In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector

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A retroviral vector system based on the human immunodeficiency virus (HIV) was developed that, in contrast to a murine leukemia virus–based counterpart, transduced heterologous sequences into HeLa cells and rat fibroblasts blocked in the cell cycle, as well as into human primary macrophages. Additionally, the HIV vector could mediate stable in vivo gene transfer into terminally differentiated neurons. The ability of HIV-based viral vectors to deliver genes in vivo into nondividing cells could increase the applicability of retroviral vectors in human gene therapy.

Until now, gene therapy protocols have often relied on vectors derived from retroviruses such as murine leukemia virus (MLV) (*1*, *2*). These vectors are useful because the genes they transduce are integrated into the genome of the target cells, a desirable feature for long-term expression. However, these retroviral vectors can only transduce dividing cells, which limits their use for in vivo gene transfer in nonproliferating cells such as hepatocytes, myofibers, hematopoietic stem cells, and neurons (*3*, *4*). The optimal gene transfer system would include a retroviral vector based on a virus, such as HIV and other lentiviruses, that can integrate into the genome of nonproliferating cells. In vitro, HIV can infect primary cultures of monocyte-derived macrophages (*5*) as well as cell cycle–arrested $CD4^+$ HeLa or T lymphoid cells (*6*). Central to this ability are karyophilic determinants contained in two virion proteins, matrix (MA) and Vpr. These proteins interact with the nuclear import machinery and mediate the active transport of the HIV preintegration complex through the nucleopore (*7*–*9*).

A three-plasmid expression system was used to generate HIV-derived retroviral vector particles by transient transfection, as described for other vectors (*10*) (Fig. 1). Plasmid pCMVΔR9, the packaging construct, contains the human cytomegalovirus (hCMV) immediate early promoter, which drives the expression of all viral proteins required in trans. This plasmid is defective for the production of the viral envelope and the accessory protein Vpu. The packaging signal (Ψ) and adjacent sequences were deleted from the 5′ untranslated region, but the 5′ splice donor site was preserved. A polyadenylation [poly(A)] site from the insulin gene was substituted for the 3′ long terminal repeat (LTR) at the end of the *nef* reading frame (*11*). This design eliminated cis-acting sequences crucial for packaging, reverse transcription, and integration of transcripts derived from the packaging plasmid (*12*). To broaden the tropism of the vector, we used a second plasmid that encodes a heterologous envelope protein for pseudotyping the particles generated by pCMVΔR9 (*13*). Two variants of this construct were used: One variant encodes the amphotropic envelope of MLV (Ampho), and the other encodes the G glycoprotein of vesicular stomatitis virus (VSV G) (*14*). The latter envelope offers the additional advantage of high stability, which allows for particle concentration by ultracentrifugation (*15*). The third plasmid, the transducing vector (pHR′), contains cis-acting sequences of HIV required for packaging, reverse transcription, and integration, as well as unique restriction sites for the cloning of heterologous complementary DNAs (cDNAs). Nearly 350 base pairs of *gag* as well as *env* sequences encompassing the Rev response element (RRE) flanked by splice signals were included in the pHR′ vector (*16*). This design had a dual purpose: first, to increase packaging efficiency, as both *gag* and *env* RNA determinants have been demonstrated to enhance this process (*17*), and second, to allow the efficient transcription and cytoplasmic export of full-length vector transcripts only in the presence of the HIV Tat and Rev regulatory proteins, both of which are encoded by the packaging plasmid, pCMVΔR9. In the absence of these transacting factors, the only detectable expression originated from the internal promoter in the vector (*18*). The *Escherichia coli* β-galactosidase (β-gal) or the firefly luciferase coding sequences were inserted into pHR′ downstream of the hCMV immediate early promoter to serve as reporter genes.

Replication-defective retroviral particles were generated by transient cotransfection of 293T human kidney cells with the three-plasmid combination (*19*). MLV-derived packaging and transducing vectors served as controls (*20*). Media from the various transfectants were first

