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Self-Inactivating Lentiviral Vectors with U3 and U5 Modifications

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Lentiviral vectors have gained much attention in recent years mainly because they integrate into nondividing host-cell genomes. For clinical applications, a safe and efficient lentiviral vector system is required. Previously, we have established a human immunodeficiency virus type 1 (HIV-1)-derived three-plasmid lentiviral vector system for viral vector production which includes a packaging vector pHP, a transducing vector pTV, and an envelope-encoding plasmid pHEF-VSVG. Cotransfection of these three plasmids into TE671 human rhabdomyosarcoma cells routinely yields 10^5–10^6 infectious units per milliliter in 24 h. Here we have extensively modified long terminal repeats (LTRs) of pTV to generate a safer lentiviral vector system. The 5′ U3 was replaced with a truncated cytomegalovirus (CMV) immediate early (IE) enhancer/TATA promoter and the 3′ U3 (except for the integration attachment site) was also deleted. These modifications resulted in a vector with 80% wild-type vector efficiency. Further deletion of 3′ U5 impaired vector function; however, this problem was solved by replacing the 3′ U5 with bovine growth hormone polyadenylation (bGHpA) sequence. The pTV vector containing all these modifications including the 5′ promoter substitution, the 3′ U3 deletion, and the substitution of 3′ U5 with bGHpA exhibited a self-inactivating (SIN) phenotype after transduction, transduced both dividing and nondividing cells at similar efficiencies, and produced vector titers twice as high as that of the wild-type construct. Thus, both safety and efficacy of the HP/TV vector have been improved by these LTR modifications. Further deletion of 5′ U5 impaired vector efficiency, suggesting that the 5′ U5 has critical roles in vector function.

INTRODUCTION

Gene therapy has great potential for treating inherited and acquired diseases. Both viral and nonviral gene transfer strategies have been employed in clinical applications, although most of them are still far from perfect (Verma and Somia, 1997). Retrovirus vectors based on Moloney murine leukemia virus (MoMLV) have been very popular, especially in *ex vivo* studies, because they integrate into host genomes permitting long-term gene expression. However, retroviruses do not integrate into nondividing cells including terminally differentiated cells (Gordon and Anderson, 1994; Marin *et al.,* 1997; Miller *et al.,* 1993). This disadvantage has been overcome by deriving vectors from lentiviruses which infect both dividing and nondividing cells (Poeschla *et al.,* 1996; Verma and Somia, 1997).

Lentiviral vectors have been tested in tissue cultures and in animals and shown to effectively transduce nondividing cells including neurons, liver, retina, and muscle (Chang *et al.,* 1999; Kafri *et al.,* 1997; Miyoshi *et al.,* 1997; Naldini *et al.,* 1996a,b; Poeschla *et al.,* 1998; Zufferey *et al.,* 1997). Nevertheless, safety has been a major concern with lentiviral vectors due to the possibility of generating replication-competent pathogenic virus. Unlike MLV vectors, cell lines for packaging lentiviral vectors are difficult to establish because several lentiviral gene products are toxic to mammalian cells (Carroll *et al.,* 1994; Kaul *et al.,* 1998; Srinivasakumar *et al.,* 1997). We have adopted a DNA cotransfection method for production of lentiviral vectors (Chang *et al.,* 1999). In this system, the lentiviral genome is divided into two plasmids, HP and TV, for safety reasons. Although we did not detect any replication-competent virus (RCV) from this HP/TV vector system (Chang *et al.,* 1999), these two plasmids still contain native HIV sequences including the two LTRs in the transducing vector pTV. Therefore, the possibility of generating RCV cannot be totally ruled out. Further removal of the HIV LTR sequences from the HP/TV vector system will greatly improve its safety.

Similar to all retroviruses, lentiviral LTR contains enhancer/promoter elements, the left integration attachment site (attL) in U3, polyadenylation signal in R, and part of the polyadenylation signal and the right integration attachment site (attR) in U5. In addition, lentiviral LTR contains a Tat-interacting TAR sequence overlapping the R region, which is essential for viral replication. In the present study, we investigated the possibility of deleting most of the U3 and U5 sequences in the LTRs of pTV to

