Efficient lentiviral transduction of liver requires cell cycling *in vivo*

Frank Park[1], Kazuo Ohashi[1], Winnie Chiu[1], Luigi Naldini[2] & Mark A. Kay[1]

Human-immunodeficiency-virus (HIV)–based lentiviral vectors are a promising tool for *in vivo* gene therapy[1]. Unlike Moloney-murine-leukaemia–based retroviruses (MLV), lentiviruses are believed to stably transduce quiescent (non-cycling) cells in various organs[2–6]. No previous studies, however, have directly established the cell-cycle status of any transduced cell type at the time of vector administration *in vivo*. *In vitro* studies using wild-type HIV or HIV-based vectors have shown that, in some cases, cell-cycle activation is required for infection, even though cellular mitosis is not an absolute requirement for integration[7–9]. Even if the block in reverse transcription is overcome in quiescent T cells, productive infection by HIV cannot be rescued in the absence of cell-cycle activation[7,10]. The potential use of these vectors for gene therapy prompted our study, which establishes a cell-cycle requirement for efficient transduction of hepatocytes *in vivo*.

We studied lentiviral transduction in quiescent and regenerating mouse livers by infusing different doses of a PGKNLSLacZ (nuclear-localized β-galactosidase transcriptionally driven by the *Pgk1* promoter) lentiviral vector into the portal vein of C57Bl/6-scid mice, some of which had a prior surgical partial hepatectomy (Figs 1 and 2). Partial hepatectomy stimulates hepatocellular regeneration such that almost all hepatocytes divide once or twice. We injected mice with a low dose of lentivirus (1×10^8 transducing units (TU); multiplicity of infection (MOI) of ~1) produced with packaging constructs containing (CMVΔR8.2) or deleted for (CMVΔR8.74) four accessory genes: *vif*, *vpr*, *vpu* and *nef*. There was no difference in hepatocyte transduction efficiency between non-hepatectomized mice that received the vector with (0.16±0.08%; n=5) or without (0.09±0.05%; n=6) the accessory proteins (Fig. 2*a*). Occasional non-parenchymal cells were also transduced (Fig. 1*c*). In contrast, a high dose (2×10^8 TU (MOI=2)) of the lentiviral vector with or without the accessory proteins increased the number of hepatocyte nuclei stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) to 0.45±0.08% (n=7) and 0.6±0.2% (n=5), respectively (Fig. 2*b*). At the highest MOI (8–10), 2.2% of hepatocyte nuclei were positive for X-gal (n=2, 2.5% and 1.9%; Fig. 2*b*).

Mice partially hepatectomized before lentiviral infusion (MOI of 1, 2 and 8–10) had a significant increase in transduction ($P<0.005$) compared with their non-hepatectomized counterparts (Fig. 2*a*,*b*), with nearly 60% of hepatocytes expressing β-galactosidase in mice given the highest dose of vector. The presence or absence of accessory proteins encoded by the HIV vectors did not alter the enhanced transduction observed with partial hepatectomy (Fig. 2*a*,*b*). Mice that were injected with VSV-G pseudotyped MLV containing the same PGKNLSLacZ expression cassette (MOI of 2; Fig. 2*c*) showed no lacZ-positive hepatic nuclei (<0.0001%; n=5), whereas mice that received a partial hepatectomy 48 hours before MLV infusion had 0.73±0.33% lacZ-positive (n=4) hepatocytes. This is consistent with previous studies using amphotropic or VSV-G pseudotyped MLV vectors[11–13].

Analysis of extrahepatic tissue showed that the brain, heart, lung, kidney and duodenum tested negative for β-galactosidase expression, but the spleen contained X-gal–positive cells (data not shown). In a previous study, non-stimulated rat livers were used to demonstrate the lentiviral transduction of quiescent hepatocytes *in vivo*[4], but we found no difference in transduction between mice and rats. When PGKNLSLacZ lentivirus (2×10^8 TU) was infused into the portal vein of mature Fisher rats, there was a similar increase in the efficiency of liver transduction (~26-fold) in non-hepatectomized and hepatectomized animals (from 0.06±0.04% (n=4) to 1.6±0.6% (n=3); Fig. 2*d*).