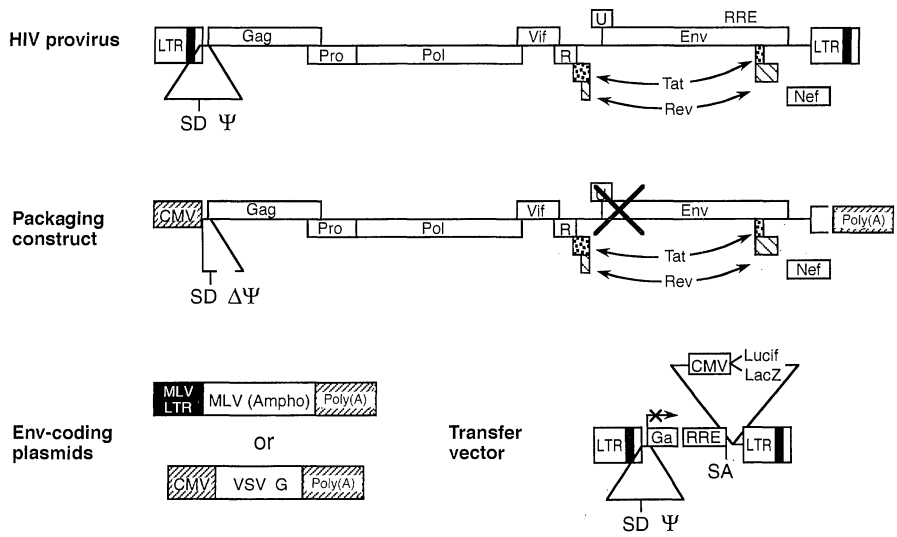

Fig. 1. Schematic representation of the HIV provirus and the three-plasmid expression system used for generating a pseudotyped HIV-based vector by transient transfection. Only the relevant portion of each plasmid is shown. For the HIV provirus, the coding region of viral proteins, including the accessory proteins, is shown. The splice donor site (SD) and the packaging signal (ψ) are indicated. In the packaging construct pCMVΔR9, the reading frames of Env and Vpu are blocked (X). In the Env-coding plasmid, the coding region of 4070a amphotropic MLV envelope is flanked by a MLV LTR and a SV40 poly(A) site. The VSV G coding region is flanked by the CMV promoter and a poly(A) site. In the transfer vector pHR′, the *gag* gene is truncated and out of frame (X), and the internal promoter CMV is used to drive expression of either β-galactosidase (*lacZ*) or luciferase cDNA. The Rev responsive element (RRE) and splice acceptor site (SA) are shown.

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assayed for transduction frequency on growing 208F rat fibroblasts (*21*). HIV-based β-gal vectors yielded titers of 0.8 (±1.7) × 10^5 (n = 3) transducing units (TU) per milliliter with the MLV(Ampho) envelope and 4 (±1.5) × 10^5 (n = 6) TU/ml with the VSV envelope. These titers are comparable with those obtained with MLV-based vectors produced by the same method—10^5 TU/ml with its own envelope, and 5 × 10^5 Tu/ml when pseudotyped with the VSV envelope—and significantly higher than those previously reported for other HIV-based vectors (*17, 22*). Potentially contributing to this increased efficiency is the incorporation of accessory HIV-1 genes into the packaging construct, including *nef* that markedly enhances virion infectivity (*23*).

Table 1. Relative transduction of cells at different stages of the cycle by HIV- and MLV-based vectors. Results are expressed relative to the transduction obtained by the vector in growing cells. Multiplicity of infection was matched for both vectors. Abbreviations: arr., arrested; d, days; repl., replated.

Infected culture	Tranduction efficiency	
	HIV-based vector	MLV-based vector
HeLa cells*		
Growing	1	1
G_1-S-arr.	0.97 ± 0.02	0.05 ± 0.01
G_2-arr.	0.71 ± 0.22	0.08 ± 0.01
208F cells†		
Growing	1	1
G_0-arr. 4 d	0.45 ± 0.02	0.11 ± 0.01
G_0-arr. 7 d	0.29 ± 0.02	0.03 ± 0.02
G_0-arr. 11 d	0.23 ± 0.01	0.02 ± 0.01
G_0-arr. 15 d	0.17 ± 0.01	0.01 ± 0.01
208F cells‡		
Growing	1	1
G_0	0.08 ± 0.02	0.05 ± 0.02
G_0 repl. 2 d	0.50 ± 0.03	0.08 ± 0.02
G_0 repl. 4 d	0.43 ± 0.04	0.08 ± 0.02
G_0 repl. 8 d	0.46 ± 0.07	0.08 ± 0.02

*Human HeLa cells were arrested in G_1-S by aphidicolin treatment or in G_2 by exposure to 40 grays (1 gray = 100 rads) of gamma radiation (*25*) and infected with β-gal vector pseudotyped with MLV (Ampho) envelope. Transduction was scored by X-Gal staining of the cultures 48 hours after infection. Results are the mean ± SEM determination from four experiments. †Rat 208F fibroblasts were plated at low density and either infected the following day (growing) or grown to confluence, switched to medium containing 5% calf serum and 2 μM dexamethasone (*3*), and further incubated for the indicated number of days (d) before infection with luciferase vectors pseudotyped with VSV G protein. Transduction was scored by measuring luminescence in cell extracts 48 hours after infection. Results are the mean ± SD of replicated determinations from a representative experiment of a total of five performed. ‡Rat 208F fibroblasts either growing or arrested in G_0 for 3 weeks were infected with β-gal vectors pseudotyped with the MLV (Ampho) envelope. Transduction was scored by X-Gal staining either 48 hours after infection (growing and G_0) or 48 hours after replating (repl.) at low density G_0 cultures trypsinized at the indicated days after infection (G_0 replated *X* d). Results are expressed relative to the number of blue cell foci obtained by infecting growing cells and are the mean ± SD of replicated determinations from a representative experiment of a total of four performed.

