

Development of a Self-Inactivating Lentivirus Vector

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We have constructed a new series of lentivirus vectors based on human immunodeficiency virus type 1 (HIV-1) that can transduce nondividing cells. The U3 region of the 5' long terminal repeat (LTR) in vector constructs was replaced with the cytomegalovirus (CMV) promoter, resulting in Tat-independent transcription but still maintaining high levels of expression. A self-inactivating (SIN) vector was constructed by deleting 133 bp in the U3 region of the 3' LTR, including the TATA box and binding sites for transcription factors Sp1 and NF- κ B. The deletion is transferred to the 5' LTR after reverse transcription and integration in infected cells, resulting in the transcriptional inactivation of the LTR in the proviruses. SIN viruses can be generated with no significant decreases in titer. Injection of viruses into the rat brain showed that a SIN vector containing the green fluorescent protein gene under the control of the internal CMV promoter transduced neurons as efficiently as a wild-type vector. Interestingly, a wild-type vector without an internal promoter also successfully transduced neurons in the brain, indicating that the HIV-1 LTR promoter is transcriptionally active in neurons even in the absence of Tat. Furthermore, injection of viruses into the subretinal space of the rat eye showed that wild-type vector transduced predominantly retinal pigment epithelium and photoreceptor cells, while SIN vector was able to transduce other types of retinal cells, including bipolar, Müller, horizontal, and amacrine cells. This finding suggests that the HIV-1 LTR can negatively influence the internal CMV promoter in some cell types. SIN HIV vectors should be safer for gene therapy, and they also have broader applicability as a means of high-level gene transfer and expression in nondividing cells.

Gene therapy approaches rely on efficient transfer of genes to the desired target cells (for reviews, see references 12, 32, 34, 35, and 48). A wide variety of viral and nonviral vectors have been developed and evaluated for their efficiency of transduction, sustained expression of the transgene, and safety. Among them, retrovirus vectors derived from oncoretroviruses such as murine leukemia virus (MLV) have been the most widely used for gene therapy applications. However, a major problem with these retrovirus vectors is the requirement for proliferation of the target cells for integration, limiting their use for gene transfer into nondividing cells such as hepatocytes, myoblasts, neurons, and hematopoietic stem cells. In contrast, lentiviruses such as human immunodeficiency virus type 1 (HIV-1) can infect nondividing cells (7, 30, 49).

We have recently developed a lentivirus vector based on HIV-1 that can transduce nondividing cells *in vitro* and *in vivo* (38). These HIV vectors are pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G); hence they can transduce a broad range of tissues and can be concentrated to high titers. We have shown that HIV vectors can stably integrate into the host cell genome and obtained long-term expression of transgenes in brain, liver, muscle, and retina (4, 24, 33, 38, 39). No cellular immune response can be detected at the site of injection. Furthermore, second injection of the HIV vector

into the animals is possible, indicating the lack of any potent humoral immune response to the vector (24).

Although HIV vectors promise great utility for gene therapy, there is concern about their safety since HIV-1 is the etiologic agent of AIDS. The major safety concern is the generation of replication-competent virus during the production of vectors. In this regard, we have minimized the possibility for generating replication-competent virus through recombination by using a three-plasmid expression system which consists of packaging, envelope, and vector constructs (38, 39). Furthermore, recent studies have demonstrated the possibility of eliminating all accessory genes (*vif*, *vpr*, *vpu*, and *nef*) from a packaging construct without losing the ability to transduce nondividing cells (24, 26, 43, 56).

Another safety concern about HIV vectors, as for MLV-based vectors, is the possibility of insertional activation of cellular oncogenes by random integration of the vector provirus into the host genome. To overcome this problem, we have constructed a self-inactivating (SIN) vector in which the viral enhancer and promoter sequences have been deleted. In this report, we show that SIN vectors can be generated and transduce nondividing cells *in vivo* with an efficacy similar to that of wild-type vectors. The transcriptional inactivation of the long terminal repeat (LTR) in the SIN provirus should prevent mobilization by replication-competent virus. This should also enable the regulated expression of genes from internal promoters by eliminating any *cis*-acting effects of the LTR. A further modification has been made in the vector construct in which the U3 region of the 5' LTR has been replaced with the cytomegalovirus (CMV) promoter, resulting in Tat-independent transcription with no decreases in viral titer. SIN vectors combined with this hybrid 5' LTR further reduce the possibility of recombination to generate replication-competent virus because there is no complete U3 sequence in the virus pro-

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duction system. These modifications add additional safety features to the HIV-1-based lentivirus vector system.