We analysed liver toxicity in rodents by measuring the serum alanine aminotransferase (ALT) concentration, a sensitive serum marker for hepatocellular injury. There was a dose-dependent, transient increase in serum ALT concentrations using the lentiviral vector not encoding accessory proteins (Fig. 3). In addition, the $8–10\times10^8$ TU dose of vector was associated with a mortality rate of 74% (20/27 mice died).

Lentiviral vectors produced with accessory proteins (MOI=1) resulted in a higher transient elevation of serum ALT levels

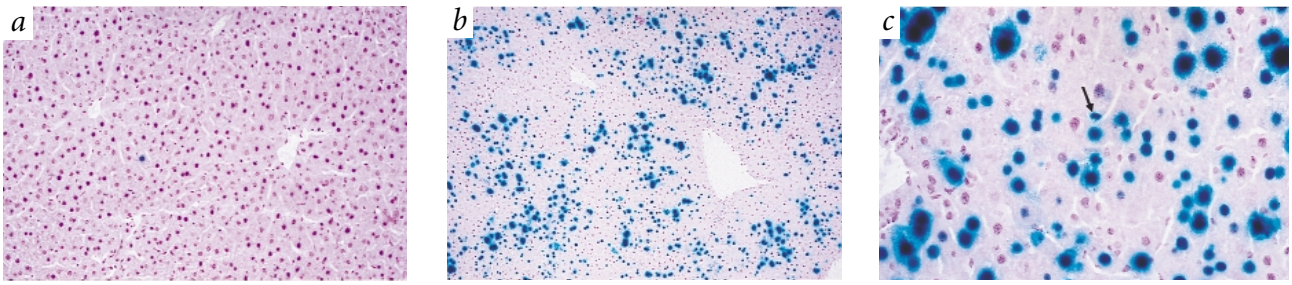

Fig. 1 β-galactosidase expression in mouse liver following lentiviral transduction. C57Bl/6-scid mice were injected with different doses of PGKNLSLacZ lentivirus packaged without accessory proteins (CMVΔR8.74) into the portal vein and liver tissue was analysed for β-galactosidase expression after three weeks. ***a***, Mouse liver without partial hepatectomy (10×10^8 TU). Original magnification, ×200. ***b***, Mouse liver with partial hepatectomy (8×10^8 TU). Original magnification ×100. ***c***, Mouse liver with partial hepatectomy (8×10^8 TU). The arrow denotes an example of a transduced nonparenchymal cell, which contains a smaller, irregularly shaped nucleus compared with the larger, circular hepatocyte nucleus. Original magnification, ×400.

[1]*Department of Pediatrics and Genetics, Stanford University, Stanford, California, USA.* [2]*Institute for Cancer Research, University of Torino Medical School, Torino, Italy. Correspondence should be addressed to M.A.K. (e-mail: markay@stanford.edu).*