The HIV-derived vector system used here is devoid of helper virus per se. Furthermore, the use of a three-plasmid combination and of a heterologous envelope, as well as the removal of multiple cis-acting sequences from the packaging vector, makes it unlikely that a replication-competent recombinant would be generated. The potential transfer of packaging functions from producer to target cells was assayed by testing for the production of the *tat* and *gag* gene products in vector-transduced cells. Neither protein was detected, which, considering the sensitivity of the assays we used (*24*), implied that the transfer of packaging functions was at least three orders of magnitude less efficient than that of vector sequences. Furthermore, conditioned medium from serially passaged transduced cells did not transfer the reporter gene to naïve cells (*24*).

HIV- and MLV-derived vectors were compared for their ability to transduce cells blocked at various stages of the cell cycle. HeLa cells were growth-arrested at the G_1-S boundary or at the G_2 phase of the cycle by aphidicolin treatment or gamma irradiation, respectively (*25*). The arrested state of the cells at the time of infection was verified by propidium iodide staining of the DNA and by flow cytometry (*18*). An HIV-based retroviral vector expressing β-gal was as efficient at transducing G_1-S– and G_2-arrested as proliferating HeLa cells, whereas its MLV counterpart was only 5 to 8% as effective (Table 1). The wider variability observed in the transduction of HeLa cells arrested by gamma irradiation was perhaps due to the cytotoxicity of the treatment.

To test whether the HIV-based vector integrates in the host cell genome, we used packaging constructs carrying mutations that inactivate integrase. HIV-1 mutants in which the expression of integrase is abrogated by the introduction of a stop codon at its 5′ end do not reverse transcribe their genome efficiently (*26*). When this mutation was introduced into the packaging construct, it completely prevented transduction by the resulting vector particles. Furthermore, whereas a β-gal vector made with the wild-type packaging construct had a transduction efficiency of 940 TU per nanogram of p24 in growing or G_1-S–arrested cells, a single amino acid change [from aspartic acid to valine at position 64 (D64V)] in the HIV-1 integrase sequence, previously demonstrated to severely decrease the activity of this enzyme but not to affect any other step of infection (*27*), reduced the efficiency to 54 and 130 TU per nanogram of p24 in growing and G1-S–arrested cells, respectively (*28*). Efficient gene transfer in both settings was thus dependent on reverse transcription as well as integration. Taken together, these results indicate that the unique features of HIV can be transferred to a replication-defective retroviral vector, allowing transduction of nonproliferating cells.

To test the transduction of cells arrested in G_0, we grew cultures of rat 208F fibroblasts to confluence and then maintained them in G_0 by density-dependent inhibition of growth in the presence of dexamethasone (*3*). The HIV-based vector was significantly more efficient than its MLV equivalent. However, its transduction rate decreased as a function of time between growth arrest and infection (Table 1). Cells growth-arrested for 4 days were transduced at levels that were 45% of those observed in dividing cells. However, in cells that had been maintained in G_0 for 15 days, the relative transduction decreased to 17%. The MLV-based vector was significantly

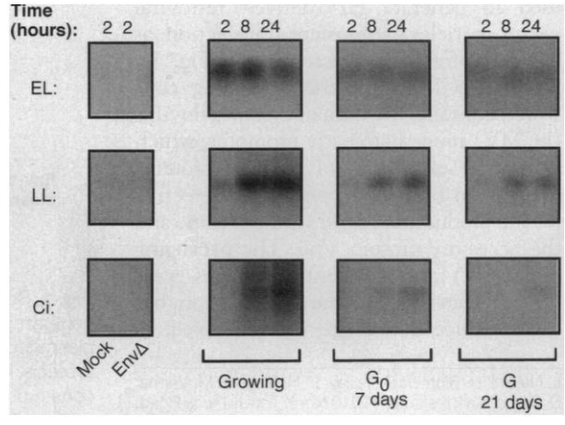

Fig. 2. Reverse transcription and nuclear import of the HIV-based vector genome in fibroblasts growing or arrested in G_0. Cultures of 208F fibroblasts were plated at low density and either infected the following day (growing) or grown to confluence and further incubated for the indicated number of days (G_0 *X* days) before infection with HIV-based luciferase vector pseudotyped or not (ΔEnv) with VSV envelope. At the indicated time in hours after infection, cells were lysed and assayed by PCR with primers specific for various products of reverse transcription, as previously described (*9, 39*). A sample of the PCR reaction was analyzed by Southern (DNA) blot with a ^{32}P-labeled HIV proviral DNA probe. EL, early products (strong stop DNA); LL, late linear products (generated after the second template switch); Ci, two-LTR circles (formed in the nucleus).