MATERIALS AND METHODS

Plasmid constructions and preparation of viral stocks. Plasmid pHR'-MCS was constructed by replacing a 420-bp *Bam*HI-*Xho*I fragment of pHR' (38) with a fragment of multiple cloning sites containing *Bam*HI, *Eco*47III, *Bst*XI, *Sac*II, *Pst*I, *Eco*RI, *Cla*I, *Xba*I, *Hpa*I, and *Xho*I sites. To restructure the HIV-1 LTR and generate the SIN LTR, PCR amplification products of pHR'-MCS, an *Mlu*I-*Bsp*EI (-454 to -145) and a *Bsp*EI-*Apa*I (-144 to +180) fragment or an *Mlu*I-*Bsp*EI (-454 to -145) and a *Bsp*EI-*Apa*I (-8 to +180) fragment, were cloned into pcDNA3.1/Zeo(+) (Invitrogen) between the *Mlu*I and *Apa*I sites to generate pHIV-LTR or pHIV-SIN, respectively (numbers in parentheses indicate positions relative to the transcriptional start site). To construct HIV vectors pLL and pLS, a 2.3-kb *Bsp*EI fragment of pHR'-MCS was cloned into the *Bsp*EI sites of pHIV-LTR and pHIV-SIN, respectively. pLS has a 133-bp deletion (-141 to -9) in the 3' LTR. pHIV- Δ U3L and pHIV- Δ U3S were constructed by replacing a *Mlu*I-*Kas*I (-454 to +183) fragment of pLL and pLS, respectively, with a *Mlu*I-*Kas*I PCR amplification product (-13 to +183) to delete the U3 region of the 5' LTR. To generate the hybrid 5' LTR with the CMV promoter, pCL and pCS were constructed by inserting a *Pvu*I-*Sac*I fragment containing the CMV promoter from pcDNA3.1/Zeo(+) into the *Pvu*I and *Mlu*I sites of pHIV- Δ U3L and pHIV- Δ U3S, respectively. The hybrid 5' LTR maintains the 23-bp distance between the TATA box of CMV promoter and the transcription start site in HIV-1 LTR. pLL-CG, pLS-CG, pCL-CG, and pCS-CG were constructed by inserting a 1.3-kb *Bam*HI-*Xho*I fragment of pKS-CMV-GFP (33) containing the CMV-green fluorescent protein (GFP) expression cassette into the same sites of pLL, pLS, pCL, and pCS, respectively. pLL-G, pCL-G, and pCS-G were constructed by deleting the CMV promoter from pLL-CG, pCL-CG, and pCS-CG, respectively.

The VSV-G-pseudotyped HIV vectors were generated by transient cotransfection of a vector construct (15 μ g) with the VSV-G-expressing construct pMD.G (5 μ g) and the packaging construct pCMV Δ R8.2 (10 μ g) into 293T cells as previously described (39). High-titer stocks of HIV vectors were prepared by ultracentrifugation. The titers of viruses were determined by infection of 293T cells or HeLa-CD4-LTR- β -gal cells (27), seeded in six-well plates at 10^5 cells/well the day before infection, with serial dilutions of the vector stocks. After overnight incubation, the cell culture medium was changed; 48 h later, the number of GFP-positive cells was scored to quantify the titer. The vector stocks were assayed for p24 antigen levels by using an HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (DuPont NEN). After concentration by ultracentrifugation, titers of 1×10^9 to 2×10^9 transducing units (TU)/ml were usually obtained. Absence of replication-competent virus was determined by the marker rescue assay and measuring p24 antigen level by ELISA as described elsewhere (39).

Southern and Northern blot analyses. Genomic DNA was isolated from cells infected with virus by the procedure of Wu et al. (51). DNA (10 μ g) was digested with the restriction enzymes *Bsp*EI and *Bam*HI, separated on a 0.7% agarose gel, and transferred to a Hybond-N+ membrane (Amersham) in $20\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Total cellular RNA was isolated from virus-infected cells by using an RNAsqueous phenol-free total-RNA isolation kit (Ambion). RNA (10 μ g) was separated on a 1% agarose-formaldehyde gel and transferred to a Hybond-N+ membrane in 25 mM potassium phosphate (pH 6.5). Membranes were UV irradiated with a UV cross-linker (Hofer), hybridized with 32 P-labeled probes generated by a Megaprime DNA labeling kit (Amersham) in Rapid-hyb buffer (Amersham) for 2 h at 65°C, washed twice in $0.1\times$ SSC-0.1% sodium dodecyl sulfate for 15 min at 65°C, and exposed to an X-Omat AR X-ray film (Kodak). The BN and GFP probes were isolated from pCL-CG as 0.43-kb *Bss*HIII-*Not*I and 0.75-kb *Eco*47III-*Xho*I fragments, respectively.

In vivo experiments. High-titer stocks of HIV vectors (1×10^9 to 2×10^9 TU/ml) were used to inject the brain and eye. The titers of CL-G and CS-G vectors were normalized for HIV-1 p24 antigen levels. Adult female Fischer 344 rats were anesthetized by intramuscular injection of ketamine-acepromazine-xylazine. Three microliters of vector stock (3×10^6 TU) was injected into the striatum and the hippocampus bilaterally ($n = 4$ for each vector) with a 5- μ l Hamilton syringe. Rats were sacrificed at 2 and 6 weeks postinjection.

Fischer 344 rat pups (age 2 to 5 days) were anesthetized by chilling on ice for 5 min. The eyeball was exposed by an incision in the eyelid, parallel to the edge of the open eyelid. Subretinal injections were performed under an operating microscope. A 0.5- μ l volume of vector stock (10^6 TU) was injected into the subretinal space ($n = 8$ for each vector), using a glass capillary connected with 5- μ l Hamilton syringe. Rats were sacrificed at 6 and 12 weeks postinjection.