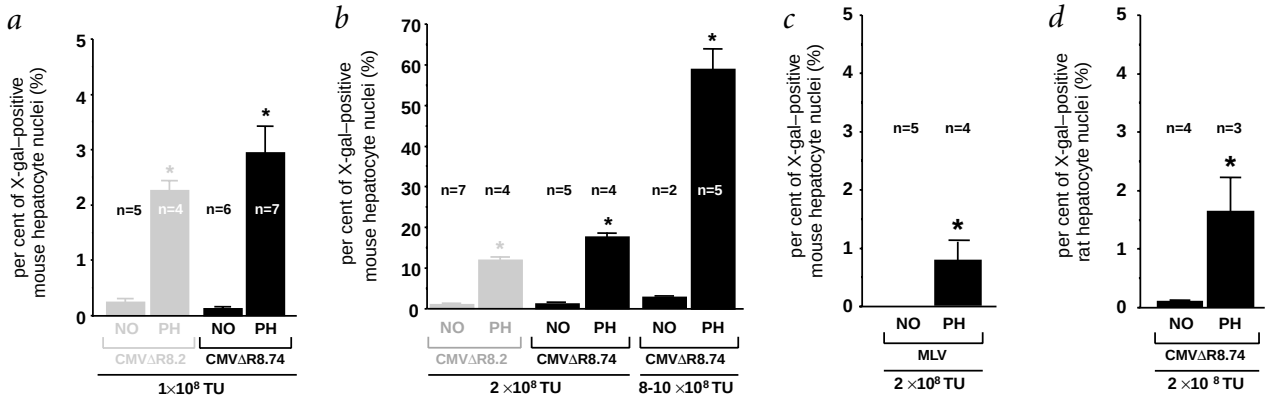

Fig. 2 Nuclear lacZ expression in the hepatocytes of mice injected with a lentiviral vector. Mice (*a*–*c*) or rats (*d*) were given different doses of MLV (***c***) or replication-defective lentivirus with (grey bars; CMVΔR8.2) or without (black bars; CMVΔR8.74) accessory proteins (***a***,***b***,***d***). X-gal–positive hepatocyte nuclei were compared in mouse (*a*–*c*) and rat (*d*) liver with (PH) or without (NO) a partial hepatectomy 48 h (in mice) and 24 h (in rats) before MLV or lentiviral administration. After one (*d*) or three (*a*–*c*) weeks later, the livers were analysed for X-gal–stained hepatocyte nuclei. The number of mice used in each group is shown on the graph. *Significant difference in X-gal–positive hepatocyte nuclei comparing hepatectomized (PH) versus non-hepatectomized (NO) mice ($P<0.005$).

(215±25 IU; n=5) compared with the same dose of vector without accessory proteins (51±16 IU; n=3). Partial hepatectomy did not affect serum ALT levels in animals that received lentivirus (Fig. 3). The observed liver injury may be a direct toxic effect of the viral particles or contaminants in the lentiviral preparation.

To determine the cell-cycle status of hepatocytes *in vivo* that were transduced by the lentiviral particles, we injected C57Bl/6-scid mice with or without a prior hepatectomy with the EF1αcyto-LacZ (2×10^8 TU) vector, which expresses cytosolic lacZ. Following vector administration, we gave mice 5′-bromo-2′-deoxyuridine (BrdU) via osmotic minipump for seven days to label proliferating nuclei (Fig. 4 and Table 1). The mild liver toxicity observed with this dose of vector was associated with a fourfold increase in the number of BrdU-positive hepatocytes, demonstrating cell-cycle activation of normally quiescent hepatocytes (Table 1). We found that 83–91% of hepatocytes expressing cytosolic β-gal were co-labelled with BrdU, irrespective of whether or not a partial hepatectomy was performed, thus establishing a requirement for DNA synthesis for efficient lentiviral transduction into mouse hepatocytes *in vivo*. Increased viral uptake was unlikely to account for the enhanced transduction observed with partial hepatectomy because of the high proportion of BrdU labelling in X-gal–positive cells in the absence of a partial hepatectomy. Without a partial hepatectomy, only 3.8% of hepatocytes were BrdU positive, whereas 83% of lacZ-positive hepatocytes were BrdU positive.

Previous studies using replication-defective lentivirus have not analysed the transduced tissue for proviral DNA integration. It is believed that circular episomal forms are incapable of integration and that integration is required for transgene expression[2,3,14,15]. To determine the integration status and copy number of lentiviral genomes in the liver, we carried out genomic Southern-blot analysis on mice three weeks after high-dose lentiviral injection. Because lentiviruses can persist as a non-integrated episomal circle[16], we determined the total copy number of integrated and episomal proviral DNA genomes in transduced livers by *Bam*HI digestion, which cuts twice in the vector (Fig. 5*b*). Densitometric analyses indicated 0.43±0.05 (n=4 mice) copies of lentiviral proviral DNA/diploid genome in animals that received a partial hepatectomy, whereas we detected a weak signal in liver of non-partially hepatectomized mice that received the same dose of virus. We found proviral DNA in spleen (Fig. 5*b*), consistent with the presence of X-gal–positive cells.