more affected by the growth arrest. In its case, the residual transducing activity reflected the fraction of cells still undergoing division, as assessed by propidium iodide staining of the cell DNA followed by flow cytometry (*29*). Whereas vector particles entered G_0-arrested and dividing cells with comparable efficiencies (*30*), they were significantly defective for reverse transcription in G_0 cells (Fig. 2), which resembles a phenomenon observed in HIV-infected quiescent T lymphocytes (*31*). Nevertheless, a stable transduction intermediate must have been established, because replating and proliferation of G_0 cells up to 8 days after infection revealed titers as high as 50% of those obtained in dividing cells (Table 1). In contrast, inducing cell division even 1 day after inoculation did not rescue the MLV-derived vector. The generation of a stable infection intermediate by the HIV-based vector offers an advantage for delivering genes into targets such as hematopoietic stem cells. Indeed, it may alleviate the need for inducing the proliferation of these cells ex vivo, a manipulation that can affect their pluripotentiality.

The decreased transduction efficiency of the HIV vector in G_0-arrested fibroblasts may partly reflect suboptimal concentrations of intracellular deoxynucleotides (*32*). Whether a similar limitation would preclude gene transfer into terminally differentiated primary cells could not be inferred from these observations and was therefore assessed directly. The HIV-based luciferase vector, pseudotyped with the VSV G protein, was tested for its ability to transduce human monocyte-derived primary macrophages (*33*). Significant levels of luciferase activity were detected in an envelope-dependent manner (Table 2). In contrast, only background levels of luciferase activity were measured in macrophages inoculated with a comparable VSV G–pseudotyped MLV-based vector (*34*). To rule out that the HIV vector was infecting a small proportion of macrophages that were proliferating, we generated mutant packaging constructs where Vpr and the nuclear localization signal (NLS) present in the MA protein were inactivated (*35*). At least one of these two elements is essential for viral infection in macrophages, because they mediate nuclear import of the HIV preintegration complex (*7–9*). A vector assembled from a mutant packaging construct in which both Vpr and the MA NLS are inactivated was severely reduced in its ability to transduce macrophages (Table 2). Similarly, NLS peptide treatment prevented transduction by a vector produced from a Vpr-defective packaging construct, thus corroborating the previously demonstrated inhibition of MA-mediated nuclear import of the HIV preintegration complex by this peptide (*9*). Neither MA-Vpr double mutations nor NLS peptide treatment affected the ability of the vectors to transduce dividing cells (*18*). The requirement for interaction with the cellular nuclear import machinery, together with the lack of significant transduction by the MLV vector, demonstrates that gene transfer by the HIV vector did occur in nonproliferating macrophages and not simply in a small proportion of dividing cells in the culture.

To test if HIV-based vectors can deliver genes in vivo, we injected highly concentrated stocks of HIV- or MLV-based β-gal vectors pseudotyped with VSV G protein bilaterally into the corpus striatum and hippocampus of adult female rat brains (*36*). Seven or 30 days later the brains were removed, sectioned, and processed for immunocytochemistry. Analysis with the light microscope showed no pathological change in the injected areas of the brains, except for a limited deposit of debris and lining-up of scavenger cells along the needle tract in brains examined 1 week after injection. These findings were even less apparent 1 month after the injection. Areas of β-gal–positive cells were detected surrounding all injected sites for both HIV-based and MLV-based vectors. In brains injected with the HIV-