Rats were intracardially perfused with cold fixing solution (4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer). The tissues were removed, further fixed in the fixing solution, and cryoprotected in 30% sucrose at 4°C overnight. The tissues were then frozen in Tissue-Tek O.C.T. compound (Sakura Finetek) on dry ice and sectioned on a sliding microtome (50- μ m-thick brain sections) or a cryostat (20- μ m-thick eye sections). Immunofluorescence staining was performed as previously described (4). The sections were analyzed by confocal laser scanning microscopy (Bio-Rad). The signals were collected, digitally color enhanced, and superimposed.

RESULTS

Construction and generation of modified HIV vectors. In the life cycle of retroviruses, the U3 region of the 3' LTR is duplicated to form the corresponding region of the 5' LTR during the process of reverse transcription and viral DNA synthesis in infected cells. This mechanism of viral replication allowed us to make two principal modifications to the HIV vector, as shown in Fig. 1. First, the U3 region of the 5' LTR was replaced with the CMV promoter. Second, 133 bp in the U3 region of the 3' LTR, which contains the TATA box and binding sites for transcription factors Sp1 and NF- κ B, were deleted. This deletion will be transferred to the 5' LTR after reverse transcription. Consequently, the transcriptional unit from the LTRs in a provirus is eliminated. This type of vector is called a SIN vector (54).

A series of vectors termed LL-CG, LS-CG, CL-CG, and CS-CG (Fig. 1) were constructed to determine whether these modifications would alter the titer of virus generated from the vectors. All vectors contain the GFP gene as a reporter with an internal CMV promoter. CL-CG and CS-CG vectors contain the CMV promoter in place of the U3 region of the 5' LTR. The virus generated from CL-CG and CS-CG should contain the same RNA genome as the virus from LL-CG and LS-CG, respectively (Fig. 1). We also constructed CL-G and CS-G vectors without an internal promoter, in which the GFP gene is under the control of the 5' LTR promoter.

VSV-G-pseudotyped vectors were generated by transient cotransfection of each vector construct with a VSV-G expression construct and a packaging construct into 293T cells. The vector virus was harvested 62 h after transfection and used to infect 293T cells to determine the viral titer by quantitation of the number of GFP-positive cells. The LL-CG vector yielded a mean titer of 6×10^5 TU/ml. As shown in Table 1, replacement of the U3 region of the 5' LTR with the CMV promoter did not reduce the viral titers. The titers of SIN vectors LS-CG and CS-CG were approximately the same as those of their wild-type counterparts, LL-CG and CL-CG, respectively. These results indicate that the small deletion in the U3 region in SIN vectors did not significantly affect titers. Lower titers of CL-G may reflect the relative inefficiency of the LTR promoter because of the absence of the *trans* activator Tat in the infected cells. A few cells were scored positive for GFP with the CS-G vector, probably due to integration events near host genome promoters since there is no promoter in the provirus. To measure viral production with CL-G and CS-G vectors, the levels of HIV-1 p24 antigen in the supernatants were measured, as these correlate with the amount of virions. The levels of p24 obtained with the CL-G and CS-G vectors were roughly equivalent to those obtained with other vectors, suggesting approximately similar levels of virus production with all vectors (Table 1). However, we cannot rule out the possibility of generating virions lacking viral genome RNA with this assay.

Expression from the hybrid 5' LTR with the CMV promoter is Tat independent. It has been shown that Tat *trans* activation of the HIV-1 LTR requires not only the Tat-responsive region (TAR) in the R region of the LTR but also the TATA box and binding sites for Sp1 and NF- κ B (2, 3, 17, 22, 31, 36, 41, 55). To verify that the hybrid CMV-LTR promoter is Tat independent, the promoter activity of the CL-G vector construct was compared with that of construct LL-G, in which the internal CMV promoter was deleted from LL-CG and therefore the GFP gene was expressed under the control of wild-type 5' LTR. These constructs were transfected into 293T or HeLa cells with either a Tat expression plasmid or a control plasmid, and levels of GFP in cell extracts were measured 48 h later by fluores-

