, K. & Yanez-Munoz, R. J. Integration-deficient lentiviral vectors: a slow coming of age. *Mol Ther* **17**, 1316-1332 (2009).

7 Azad, A. A. Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells? *Biochem Biophys Res Commun* **267**, 677-685 (2000).

8 Goh, W. C. *et al.* HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat Med* **4**, 65-71 (1998).

9 Hassaine, G. *et al.* The tyrosine kinase Hck is an inhibitor of HIV-1 replication counteracted by the viral vif protein. *J Biol Chem* **276**, 16885-16893 (2001).

10 Naldini, L. *et al.* In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263-267 (1996).

11 Reiser, J. *et al.* Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci U S A* **93**, 15266-15271 (1996).

12 Zufferey, R. *et al.* Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* **72**, 9873-9880 (1998).

13 Kim, V. N., Mitrophanous, K., Kingsman, S. M. & Kingsman, A. J. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1.

J Virol **72**, 811-816 (1998).

14 Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H. & Verma, I. Development of a self-inactivating lentivirus vector. *J Virol* **72**, 8150-8157 (1998).

15 Hoover, T. *et al.* A nuclear matrix-specific factor that binds a specific segment of the negative regulatory element (NRE) of HIV-1 LTR and inhibits NF-κB activity. *Nucleic Acids Res* **24**, 1895-1900 (1996).

16 Lu, Y., Stenzel, M., Sodroski, J. G. & Haseltine, W. A. Effects of long terminal repeat mutations on human immunodeficiency virus type 1 replication. *J Virol* **63**, 4115-4119 (1989).

17 Rosen, C. A., Sodroski, J. G. & Haseltine, W. A. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. *Cell* **41**, 813-823 (1985).

18 Shaw, G. M. *et al.* Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. *Science* **226**, 1165-1171 (1984).

19 Ginn, S. L., Fleming, J., Rowe, P. B. & Alexander, I. E. Promoter interference mediated by the U3 region in early-generation HIV-1–derived lentivirus vectors can influence detection of transgene expression in a cell-type and species-specific manner. *Hum Gene Ther* **14**, 1127-1137 (2003).

20 Hacein-Bey-Abina, S. *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415-419 (2003).

21 Howe, S. J. *et al.* Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* **118**, 3143-3150 (2008).

22 Montini, E. *et al.* Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* **24**, 687-696 (2006).

23 Modlich, U. *et al.* Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Mol Ther* **17**, 1919-1928 (2009).

24 Zychlinski, D. *et al.* Physiological Promoters Reduce the Genotoxic Risk of Integrating Gene Vectors. *Mol Ther* (2008).

25 Masuda, T., Kuroda, M. & Harada, S. Specific and independent recognition of U3 and U5 att sites by human immunodeficiency virus type 1 integrase in vivo. *J Virol*

72, 8396-8402 (1998).

26 Wiskerchen, M. & Muesing, M. A. Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J Virol* **69**, 376-386 (1995).

27 Katz, R. A. *et al.* Requirement for a conserved serine in both processing and joining activities of retroviral integrase. *Proc Natl Acad Sci U S A* **89**, 6741-6745 (1992).

28 Engelman, A., Englund, G., Orenstein, J. M., Martin, M. A. & Craigie, R. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. *J Virol* **69**, 2729-2736 (1995).

29 Leavitt, A. D., Shiue, L. & Varmus, H. E. Site-directed mutagenesis of HIV-1 integrase demonstrates differential effects on integrase functions in vitro. *J Biol Chem* **268**, 2113-2119 (1993).

30 LaFemina, R. L. *et al.* Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. *J Virol* **66**, 7414-7419 (1992).

31 Apolonia, L. *et al.* Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol Ther* **15**, 1947-1954 (2007).

32 Nightingale, S. J. *et al.* Transient gene expression by nonintegrating vectors. *Mol Ther* **13**, 1121-1132 (2006).

33 Baum, C. What are the consequences of the fourth case? *Mol Ther* **15**, 1401-1402 (2007).

34 Couzin, J. & Kaiser, J. As Gelsinger Case Ends,Gene Therapy Suffers Another Blow. *Science* **307**, 1028 (2005).

35 Dave, U. P., Jenkins, N. A. & Copeland, N. G. Gene Therapy Insertional Mutagenesis Insights. *Science* **303**, 333 (2004).