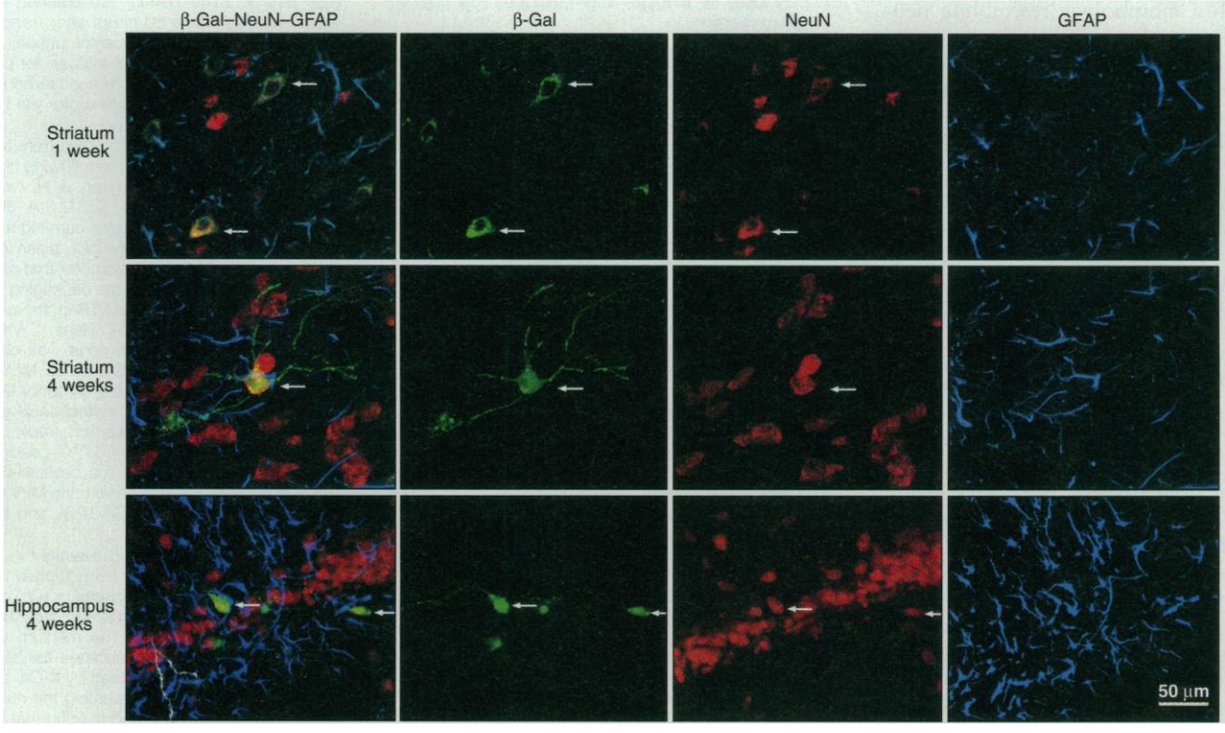

Fig. 3. The in vivo transduction of adult rat neurons. Confocal microscope images of sections from brains injected with HIV-based β-gal vectors stained by immunofluorescence for β-gal, NeuN, and glial fibrillary acidic protein (GFAP). The images obtained from each individual staining and from their overlap are shown, as indicated on the top. Representative fields of the area surrounding the injection site are shown for a section from striatum 1 week and 4 weeks and from hippocampus 4 weeks after injection of the vector. Several cells doubly labeled for β-gal and NeuN (arrows) are evident in the sections. The overall pattern was reproduced in all five animals (three examined after 7 days, and two after 30 days) injected with the HIV-based vector.

Table 2. Transduction of human monocyte-derived macrophages. Primary cultures of human macrophages prepared from different donors were incubated with HIV-based luciferase vectors pseudotyped with VSV G protein and generated either from wild-type (pCMVΔR9) or mutant packaging plasmids carrying inactivating mutations in the *vpr* gene (ΔVpr) or both in the *vpr* gene and the MA NLS [ΔVpr ΔNLS MA (*33, 34*)]. Macrophage cultures were incubated with 100 μM of peptide whose sequence corresponded either to the SV40 T antigen NLS to block NLS-dependent nuclear import, or to its reverse, inactive orientation (Rev. NLS), starting 1 hour before and throughout infection, as previously described (*8*). Luminescence was measured in cell extracts 48 hours after infection. Transduction was dependent on active nuclear import of the vector in target cells, as it was inhibited by mutations inactivating Vpr and the MA NLS in the packaging plasmids and when infected cells were incubated with NLS peptide.

Packaging plasmid	Cell treatment	Luminescence (RLU)*	
		Donor 1	Donor 2
Wild type†	—	0	0
Wild type	Rev. NLS	17,873	17,785
Wild type	NLS	16,225	15,322
ΔVpr	Rev. NLS	8,447	9,687
ΔVpr	NLS	1,141	1,348
ΔVpr ΔNLS MA	—	3,501	2,787

*Luminescence in relative units above background of 50 μl of infected macrophages extract. †As a control, this plasmid was not pseudotyped with VSV G protein.

based vector, a variety of β-gal–positive cells with a morphology resembling neurons, oligodendrocytes, and astrocytes could be detected (*18*). To further identify the cell types transduced by both vectors, we used confocal microscopy after immunofluorescence staining with antibodies specific for β-gal, glial fibrillary acidic protein (GFAP, a marker for astrocytes), and NeuN (a marker for terminally differentiated neurons) (*37*). Sections from brains injected with the MLV-based vector contained cells either labeled only for β-gal or for both β-gal and GFAP (*18*). The MLV vector was unable to transduce neurons because no cells labeled for both β-gal and NeuN were detected. In contrast, the striatum of animals injected with the HIV-based vector showed multiple cells double-labeled for β-gal and NeuN (Fig. 3, top panel), demonstrating the ability of the HIV-based vector to infect and transduce genes in terminally differentiated neurons. NeuN and β-gal double-positive cells were also detected in the hippocampus of brains injected with the HIV vector. As expected, the HIV-based vector was also able to transduce astroglial cells (*18*). The expression of β-gal in neurons in the striatum and the hippocampus could be detected after a 30-day period, the longest time tested (Fig. 3, bottom two panels).