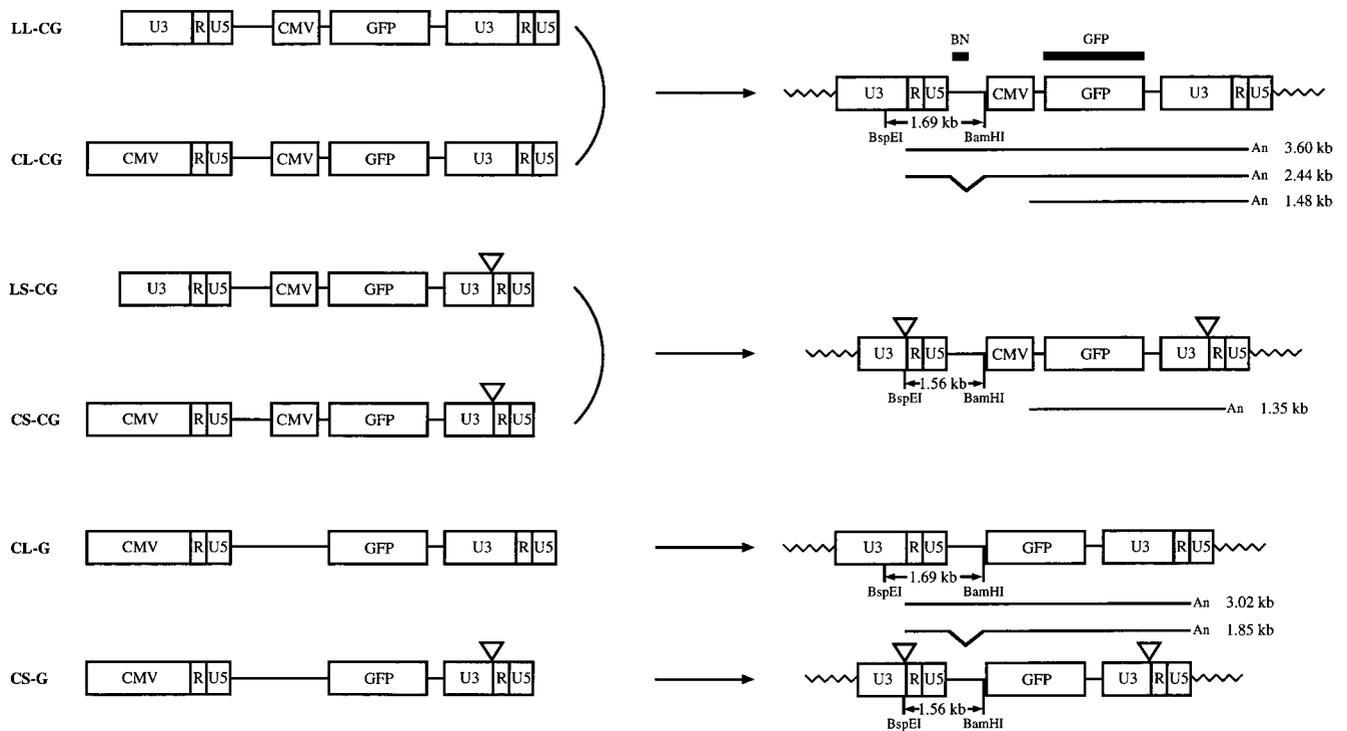


FIG. 1. Structures of HIV vector constructs and corresponding proviruses. (Left) HIV vector constructs. Each vector construct is cotransfected with the packaging and VSV-G expression constructs into 293T cells. Viral transcription initiates at the U3/R border in the 5' LTR and terminates at the R/U5 border in the 3' LTR. The viral RNA is packaged into virions. Virus is harvested and used to infect target cells. Triangles represent deleted U3 region. (Right) Structures of integrated proviruses. In infected cells, the U3 region of the 3' LTR is used as a template for the synthesis of the U3 region in both LTRs during the process of reverse transcription of the viral RNA into double-stranded DNA. As a result, the U3 region of the 3' LTR is duplicated and transferred to the 5' LTR in the integrated provirus. The *BspEI*-*Bam*HI restriction fragments expected in Southern blot analysis (Fig. 2) and the RNA transcripts expected in Northern blot analysis (Fig. 3) are shown below each proviral structure with their sizes. The locations of the BN and GFP probes used in Southern blot and Northern blot analyses are also indicated.

cence spectroscopy (40). Table 2 shows that the GFP expression in cells transfected with LL-G was increased three- to sixfold in the presence of Tat. The low level of *trans* activation by Tat in 293T cells is probably caused by the higher level of expression from the LTR due to *trans* activation by the adenovirus E1A gene product expressed in 293T cells (37). On the other hand, CL-G was not responsive to Tat, though levels of expression were similar to those obtained with LL-G in the presence of Tat. Thus, the replacement of U3 region of the 5' LTR with the CMV promoter resulted in loss of Tat responsiveness, but the basal activity was comparable to that of wild-type LTR in the presence of Tat in 293T and HeLa cells.

Characterization of proviral structure and transcription. The structures of proviruses were characterized by Southern blot analysis. HeLa-CD4-LTR- β -gal cells (27) were infected with each vector and cultured for 36 days with 11 passages. Genomic DNA was isolated from infected cells, digested with the restriction enzymes *BspEI* and *Bam*HI, and hybridized with a BN probe (Fig. 2). The predicted structures of proviruses are shown in Fig. 1. *BspEI* cleaves once in each LTR, immediately upstream of the deleted region in SIN vectors, and *Bam*HI cleaves once downstream of the 5' LTR. Therefore, a 1.69-kb *BspEI*-*Bam*HI fragment should be generated from provirus containing wild-type 5' LTR when hybridized with the BN probe. If the 133-bp deletion present in the 3' LTR of SIN vector is transferred to the 5' LTR of the integrated provirus, a 1.56-kb *BspEI*-*Bam*HI fragment should be generated in place of the 1.69-kb fragment. As shown in Fig. 2, the expected fragments were generated from each provirus. Analysis of infected 293T cells yielded identical results (data not shown).