We detected episomal, but not integrated, genomes as discreet bands with *Xho*I digestion (Fig. 5*c*). Approximately 15% (or 0.09 copies/cell) of the proviral DNA was present as one- or two-LTR circles (Fig. 5*b*). By subtracting the number of circular forms from the total average amount of proviral DNA (0.43–0.09), the number of integrated copies of lentivirus per cell was approximately 0.34 in liver. After correction for the approximately 35% of DNA derived from non-parenchymal liver cells, we calculated the number of copies of lentivirus per hepatocyte to be approximately 0.53, which correlated with the 58% transduction efficiency (Figs 1*b*,*c* and 2). We conclude that most lentiviral DNA was integrated into the host genome, and that the gene copy number and the number of transduced cells detected by lacZ expression were concordant, suggesting there was one proviral DNA copy per transduced hepatocyte.

Our results conflict those of a previous study[4] that concluded only lentiviral vectors packaged with accessory proteins, and not MLV-based viral vectors, transduce quiescent hepatocytes *in vivo*. The non-linear, dose-dependent increase in lentiviral transduction and associated increase in liver injury and hepatocellular regeneration (Table 1) raises the possibility that the direct intraparenchymal injection of vector[4] may have induced physical or chemical injury from a high local concentration of the vector. This may have inad-

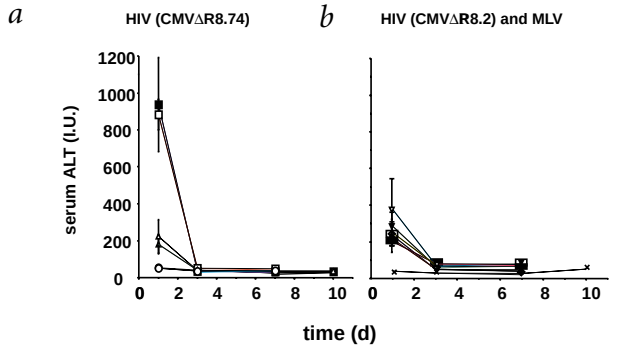

Fig. 3 Liver toxicity measured by serum alanine aminotransferase (ALT) concentrations. Serum samples from C57Bl/6-scid mice (with or without prior partial hepatectomy) were analysed for liver injury following direct portal vein infusion of different doses of PGKNLSLacZ MLV retrovirus, and lentivirus with (CMVΔR8.2) or without (CMVΔR8.74) accessory genes. ***a***, Mice with (open shapes) or without (filled shapes) partial hepatectomy at different doses of lentivirus (CMVΔR8.74): circles, 1×10^8 TU; triangles, 2×10^8 TU; squares, $8–10\times10^8$ TU. ***b***, Mice with (open shapes) or without (closed shapes) partial hepatectomy at different doses of lentivirus (CMVΔR8.2) or MLV: diamonds, 1×10^8 TU (CMVΔR8.2); triangles, 2×10^8 TU (CMVΔR8.2); rectangles, 2×10^8 TU (MLV); X, vehicle control (phosphate-buffered saline without calcium and magnesium plus 8 μg/ml polybrene). There were 3–5 mice per group.

Table 1 • BrdU and lacZ quantitation in mouse hepatocytes

Protocol	% of BrdU-labelled hepatocytes	% of lacZ hepatocytes that are BrdU positive
vehicle without partial hepatectomy (n=3)	0.9% (0.1)	NA
lentivirus without partial hepatectomy (n=4)	3.8% (0.5)	83.3%
vehicle plus partial hepatectomy (n=3)	50.4% (1.9)	NA
lentivirus plus partial hepatectomy (n=3)	75.2% (6.2)	91.1%

The average number of BrdU- or BrdU/lacZ-positive hepatocytes is given with the standard error in parentheses. $P<0.05$ for vehicle versus lentiviral vector administration for both non-hepatectomized and partial hepatectomized animals. NA, not applicable.