Our results lend strong credence to the idea that HIV-based vectors transduce genes efficiently and can be used for in vivo gene delivery. Because retroviruses integrate in the genome of the target cells, repeated transduction is unnecessary. Therefore, in contrast to an adenoviral vector capable of in vivo gene delivery, problems linked to the humoral response to injected viral antigens can be avoided (*38*). Furthermore, the vectors described here are replication defective; consequently, the transduced cells lack viral protein that could trigger a cellular immune response. A major goal of our work was to establish a proof of principle that lentiviral vectors can be used for stable in vivo gene delivery in nondividing cells. For human experimentation, it may be more prudent to develop vectors derived from nonhuman lentiviruses such as simian immunodeficiency virus, bovine immunodeficiency virus, or equine infectious anemia virus. We believe that the generation of safe and efficacious lentiviral vectors will significantly advance the prospects of human gene therapy.

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16. Plasmid pHR′ was constructed by cloning a fragment of the *env* gene encompassing the RRE and a splice acceptor site between the two LTRs of the HIV-1 proviral DNA. The *gag* gene was truncated and its reading frame blocked by a frameshift mutation. pHR′-CMVLacZ was generated by cloning a 3.6-kbp Sal I–Xho I fragment containing the CMV promoter and the *E. coli lacZ* gene (encoding β-gal) from plasmid pSLX-CMVLacZ (*20*). pHR′-CMVLucif was made by replacing a Bam HI–Xho I fragment in pHR′-CMVLacZ, containing the *lacZ* gene, with a 1.7-kbp Bam HI–Xho I fragment from pGEM-luc (Promega) containing the firefly luciferase gene.
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19. A total of 40 μg of plasmid DNA was used for the transfection of a 10-cm-diameter plate of 293T cells in the following proportions: 10 μg of pCMVΔR9, 20 μg of pHR′, and 10 μg of *env* plasmid, as described [C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987)]. Conditioned medium was harvested 48 to 60 hours after transfection, subjected to low-speed centrifugation, filtered through 0.45-μm filters, and assayed for p24 Gag antigen by enzyme-linked immunosorbent assay (ELISA) (DuPont). The average vector yield was 50 to 80 ng of p24 per milliliter.
20. MLV-based vectors were produced from transient transfection in 293T cells of the following plasmids. pSLX-CMVLacZ [R. Scharfmann, J. H. Axelrod, I. M. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4626 (1991)] is a MLV-derived vector carrying a hCMV-driven *E. coli lacZ* gene. The pCL plasmid series carries a hybrid CMV-LTR promoter that allows for CMV-driven transcription in the packaging cell and reconstitution of a functional LTR in the target cell (R. Naviaux, E. Costanzi, M. Haas, I. Verma, in preparation). The luciferase gene was cloned in vector pCLNCX, creating pCLNCLuc. MLV-based vectors with the cognate MLV (Ampho) envelope were produced by the cotransfection of either of the vector plasmids with the amphotropic packaging plasmid pCL-Ampho. VSV G–pseudotyped vectors were produced by the cotransfection of either of the vector plasmids with the MLV *gag-pol* packaging plasmid pCMV-GAGPOL and the VSV G plasmid.
21. Rat 208F cells were infected overnight in six-well plates with serial dilutions of conditioned medium from 293T transient transfectants or with concentrated viral stocks in culture medium supplemented with polybrene (8 μg/ml). The medium was replaced, the cells further incubated for 36 hours, and expression of β-gal scored by X-Gal staining. Titers were calculated by counting the number of foci of blue cells per well and dividing that number by the dilution factor. Transduction of the reporter gene was only observed when the packaging vector and Env-coding plasmid had been cotransfected in 293T cells; no transduction was observed when either plasmid was omitted or when the HIV-based vector was cotransfected with an MLV-

based packaging plasmid or vice versa. Virtually all cells in a well could be transduced when a multiplicity of infection (MOI) >1 was used. When the luciferase vector was used, transduction was assayed by washing the cultures twice with tris-buffered saline (TBS), extracting the cells with 200 μl per well of 0.5% NP-40 in TBS containing 5 mM $MgCl_2$, and assaying a 50-μl sample for luminescence in a luminometer. The HIV-based luciferase vector transduced 930 ± 240 (n = 4) relative luminescence units (RLU) per microliter of infected transfectant-conditioned medium with VSV envelope, and 460 ± 110 (n = 2) RLU with MLV (Ampho) envelope. MLV-based luciferase vector pseudotyped with VSV envelope transduced 920 RLU/μl.