We next analyzed the transcriptional activities of proviruses. Total cellular RNA was isolated from HeLa-CD4-LTR- β -gal cells infected with each virus described above, and Northern blot analysis was performed with the GFP gene as a probe. As shown in Fig. 3, expression of the expected transcripts depicted in Fig. 1 was observed for each of the proviruses. In the provirus derived from the LL-CG or CL-CG vector, three transcripts were produced: two viral transcripts initiated in the 5' LTR, a 3.60-kb full-length form and a 2.44-kb spliced form; and a 1.48-kb transcript initiated in the internal CMV promoter. All transcripts terminated at the polyadenylation signal in the R region of the 3' LTR. The full-length and spliced forms were confirmed by using the BN probe, which detects only the full-length form (data not shown; see Fig. 1 for structures). The level of transcripts expressed from the internal CMV promoter is higher than that of transcripts initiated in

TABLE 1. Titers of HIV vectors

Vector	Titer on 293T cells (TU/ml) ^a	Relative p24 levels ^b
LL-CG	5.7×10^5	1.00
CL-CG	9.3×10^5	1.42
LS-CG	5.0×10^5	0.56
CS-CG	8.2×10^5	1.39
CL-G	2.1×10^4	1.51
CS-G	<10	1.26

^a Average of at least three independent experiments.

^b Relative to the p24 level of the LL-CG vector.

TABLE 2. Transcriptional activities of LL-G and CL-G constructs^a

Construct	Tat	Relative GFP expression	
		293T	HeLa
pLL-G	-	1.0	1.0
	+	3.0	6.2
pCL-G	-	4.9	7.3
	+	3.9	9.4

^a 293T or HeLa cells were seeded at a concentration 2.5×10^5 /six-well dish and transfected on the following day with 0.5 μ g of vector construct together with 0.5 μ g of the Tat expression plasmid pSV2 tat72 (16) or the control plasmid pBluescript II (Stratagene), using Lipofectamine (GibcoBRL). Cells were harvested 48 h after transfection, and the levels of GFP were measured by fluorescence spectroscopy. The results are expressed relative to the GFP level of pLL-G in the absence of Tat and are means of triplicate transfections.

the 5' LTR. In the case of SIN vectors LS-CG and CS-CG, transcripts expressed from the 5' LTR were undetectable and a 1.35-kb transcript expressed from the internal promoter was detected. This transcript is shorter than the corresponding transcript from the LL-CG or CL-CG provirus because of the 133-bp deletion in the U3 region of the 3' LTR. The provirus derived from the CL-G vector produced detectable levels of the full-length and spliced viral transcripts. As expected, no RNA transcripts were detected in the cells infected with the CS-G vector. The same results were obtained with 293T cells infected with each virus (data not shown).

Rescue of virus from cells infected with SIN vectors. To determine if there was any transcriptional activity from the 5' LTR of the SIN provirus, we used a more sensitive virus rescue assay. The VSV-G-expressing construct and the packaging construct were transfected into 293T cells previously infected with SIN vectors. If RNA was initiated in the 5' LTR of the SIN provirus, it would be packaged into viral particles and released into the medium. Such virus would then be detected by infection of virginal 293T cells. The results showed that 3 to 8 TU of virus per ml was rescued from cells previously infected with the LS-CG or CS-CG vector, whereas 5×10^4 to 8×10^4 TU/ml was rescued from cells infected with the LL-CG or CL-CG vector (Table 3). It is unlikely that the rescued virus arose through recombination during transfection and regenerated a functional 5' LTR because there is no complete U3 sequence, especially in the case of CS-CG. In fact, the virus preparations used for infection were shown to be free of rep-

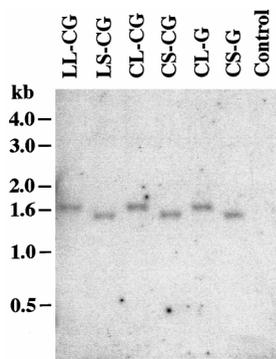


FIG. 2. Southern blot analysis of the integrated proviral structure. Genomic DNA isolated from HeLa-CD4-LTR- β -gal cells infected with HIV vectors was digested with *Bsp*EI and *Bam*HI. The blot was hybridized with the BN probe. The vector used for infection is indicated above each lane. Control, uninfected HeLa-CD4-LTR- β -gal cells. The expected sizes of fragments are shown in Fig. 1. Size markers are indicated on the left.

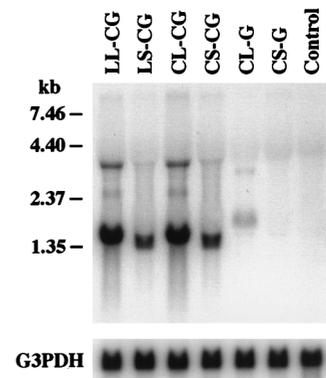


FIG. 3. Northern blot analysis of the proviral expression. Total cellular RNA was isolated from HeLa-CD4-LTR- β -gal cells infected with HIV vectors. The blot was hybridized with the GFP probe and rehybridized with the human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe (Clontech). The vector used for infection is indicated above each lane. Control, uninfected HeLa-CD4-LTR- β -gal cells. The expected size of each transcript is shown in Fig. 1. Size markers are indicated on the left.

lication-competent virus by the marker rescue assay (39). Viruses were able to be rescued presumably because SIN vectors integrated near an active cellular promoter and expressed the proviral genome, as seen with the CS-G vector (Table 1). On the basis of Northern blot analysis and virus rescue, it is apparent that effective transcription from the 5' LTR of the SIN provirus was inactivated.