vertently increased the number of hepatocytes progressing into the cell cycle, allowing for gene transfer. If lentiviruses can persist intracellullarly for a longer period of time compared with MLV (refs 17,18), it is conceivable that lentiviral but not MLV-mediated transduction may continue to occur for an undefined time interval after viral entry. In support of this hypothesis, we found that mice given a partial hepatectomy 24 hours after portal vein injection of lentivirus (MOI of 2 with accessory proteins) had a twofold increase ($P<0.05$) in transduction compared with their non-hepatectomized counterparts (from 0.45±0.08% (n=7) to 0.9±0.19% (n=6)). We saw no X-gal–positive hepatocytes in mice injected with MLV (with or without a partial hepatectomy) 24 hours after viral injection (n=5 per group). This suggests that, unlike MLV-based vectors, lentiviral pre-integration complexes transduce hepatocytes more than 24 hours after vector infusion. This may explain increased hepatic transduction efficiencies observed with lentivirus compared with MLV when equivalent doses of virus containing the same envelope protein were administered to animals.

In addition to demonstrating a cell-cycle requirement for *in vivo* hepatocyte transduction, our study has additional implications for gene therapy. First, intravascular vector administration resulted in β-gal expression in splenic and other non-parenchymal liver cells, which was not observed when recombinant adeno-associated virus was infused into the portal vein[19]. Vector-mediated expression from these antigen-presenting cells may influence the humoral or cellular immunity toward the transgenic protein. Second, our study demonstrates that HIV accessory proteins can be omitted, enhancing the biosafety of lentiviral-based vectors. Third, because we saw enhancement of lentiviral transduction in hepatocytes following progression into the cell cycle, administration of growth factors such as HGF or KGF could promote hepatocellular cycling, resulting in efficient gene transfer to the liver[20,21].

Methods

Animal preparation. Female C57Bl/6-scid mice (6–8 weeks of age; the Jackson Laboratory) and female Fisher rats (10–11 weeks of age; Charles River) were purchased. We performed all animal protocols according to the Stanford University guidelines. Mice were anaesthetized with methoxyflurane (Metafane; Mallinckrodt Veterinary) and rats anaesthetized with ethyl ether (VWR Scientific Products). We injected HIV lentiviruses into the portal vein at a volume of 0.4–0.5 ml for mice and 1 ml for rats over a period of 120 s using a 30-gauge needle. A two-thirds partial hepatectomy was performed 24 h before lentiviral injection in rats[22], and 48 h in mice[23], representing the maximal period of liver regeneration for these species. In some experiments an osmotic minipump (model 2001, Alzet) was placed subcutaneously to administer BrdU (1 mg/d) for 7 d. We determined serum ALT concentrations using a colorimetric diagnostic kit (Sigma).

Plasmid constructs for vector production. We used two helper packaging constructs as described: pCMVΔR8.2 contains the four accessory genes *vif*, *nef*, *vpr* and *vpu* (ref. 24), and pCMVΔR8.74 is deleted for these four genes[2]. pMD.G contains the expression sequence for the pseudotyped VSV-G envelope. pHR2PGKNLSLacZ plasmid, encoding the nuclear-localized *lacZ* driven by the mouse *Pgk1* promoter, has been described[2]. pHR2EF1αcytoLacZ, expressing a cytosolic *lacZ* from the ubiquitous *Eif1a* promoter, was produced by digesting pHR2PGKNLSLacZ with *Bam*HI/*Xho*I and inserting the full-length *Eif1a* promoter fragment (*Bam*HI/blunted *Not*I; 2.4 kb) to generate the plasmid construct pHR2EF1α. To make pHR2EF1αcytoLacZ, an *Eco*RI/*Eco*RI fragment (3.0 kb) from pBScytoLacZ was gel isolated and ligated into the pHR2EF1α plasmid construct.