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24. Presence of helper virus and transfer of the HIV *tat* gene were measured by inoculating HeLa P4.2 cells with HIV vector, pseudotyped or not with MLV (Ampho) envelope, and staining with X-Gal after 48 hours. P4.2 cells express CD4 and contain an integrated *lacZ* reporter gene driven by the HIV LTR [P. Charneau *et al.*, *J. Mol. Biol.* **241**, 651 (1994)]. Positive scoring indicated expression of the *tat* gene. The detection limit of the assay was 20 *tat*–transducing units per milliliter of test medium. For comparison, envelope-defective HIV viruses, pseudotyped or not with MLV (Ampho) envelope, were generated by transfecting plasmid pΔER9 in 293T cells. When normalized for p24 Gag antigen, MLV (Ampho)–pseudotyped HIV virus transduced Tat with an efficiency of 630 TU per nanogram of p24 Gag antigen, nonpseudotyped envelope-defective virus had a barely detectable activity of 0.023 TU per nanogram of p24, and no transduction of Tat was detected with a maximal dose tested of HIV vector corresponding to 1.2 μg of p24 Gag antigen, either with or without envelope. On the other hand, when assayed for the transduction of β-gal into naïve HeLa cells, HIV vector pseudotyped with MLV (Ampho) envelope had an average efficiency of 115 TU per nanogram of p24 and of 940 TU/ng when pseudotyped with VSV G. Transfer of the HIV *gag* gene was assayed by measuring p24 Gag antigen in extracts of HeLa cultures serially passaged after infection with the viral equivalent of 1 μg of p24 of both MLV (Ampho)–pseudotyped HIV vector and virus. The detection limit was ≥1 pg per milliliter of extract. Cells infected with envelope-defective, pseudotyped HIV virus consistently gave readings above 20 ng/ml. No Gag antigen was detected in extracts of vector-transduced cells. The same held true when HeLa cells transduced with the pHR′-LacZ vector were selected for β-gal expression by live fluorescence-activated cell sorting after fluorescein-di-β-D-galactopyranoside (FDG) staining [G. P. Nolan, S. Fiering, J.-F. Nicolas, L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2603 (1988)]. More than 65% of selected cells expressed the marker gene and were serially passaged. Their supernatant scored negative both for p24 Gag content and for transfer of the *lacZ* gene to 208F fibroblasts.
25. For infection of growing cultures, HeLa cells (American Type Culture Collection) were seeded at 1.6 × 10^5 cells per well in a six-well tray the day before infection. G_1-S–arrested cultures were prepared by seeding 2 × 10^5 cells per well 2 days before infection and adding aphidicolin (15 μg/ml) 24 hours before infection and then daily throughout the experiment. Aphidicolin inhibits DNA polymerases α and δ [J. A. Huberman, *Cell* **23**, 647 (1981); J. J. Byrnes, *Mol. Cell. Biochem.* **62**, 13 (1984)], thereby arresting cells in the G_1-S phase of the cycle. G_2-arrested cells were exposed for 20 min to a ^{61}Co source calibrated at 2 grays per minute (1 gray = 100 rads) 1 day before infection and seeded at 4 × 10^5 cells per well. The gamma radiation induces double-strand DNA breaks, thus blocking cells in G_2 [M. B. Kastan *et al.*, *Cell* **71**, 587 (1992)]. Similar results were obtained for all MOIs tested (from 0.001 to 1) and for the transduction of luciferase. Virtually all cells in a well could be transduced with MOI > 1 both when growing and when growth-arrested.
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28. Mutant packaging plasmids were constructed by substituting in pCMVΔR9 a Bcl I–Sal I cassette containing the mutations from plasmids pΔINR8 (*9*) and pHIV-Hygro-D64V (*27*). Although residual transducing activity was scored for the vector assembled from the packaging plasmid carrying the D64V integrase mutation, β-gal–positive cells showed on average significantly weaker staining than those transduced by the wild-type vector and were apparently unable to form foci of stably transduced cells. This is also consistent with the residual activity observed for the D64V integrase mutation in the context of the HIV-1 genome (*27*) and probably reflects expression from episomal DNA.
29. The fraction of cells in S phase was 40 to 50% in growing cells, and from 10 down to 2% after reaching confluence, depending on the elapsed time, as assayed by propidium iodide staining and flow cytometry (*18*).
30. Entry of the HIV-based vector in cultures of 208F cells growing and growth-arrested for 21 days was assayed by measuring the envelope-dependent uptake of p24 Gag protein. Cultures were incubated overnight with 33 ng of p24 Gag antigen of HIV-based vector either pseudotyped or not, washed, trypsinized, and extracted for measuring p24 content by ELISA. Growing cells contained 553 ± 50 pg of p24 after incubation with pseudotyped vector, and 100 ± 25 pg of p24 after incubation with particles with no envelope; G_0 cells contained 592 ± 120 pg of p24 after incubation with pseudotyped vector, and 90 ± 15 pg of p24 after incubation with particles with no envelope.