36 Li, Z. *et al.* Murine Leukemia Induced by Retroviral Gene Marking. *Science* **296**, 497 (2002).

37 Seggewiss, R. *et al.* Acute myeloid leukemia is associated with retroviral gene transfer to hematopoietic progenitor cells in a rhesus macaque. *Blood* **107**, 3865-3867 (2006).

38 Philippe, S. *et al.* Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. *Proc Natl Acad Sci USA* **103**,

17684-17689 (2006).

39 Li, L. *et al.* Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. *EMBO J* **20**, 3272-3281 (2001).

40 Chun, T.-W. *et al.* Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183-188 (1997).

41 Shoemaker, C. *et al.* Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: Implications for retrovirus integration. *Proc Natl Acad Sci USA* **77**, 3932-3936 (1980).

42 Farnet, C. M. & Haseltine, W. A. Circularization of human immunodeficiency virus type 1 DNA in vitro. *J Virol* **65**, 6942-6952 (1991).

43 Gianni, A. M., Smotkin, D. & Weinberg, R. A. Murine leukemia virus: detection of unintegrated double-stranded DNA forms of the provirus. *Proc Natl Acad Sci U S A* **72**, 447-451 (1975).

44 Jacque, J. M. & Stevenson, M. The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. *Nature* **441**, 641-645 (2006).

45 Shank, P. R. *et al.* Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two species of circular DNA. *Cell* **15**, 1383-1395 (1978).

46 Gilboa, E., Mitra, S. W., Goff, S. & Baltimore, D. A detailed model of reverse transcription and tests of crucial aspects. *Cell* **18**, 93-100 (1979).

47 Junghans, R. P., Boone, L. R. & Skalka, A. M. Products of reverse transcription in avian retrovirus analyzed by electron microscopy. *J Virol* **43**, 544-554 (1982).

48 Dina, D. & Benz, E. Structure of murine sarcoma virus DNA replicative intermediates synthesized in vitro. *J Virol* **33**, 377-389 (1980).

49 Ringold, G. M., Yamamoto, K. R., Shank, P. R. & Varmus, H. E. Mouse mammary tumor virus DNA in infected rat cells: characterization of unintegrated forms. *Cell* **10**, 19-26 (1977).

50 Ira, G. *et al.* DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* **431**, 1011-1017 (2004).

51 Jazayeri, A. *et al.* ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* **8**, 37-45 (2006).

52 Al-Minawi, A. Z., Saleh-Gohari, N. & Helleday, T. The ERCC1/XPF endonuclease

is required for efficient single-strand annealing and gene conversion in mammalian cells. *Nucleic Acids Res* **36**, 1-9 (2008).

53 Englund, G., Theodore, T. S., Freed, E. O., Engelman, A. & Martin, M. A. Integration Is Required for Productive Infection of Monocyte-Derived Macrophages by Human Immunodeficiency Virus Type 1 *J Virol* **69**, 3216-3219 (1995).

54 Pauza, D. C., Trivedi, P., McKechnie, T. S., Richman, D. D. & Graziano, F. M. 2-LTR circular viral DNA as a marker for human immunodeficiency virus type 1 infection in vivo. *Virology* **205**, 470-478 (1994).

55 Brussel, A. & Sonigo, P. Evidence for Gene Expression by Unintegrated Human Immunodeficiency Virus Type 1 DNA Species. *J Virol* **78**, 11263-11271 (2004).

56 Butler, S. L., Johnson, E. P. & Bushman, F. D. Human immunodeficiency virus cDNA metabolism: notable stability of two-long terminal repeat circles. *J Virol* **76**, 3739-3747 (2002).