We generated the pLPGKNLSLacZ plasmid for production of an MLV vector by digesting pLXSHD (ref. 25) with *Xho*I/*Hin*dIII and inserting an annealed oligo linker that had novel restriction sites (*Not*I and *Pme*I), producing pLPN. pLPN was digested with *Pme*I and ligated with a fragment (blunted *Xho*I/blunted *Sac*I; 3.8 kb) containing the *Pgk1* promoter upstream of a nuclear-localized *lacZ* from pHR2PGKNLSLacZ, producing the final product, pLPGKNLSLacZ.

Viral production and assays. We transiently transfected 293T and 293TGP cells (a gift from G.P. Nolan) to produce replication-defective HIV-1 lentivirus and MLV, respectively. We seeded $9–12\times10^6$ cells in 15-cm-diameter dishes 24 h before transfection. We added chloroquine to the plates at a final concentration of 25 μM before the transfection of DNA. We used 40 μg plasmid for the transfection of one dish for HIV by the calcium phosphate coprecipitation method[26]: 7 μg envelope plasmid pMD.G, 13 μg packaging plasmid pCMVΔR8.74 or pCMVΔR8.2 and 20 μg transfer plasmid (pHR2PGKNLSLacZ or pHR2EF1αcytoLacZ). For MLV, we used 27 μg plasmid: 7 μg pMD.G and 20 μg pLPGKNLSLacZ. We replaced the medium (22 ml) after 10–12 h, collected the medium after 28–30 h, cleared by low-speed ultracentrifugation and filtered through 0.22-μm pore-size cellulose filters. The virus was concentrated by centrifuging the medium at 19,500 r.p.m. for 2.33 h at 20 °C. We resuspended the pellet in phosphate-

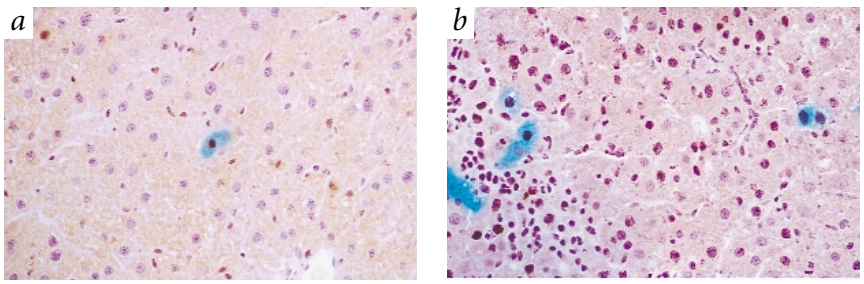

Fig. 4 Co-localization of BrdU and cytoplasmic lacZ labelling in mouse liver. Mice were infused with 2×10^8 TU EF1αcytoLacZ lentivirus (no accessory proteins) that expressed the cytoplasmic lacZ and then administered BrdU via subcutaneous osmotic minipumps for 7 d before sacrifice. We stained liver sections for both β-galactosidase expression (cytoplasmic blue staining) and BrdU incorporation (brown nuclei). ***a***, Liver section from a mouse which did not receive partial hepatectomy, demonstrating co-localization of BrdU and cytoplasmic lacZ. Note the lack of non-BrdU-labelled hepatocyte nuclei (purple nuclei) in non-hepatectomized mouse liver. ***b***, Liver section from a mouse which received partial hepatectomy before lentiviral injection. Four hepatocytes are shown to be co-localized with BrdU and cytoplasmic lacZ; one hepatocyte is shown which expressed β-galactosidase independent of cell cycling. Original magnification, ×400. The total number of X-gal–positive cells was slightly lower than that achieved with the same dose of a nuclear-localized lacZ. This has been our experience with several vectors and was likely due to the lower sensitivity of the former.