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33. Peripheral blood monocytes were prepared from the buffy coats of healthy donors as previously described (*8*) and cultured in RPMI containing 10% human serum for 2 to 4 weeks before infection. Cultures were infected without or with HIV-based and MLV-based luciferase vectors pseudotyped or not with VSV envelope. For the HIV-based vector, 150 ng of p24 equivalent were used per each inoculum.
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35. Mutant packaging plasmids were constructed by substituting in pCMVΔR9 either or both of a Bcl I–Sal I cassette from plasmid pΔVprR8, whose *vpr* gene has a truncated reading frame, and a Cla I–Bcl I cassette from $pMA_{KK}27_{TT}R7$, in which two threonines (T) replace two lysines (K) in the NLS of MA. The construction and biological properties of these mutants have been described (*8*, *9*). Occasionally, mutant vectors showed a less pronounced phenotype in the macrophages from one donor, perhaps because of variation in the state of growth arrest of the cells. High-pressure liquid chromatography (HPLC)–purified peptides had the following sequence: PKKKRKVEDPYC (NLS peptide) and PDEVKRKKKPYC (reverse). Abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; K, Lys; P, Pro; R, Arg; V, Val; and Y, Tyr.
36. The details of vector preparation will be published elsewhere (L. Naldini *et al.*, in preparation). After concentration by ultracentrifugation, titers of 1 × 10^6 to 3 × 10^8 TU/ml on 208F cells were obtained. All animal procedures were performed according to institution-approved protocols and in a biosafety level 3 environment. Adult female Fischer 344 rats were anesthetized [ketamine (44 mg per kilogram of body weight), acepromazine (0.75 mg/kg), and xylazine (4 mg/kg) in 0.9% NaCl, intraperitoneally], positioned in a stereotactic head frame, and slowly injected with 2 μl of vector stock into the striatum [anteroposterior (AP), +0.2; mediolateral (ML), ±3.5; dorsoventral (DV), −4.5] and hippocampus (AP, −3.5, ML, 3.0; DV, −4.0) bilaterally. Seven or 30 days after injection the rats were deeply anesthetized and perfused with 4% cold paraformaldehyde and 0.2% glutaraldehyde intracardially. The brains were removed, postfixed 24 hours, saturated in 30% sucrose, and sectioned on a freezing microtome (40-μm sections). Light microscopy sections were stained with avidin-biotin peroxidase (Vectastain Elit, Vector Labs) and diaminobenzidine. Immunofluorescence triple labeling was conducted with rabbit antibody to β-gal (anti–β-gal) (1:1000, Cortex), mouse monoclonal anti-NeuN (1:4), and guinea pig anti-GFAP (1:250, Advanced Immunochemical). Secondary antibodies coupled to fluorescent markers CY5, dichlorotriazinyl amino fluorescein, and Texas Red were used at 1:250 dilution. Slices were mounted with diazobicycloоctane/polyvinyl alcohol mounting medium and analyzed by confocal scanning laser microscopy (Bio-Rad MRTC600). Fluorescent signals were collected, digitally color-enhanced, and superimposed. False-color images were generated electronically with Adobe Photoshop (Adobe System).
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39. For the polymerase chain reaction (PCR) assay, cultures were incubated with vector concentrated by ultracentrifugation and pretreated with deoxyribonuclease I (DNase I) (20 μg/ml for 2 hours at 37°C), washed, trypsinized, and extracted for PCR as previously described (*9*). To adjust for the different content of cellular DNA in growing and confluent cultures, each PCR reaction contained an equal volume (2 μl for EL and LL, 7.5 μl for Cl) of both growing and G_0-arrested cell extract, either one of which had been infected. The sequence of HIV-specific primers are as follows [positions of nucleotides in the $HIV\text{-}1_{HXB2D}$ sequence, according to L. Ratner *et al.*, *Nature* **313**, 277 (1985), are indicated in parentheses]. LTR5: GGCTAACTAGGGAACCCACTGCTT (496 to 516); LTR6: CTGCTAGAGATTTTCCACACTGAC (635 to 612); 5NC2: CCGAGTCCTGCGTCGAGAGAGC (698 to 677); LTR8: TCCCAGGCTCAGATCTGGTCTAAC (488 to 465 and 9572 to 9549); and LTR9: GCCTCAATAAAGCTTGCCTTG (522 to 542 and 9606 to 9626). LTR5 plus LTR6 amplifies minus-strand strong stop DNA, LTR5 plus 5NC2 amplifies double-stranded molecules generated after the second template switch, and LTR8 plus LTR9 amplifies two LTR circles. A series of logarithmic dilutions of pHR′ plasmid used as a template showed linearity of the PCR reaction over the early time points.
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