57 Gillim-Ross, L., Cara, A. & Klotman, M. E. HIV-1 extrachromosomal 2-LTR circular DNA is long-lived in human macrophages. *Viral Immunol* **18**, 190-196 (2005).

58 Nakajima, N., Lu, R. & Engelman, A. Human immunodeficiency virus type 1 replication in the absence of integrase-mediated DNA recombination: definition of permissive and nonpermissive T-cell lines. *J Virol* **75**, 7944-7955 (2001).

59 Pierson, T. C. *et al.* Intrinsic stability of episomal circles formed during human immunodeficiency virus type 1 replication. *J Virol* **76**, 4138-4144 (2002).

60 Sharkey, M. E. *et al.* Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. *Nat Med* **6**, 76-81 (2000).

61 Stevenson, M. *et al.* Integration is not necessary for expression of human immunodeficiency virus type 1 protein products. *J Virol* **64**, 2421-2425 (1990).

62 Case, S. S. *et al.* Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc Natl Acad Sci U S A* **96**, 2988-2993 (1999).

63 Haas, D. L., Case, S. S., Crooks, G. M. & Kohn, D. B. Critical factors influencing stable transduction of human CD34(+) cells with HIV-1-derived lentiviral vectors. *Mol Ther* **2**, 71-80 (2000).

64 Li, S. *et al.* Stable transduction of myogenic cells with lentiviral vectors expressing a minidystrophin. *Gene Ther* **12**, 1099-1108 (2005).

65 Mali, P. *et al.* Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. *Stem Cells* **26**, 1998-2005 (2008).

66 Saenz, D. T. *et al.* Unintegrated lentivirus DNA persistence and accessibility to expression in nondividing cells: analysis with class I integrase mutants. *J Virol* **78**, 2906-2920 (2004).

67 Vargas, J., Jr., Gusella, G. L., Najfeld, V., Klotman, M. E. & Cara, A. Novel integrase-defective lentiviral episomal vectors for gene transfer. *Hum Gene Ther* **15**, 361-372 (2004).

68 Balague, C. *et al.* Sustained high-level expression of full-length human factor VIII and restoration of clotting activity in hemophilic mice using a minimal adenovirus vector. *Blood* **95**, 820-828 (2000).

69 McCown, T. J. Adeno-associated virus-mediated expression and constitutive secretion of galanin suppresses limbic seizure activity in vivo. *Mol Ther* **14**, 63-68 (2006).

70 Miao, C. H., Thompson, A. R., Loeb, K. & Ye, X. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. *Mol Ther* **3**, 947-957 (2001).

71 Nakai, H. *et al.* Adeno-associated viral vector-mediated gene transfer of human blood coagulation factor IX into mouse liver. *Blood* **91**, 4600-4607 (1998).

72 Pastore, L. *et al.* Use of a liver-specific promoter reduces immune response to the transgene in adenoviral vectors. *Hum Gene Ther* **10**, 1773-1781 (1999).

73 Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H. & Verma, I. M. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* **17**, 314-317 (1997).

74 Park, F., Ohashi, K., Chiu, W., Naldini, L. & Kay, M. A. Efficient lentiviral transduction of liver requires cell cycling in vivo. *Nat Genet* **24**, 49-52 (2000).

75 Fabes, J. *et al.* Accumulation of the inhibitory receptor EphA4 may prevent regeneration of corticospinal tract axons following lesion. *Eur J Neurosci* **23**, 1721-1730 (2006).

76 Karwacz, K. *et al.* Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy. *J Virol* **83**, 3094-3103 (2009).

77 Negri, D. R. M. *et al.* Successful immunization with a single injection of

non-integrating lentiviral vector. *Mol Ther* **15**, 1716-1723 (2007).

78 Rahim, A. A. *et al.* Efficient gene delivery to the adult and fetal CNS using pseudotyped non-integrating lentiviral vectors. *Gene Ther* **16**, 509-520 (2009).