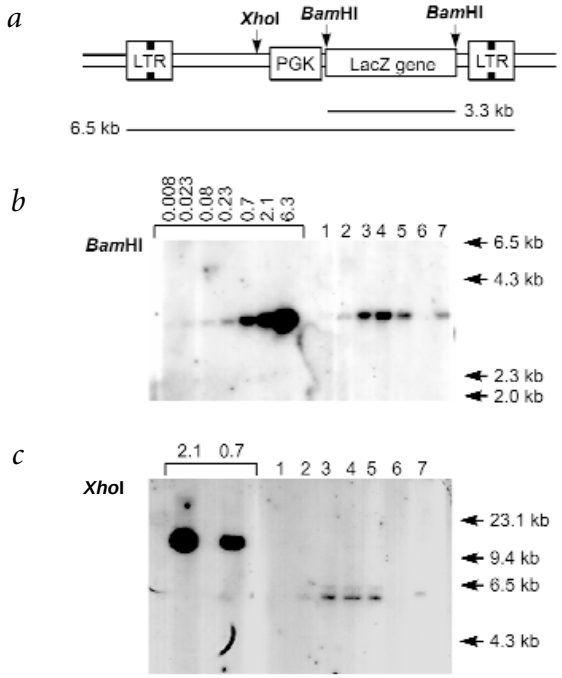

Fig. 5 Genomic Southern-blot analysis. Mice were infused with 8–10×10^8 TU of PGKNLSLacZ and then three weeks later total DNA was isolated from liver for genomic Southern analysis. We used genomic DNA (10 μg) for Southern-blot analysis. The radiolabelled *lacZ* fragment (*Bam*HI) was used as a probe. ***a***, Restriction map of the vector showing the restriction sites for *Bam*HI and *Xho*I/*Bam*HI with their expected sizes following digestion. ***b***, *Bam*HI digest for estimation of total proviral DNA content. Lane 1, no partial hepatectomy; lanes 2–5, different mice receiving a partial hepatectomy 48 h before gene transfer; lane 6, brain; lane 7, spleen. The concentration curve on the left (6.3–0.008 copies/cell) was derived from adding plasmid DNA to mouse genomic DNA. ***c***, *Xho*I digestion to determine the circular proviral DNA forms. The lane designations are the same as for (*b*). One (5.9 kb) and two (6.5 kb)-LTR circles are visible. Arrows on the right are molecular wieght size markers.

buffered saline without calcium and magnesium (PBS) and then re-centrifuged at 19,500 r.p.m. for 2.33 h. The final pellet was resuspended in PBS in the presence of polybrene (8 μg/mL).

To determine the titre of MLV and HIV vector stocks, we used serial dilution of concentrated virus to infect 5×10^5 HeLa cells in a 6-well plate in the presence of polybrene (8 μg/ml). The titres are given in the text as transducing units (TU). HIV p24 Gag antigen concentration was determined by ELISA (Alliance; Dupont-NEN). The absence of replication-competent virus in each vector batch was assessed by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for three weeks. In all cases tested, p24 antigen was undetectable (detection limit, 3 pg/ml).

β-galactosidase expression. Liver, spleen, brain, heart, lung, kidney and duodenum were snap frozen in OCT buffer on dry ice. We stained sections (10 μm) for β-galactosidase (β-gal) expression using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal; Fisher Scientific).

BrdU incorporation. Liver and duodenum were frozen in OCT buffer on dry ice. We used duodenum as a positive control for DNA synthesis (that is, BrdU incorporation). We mounted sections (7 μm) of the tissue onto glass slides. Endogenous peroxidase activity was removed by incubation with 0.3% hydrogen peroxide and the tissue blocked in 10% normal rabbit serum (Vector Labs) overnight at 4 °C. We incubated tissue sections with primary antibody against BrdU (1:4,000) for 1 h and secondary antibody (1:500) for 30 min. We used 3′-3′-diaminobenzidine (DAB; Sigma) as the brown substrate for precipitation of the BrdU sample. The tissue sections were then counterstained with haematoxylin, dehydrated and coverslipped for analysis.

For liver tissue, we analysed X-gal and BrdU labelling in two or three separate lobes for each animal (in total, 30–40 random areas of the sections from 2–3 liver lobes; 2,000 nuclei from each animal).

Statistical analysis. Student's *t*-test was used to analyse differences in vehicle (control) versus experimental groups. $P<0.05$ was considered significant.

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