In vivo delivery of modified vectors. To transduce terminally differentiated neurons in vivo, high-titer stocks (1×10^9 to 2×10^9 TU/ml) of the CS-CG and CL-CG vectors were injected into the striatum and hippocampus of adult rat brains. The CL-G and CS-G vectors, normalized for equal amounts of p24 antigen, were also injected to detect the expression from the 5' LTR of wild-type and SIN vectors. At 2 and 6 weeks postinjection, the rats were sacrificed and the brains were cryosectioned and analyzed for GFP expression by fluorescence microscopy. As shown in Fig. 4, the CS-CG vector was found to transduce at efficiencies comparable to those of the CL-CG vector. Interestingly, the CL-G vector gave similar transduction efficiencies as well. This observation is consistent with previous studies showing high levels of expression from the HIV-1 LTR through constitutively active NF- κ B in neurons (10, 25, 42). In brains injected with the CS-G vector, very few GFP-positive cells could be detected, and only at higher magnification. This was most likely due to proviral integration near a cellular promoter and expression of GFP, as seen in infection in vitro. There were no significant differences in the GFP

TABLE 3. Titers of viruses rescued by transient transfection of packaging and VSV-G constructs^a

Vector	Titer on 293T cells (TU/ml)	
	Expt 1	Expt 2
LL-CG	7×10^4	5×10^4
CL-CG	8×10^4	7×10^4
LS-CG	3	8
CS-CG	3	3

^a The VSV-G expression construct pMD.G (5 μ g) and packaging construct pCMV Δ R8.2 (15 μ g) were transfected into 293T cells previously infected with each vector. Virus stocks were prepared 48 h after transfection and used to infect 293T cells. Titers were measured by counting the number of GFP-positive cells 3 days after infection.