79 Yanez-Munoz, R. J. *et al.* Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* **12**, 348-353 (2006).

80 Jackson, S. P. & Bartek, J. The DNA-damage response in human biology and disease. *Nature* **461**, 1071-1078 (2009).

81 Pardo, B., Gomez-Gonzalez, B. & Aguilera, A. DNA repair in mammalian cells: DNA double-strand break repair: how to fix a broken relationship. *Cell Mol Life Sci* **66**, 1039-1056 (2009).

82 Gu, J. & Lieber, M. R. Mechanistic flexibility as a conserved theme across 3 billion years of nonhomologous DNA end-joining. *Genes Dev* **22**, 411-415 (2008).

83 van den Bosch, M., Lohman, P. H. & Pastink, A. DNA double-strand break repair by homologous recombination. *Biol Chem* **383**, 873-892 (2002).

84 Elstrodt, F. *et al.* BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res* **66**, 41-45 (2006).

85 Liu, N. *et al.* XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol Cell* **1**, 783-793 (1998).

86 Adair, G. M. *et al.* Role of ERCC1 in removal of long non-homologous tails during targeted homologous recombination. *Embo J* **19**, 5552-5561 (2000).

87 Xu, K., Ma, H., McCown, T. J., Verma, I. & Kafri, T. Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. *Mol Ther* **3**, 97-104 (2001).

88 Cockrell, A. S., Ma, H., Fu, K., McCown, T. J. & Kafri, T. A trans-lentiviral packaging cell line for high-titer conditional self-inactivating HIV-1 vectors. *Mol Ther* **14**, 276-284 (2006).

89 Kantor, B., Ma, H., Webster-Cyriaque, J., Monahan, P. E. & Kafri, T. Epigenetic activation of unintegrated HIV-1 genomes by gut-associated short chain fatty acids and its implications for HIV infection. *Proc Natl Acad Sci U S A* **106**, 18786-18791 (2009).

90 Cepko, C. L., Roberts, B. E. & Mulligan, R. C. Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell* **37**, 1053-1062 (1984).

91 Hirt, B. Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* **26**, 365-369 (1967).

92 Yoder, K. E. & Fishel, R. PCR-based detection is unable to consistently distinguish HIV 1LTR circles. *J Virol Methods* **138**, 201-206 (2006).

93 Shun, M. C., Daigle, J. E., Vandegraaff, N. & Engelman, A. Wild-type levels of human immunodeficiency virus type 1 infectivity in the absence of cellular emerin protein. *J Virol* **81**, 166-172 (2007).

94 Kilzer, J. M. *et al.* Roles of host cell factors in circularization of retroviral DNA. *Virology* **314**, 460-467 (2003).

95 Vigna, E. & Naldini, L. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J Gene Med* **2**, 308-316 (2000).

96 Hamaguchi, I. *et al.* Lentivirus vector gene expression during ES cell-derived hematopoietic development in vitro. *J Virol* **74**, 10778-10784 (2000).

97 Philpott, N. J. & Thrasher, A. J. Use of nonintegrating lentiviral vectors for gene therapy. *Hum Gene Ther* **18**, 483-489 (2007).

98 Svarovskaia, E. S. *et al.* Azido-containing diketo acid derivatives inhibit human immunodeficiency virus type 1 integrase in vivo and influence the frequency of deletions at two-long-terminal-repeat-circle junctions. *J Virol* **78**, 3210-3222 (2004).

99 Ma, H. & Kafri, T. A single-LTR HIV-1 vector optimized for functional genomics applications. *Mol Ther* **10**, 139-149 (2004).

100 Kafri, T., van Praag, H., Gage, F. H. & Verma, I. M. Lentiviral vectors: regulated gene expression. *Mol Ther* **1**, 516-521 (2000).

101 Hu, B., Yang, H., Dai, B., Tai, A. & Wang, P. Nonintegrating lentiviral vectors can effectively deliver ovalbumin antigen for induction of antitumor immunity. *Hum Gene Ther* **20**, 1652-1664 (2009).