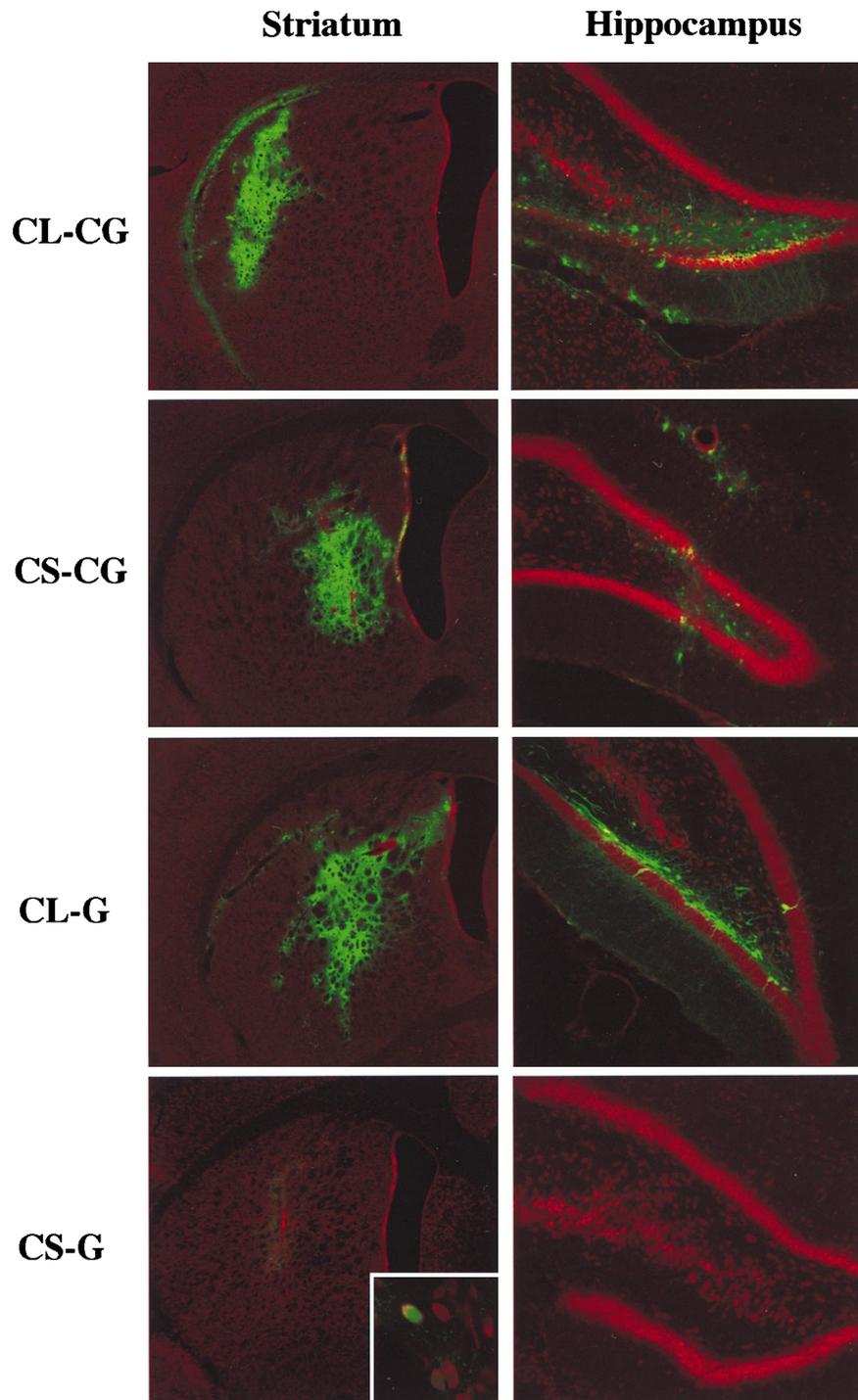


FIG. 4. Expression of GFP in the striatum and hippocampus of adult rat brains 6 weeks after injection of HIV vectors. Sections were counterstained with propidium iodide. The vectors used for injection are indicated on the left. Inset, higher magnification of a GFP-positive cell.

transduction frequencies of the vectors between 2 and 6 weeks postinjection (data not shown). The nature of transduced cells was determined by immunofluorescence staining with three markers: NeuN for neurons, glial fibrillary acidic protein for astrocytes, and RIP for oligodendrocytes. The results showed that the majority of cells transduced with each vector were terminally differentiated neurons expressing NeuN, consistent

with our previous observation (4, 38, 39). Additional immunofluorescence staining showed that cells transduced with the CS-CG vector were frequently labeled with choline acetyltransferase, which is a marker of cholinergic neurons (data not shown). In contrast, the CL-CG vector-transduced cells were rarely labeled with this marker.

The CL-CG and CS-CG vectors were also injected into the

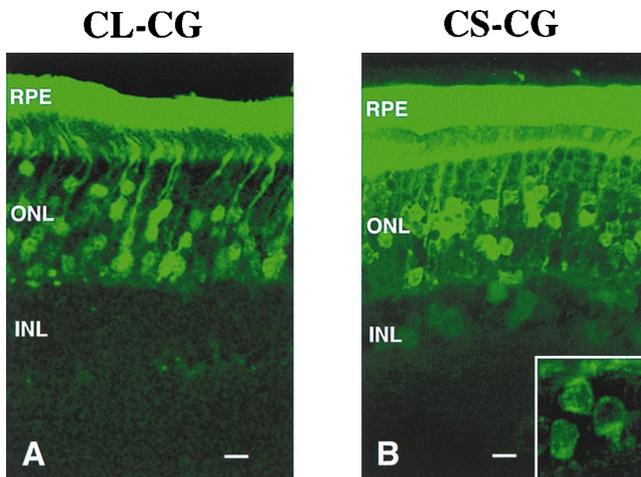


FIG. 5. Expression of GFP in the retina of rat pups 12 weeks after injection of the CL-CG vector (A) and the CS-CG vector (B). Inset, higher magnification of GFP-positive inner nuclear layer (INL) cells. Scale bars, 10 μ m. RPE, retinal pigment epithelium; ONL, outer nuclear layer.

subretinal space of rat eyes, and transduction was evaluated 6 and 12 weeks later. Retinal cell types can be readily identified by their positions in the different retinal layers and by their distinctive morphologies. In the eyes injected with the wild-type vector CL-CG, GFP was predominantly expressed in retinal pigment epithelium and photoreceptor cells (Fig. 5A). On the other hand, the SIN vector CS-CG efficiently transduced not only retinal pigment epithelium and photoreceptor cells but also other retinal cells in inner nuclear layer, including bipolar, Müller, horizontal, and amacrine cells (Fig. 5B). This result together with that for the brain suggests that transcription from the HIV-1 LTR may have inhibitory effects on transcription from the internal promoter in some cell types. There were no significant differences in the patterns and transduction frequencies of the vectors between 6 and 12 weeks postinjection (data not shown).

DISCUSSION

HIV-1, unlike other retroviruses such as MLV, is characterized by a complex genome that encodes two regulatory proteins (Tat and Rev) and four accessory proteins (Vif, Vpr, Vpu, and Nef) in addition to the common *gag*, *pol*, and *env* gene products. One of the accessory proteins, Tat, is an essential nuclear protein that augments levels of viral RNA by increasing transcriptional initiation and/or elongation (reviewed in reference 23). To manifest its function, Tat binds to a nascent RNA stem-loop structure of TAR, located at the 5' end of all viral transcripts. Tat *trans* activation has also been reported to require the TATA box and additional sequences in the 5' LTR, including binding sites for Sp1 and NF- κ B (2, 3, 17, 22, 31, 36, 41, 55). This is consistent with our result that replacement of the U3 region of the 5' LTR with the CMV promoter resulted in Tat-independent transcription (Table 2). In the absence of Tat, this hybrid CMV-LTR promoter can drive high levels of expression comparable to those of the wild-type LTR in the presence of Tat. This allows production of HIV vectors in a system devoid of Tat. Indeed, Kim et al. (26) have recently reported that an HIV vector containing a similar promoter swap could generate virus with no significant decrease in titer in the absence of Tat. Recent studies have demonstrated the possibility of eliminating all accessory genes from a packaging