102 Blomer, U., Naldini, L., Verma, I. M., Trono, D. & Gage, F. H. Applications of gene therapy to the CNS. *Hum Mol Genet* **5 Spec No**, 1397-1404 (1996).

103 Butler, S. L., Hansen, M. S. T. & Bushman, F. D. A quantitative assay for HIV DNA integration in vivo. *Nat Med* **7**, 631-634 (2001).

104 Folger, K. R., Wong, E. A., Wahl, G. & Capecchi, M. R. Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous

recombination between injected plasmid DNA molecules. *Mol Cell Biol* **2**, 1372-1387 (1982).

105 Leavitt, A. D., Robles, G., Alesandro, N. & Varmus, H. E. Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. *J Virol* **70**, 721-728 (1996).

106 Cornu, T. I. & Cathomen, T. Targeted genome modifications using integrase-deficient lentiviral vectors. *Mol Ther* **15**, 2107-2113 (2007).

107 Lombardo, A. *et al.* Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol* **25**, 1298-1306 (2007).

108 Radecke, S., Radecke, F., Cathomen, T. & Schwarz, K. Zinc-finger Nuclease-induced Gene Repair With Oligodeoxynucleotides: Wanted and Unwanted Target Locus Modifications. *Mol Ther* (2010).

109 Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872 (2007).

110 Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920 (2007).

111 Hahn, W. C. *et al.* Enumeration of the simian virus 40 early region elements necessary for human cell transformation. *Mol Cell Biol* **22**, 2111-2123 (2002).

112 Ruggero, D. The role of Myc-induced protein synthesis in cancer. *Cancer Res* **69**, 8839-8843 (2009).

113 Coutant, F., Frenkiel, M. P., Despres, P. & Charneau, P. Protective antiviral immunity conferred by a nonintegrative lentiviral vector-based vaccine. *PLoS One* **3**, e3973 (2008).

114 McWilliams, M. J. *et al.* Mutations in the 5' end of the human immunodeficiency virus type 1 polypurine tract affect RNase H cleavage specificity and virus titer. *J Virol* **77**, 11150-11157 (2003).

115 Julias, J. G. *et al.* Effects of mutations in the G tract of the human immunodeficiency virus type 1 polypurine tract on virus replication and RNase H cleavage. *J Virol* **78**, 13315-13324 (2004).

116 Makinen, P. I. *et al.* Stable RNA interference: comparison of U6 and H1 promoters in endothelial cells and in mouse brain. *J Gene Med* **8**, 433-441 (2006).

117 Follenzi, A. *et al.* Targeting lentiviral vector expression to hepatocytes limits

transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. *Blood* **103**, 3700-3709 (2004).

118 Bonci, D. *et al.* 'Advanced' generation lentiviruses as efficient vectors for cardiomyocyte gene transduction in vitro and in vivo. *Gene Ther* **10**, 630-636 (2003).

119 Fleury, S. *et al.* Multiply attenuated, self-inactivating lentiviral vectors efficiently deliver and express genes for extended periods of time in adult rat cardiomyocytes in vivo. *Circulation* **107**, 2375-2382 (2003).

120 VandenDriessche, T. *et al.* Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood* **100**, 813-822 (2002).

121 Paxinos, G. & Watson, C. *The rat brain in stereotaxic coordinates*. 2nd edn, (Academic Press, 1986).

122 Brown, B. D. *et al.* In vivo administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. *Blood* **109**, 2797-2805 (2007).

123 Pierson, T. C. *et al.* Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. *J Virol* **76**, 8518-8531 (2002).

124 Spina, C. A., Guatelli, J. C. & Richman, D. D. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. *J Virol* **69**, 2977-2988 (1995).

125 Liu, B. H., Wang, X., Ma, Y. X. & Wang, S. CMV enhancer/human PDGF-beta promoter for neuron-specific transgene expression. *Gene Ther* **11**, 52-60 (2004).

126 Mitchell, R. S. *et al.* Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* **2**, E234 (2004).

127 Schroder, A. R. *et al.* HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**, 521-529 (2002).