construct without losing the ability to transduce nondividing cells (24, 26, 43, 56), although some accessory proteins appeared to be required for maximum transduction of specific tissues (e.g., Vpr for macrophages and Vpr and/or Vif for liver) (24, 56). Taken together, these observations suggest the possibility of eliminating all regulatory and accessory genes except *rev* from the HIV vector production system. Rev, together with the Rev response element in the vector, is required for efficient export of full-length vector transcripts to the cytoplasm. However, it may be possible to substitute Rev function by replacement with a protein serving a similar function (28).

The transcription of HIV-1 is directed by regulatory sequences in the 5' LTR. The core element in the U3 region, containing a canonical TATA box and three Sp1 binding sites, is essential for basal promoter activity and viral replication (2, 3, 17, 22, 41, 55). Immediately upstream of the Sp1 binding sites, tandem binding sites for NF- κ B constitute an activation-dependent enhancer element (31, 36). Elimination of all Sp1 and NF- κ B binding sites results in essentially total inactivation of viral replication (29, 44). Although a number of enhancer elements that modulate transcriptional activity have been identified upstream of the NF- κ B binding sites, none of them can compensate for the loss of Sp1 and NF- κ B binding sites. In the SIN HIV vector described in this study, as expected, the deletion of the TATA box and binding sites for Sp1 and NF- κ B resulted in transcriptional inactivation of the LTR in the provirus in infected cells *in vitro* and *in vivo*. A notable aspect of the expression from the HIV-1 LTR is its low basal activity in the absence of Tat. In addition, Tat is not packaged into virions. Therefore, in most cells infected with HIV vectors, low levels of expression from the LTR would be expected because of the absence of Tat. However, we have demonstrated high-level expression from the LTR in the brain (Fig. 4), consistent with the finding that transgenic mice expressed an HIV-1 LTR-driven reporter gene at high levels in the brain (10). It has been reported that strong HIV-1 LTR promoter activity in neurons is mainly due to the presence of constitutively active NF- κ B (25, 42). One safety concern about HIV vectors is the activation of proto-oncogenes through the LTR resulting from random integration of provirus into the host genome. In this regard, the SIN vector should reduce the potential of insertional activation. The transcriptional inactivation of the LTR in the SIN provirus should also prevent mobilization by replication-competent virus and minimize any risk of vector virus spread. It can also be argued that the rearranged viruses may arise through recombination during transfection and regenerate a wild-type LTR. Since SIN vectors combined with the hybrid CMV-LTR promoter (e.g., CS-CG) contain a single U3 region with a deletion, there is no complete U3 sequence in the virus production system: recombination to regenerate a wild-type U3 is not possible with such vectors. Furthermore, this combined modification minimizes the risk of recombination to generate replication-competent virus, although we have not detected replication-competent virus so far.

The application of MLV-based SIN vectors has been limited, since deletion of the TATA box resulted in low titers (9, 20, 21, 45, 53, 54). This is probably because the region of the TATA box in the 3' LTR plays an important role in processing of the 3' end of the viral RNA through secondary-structure interactions. Therefore, most SIN MLV vectors had a deletion only in the enhancer region, and transcriptional inactivation of the LTR was relatively inefficient (9, 20, 45, 54). In HIV-1, sequences within the U3 region, especially between the TATA box and the transcription initiation site, have been shown to be required for efficient 3' end processing (6, 8, 13, 18, 46, 47). These sequences direct the stable binding of cleavage and

polyadenylation specificity factor, the factor responsible for recognition of the AAUAAA hexamer, to the poly(A) site and enhance the efficiency of 3'-end processing (19). However, Northern blot analysis of RNA from 293T cells transfected with the SIN vector construct showed no decreased levels of transcripts (data not shown) compared with the wild-type vector, consistent with no significant reduction in viral titer (Table 1). This finding suggests that the deletion of U3 sequences in the SIN vector may not affect the efficiency of 3'-end processing.

The results obtained from *in vivo* experiments revealed that the SIN vector can improve the expression of transgene in cholinergic neurons compared to the wild-type vector. Similarly, additional retinal cell types also showed high levels of transgene expression with the SIN vector (Fig. 5). It seems likely that the expression from the internal CMV promoter is negatively influenced by expression from the HIV-1 LTR in some cell types. This phenomenon, called transcriptional interference, has been reported for other retrovirus vectors (1, 5, 11, 14, 15, 45, 50, 52). Our results suggest that deletion of the U3 region of the LTR in HIV vectors may result in efficient expression from internal promoters in some tissues. Furthermore, SIN vectors should be particularly useful to introduce tissue-specific or regulatable promoters, since the LTR can have no *cis*-acting influence on these internal promoters. SIN HIV vectors should not only increase the safety of HIV vector-mediated gene therapy but also have general utility for high-efficiency transduction of genes into nondividing cells.

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