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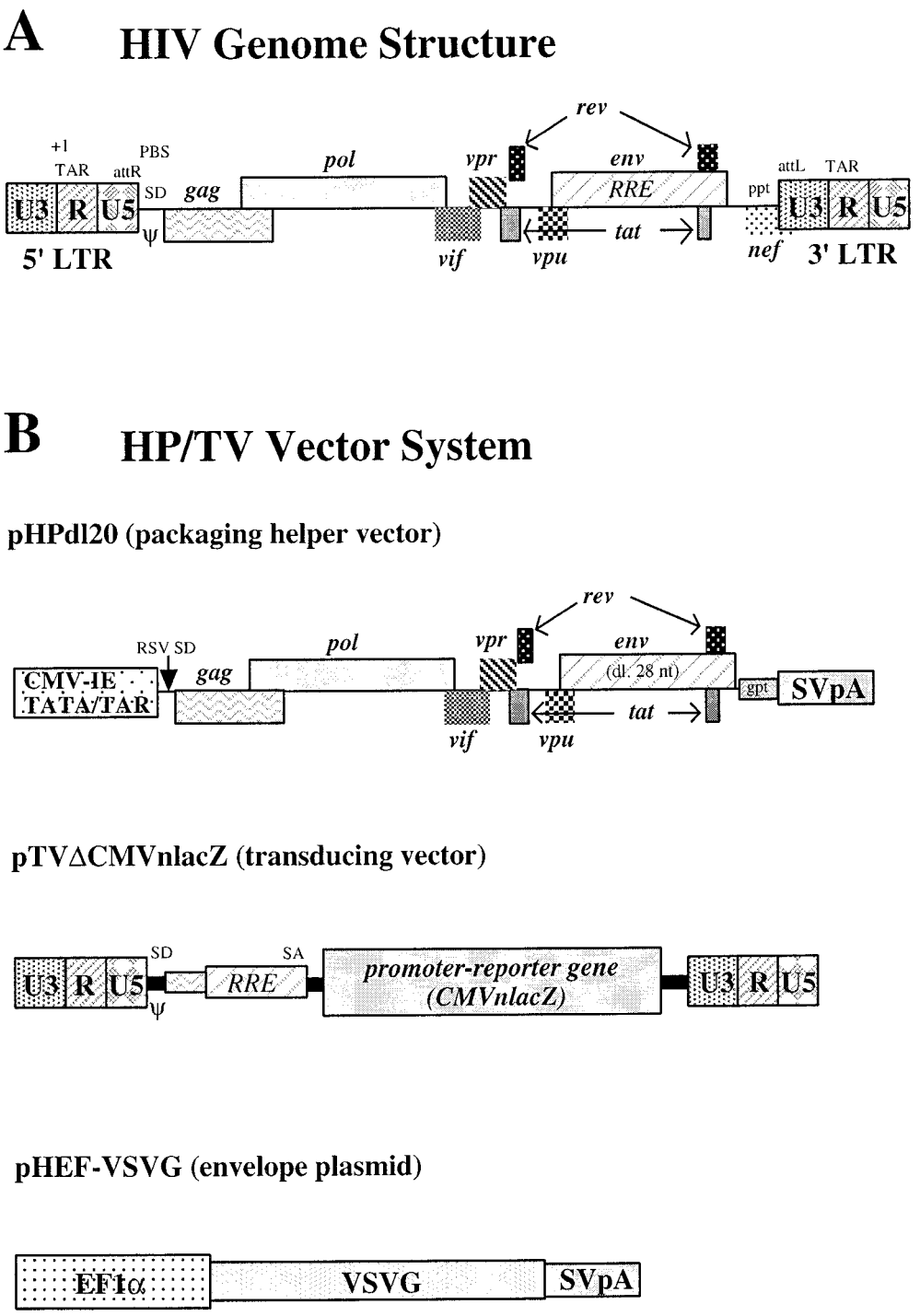


FIG. 1. HIV-1-derived lentiviral vector system. (A) HIV-1 genome structure. att, integrase attachment site; SD, splice donor; ψ, packaging signal; ppt, polypurine tract. (B) HP/TV vector system. In pHPdl20, 28 nucleotides in env were deleted as previously described (Chang *et al.,* 1999).

generate an improved lentiviral self-inactivated (SIN) vector.

RESULTS

The HP/TV lentiviral vector system

A three-plasmid lentiviral vector system has been established as illustrated in Fig. 1. pHPdl20 contains a chimeric promoter made of CMV-IE enhancer/TATA, an artificial leader sequence made of HIV TAR, and a Rous sarcoma virus (RSV) 5′ splice site with a mutated RSV gag AUG, HIV-1 gag-pol, vif, vpr, vpu, tat, rev, a mutated env, and an SV40 polyadenylation signal. Transducing vector pTV contains 5′ and 3′ HIV-1 LTRs, part of gag, and part of env including RRE, an internal promoter (CMV-IE), and a reporter gene (nlacZ). Also, an envelope-

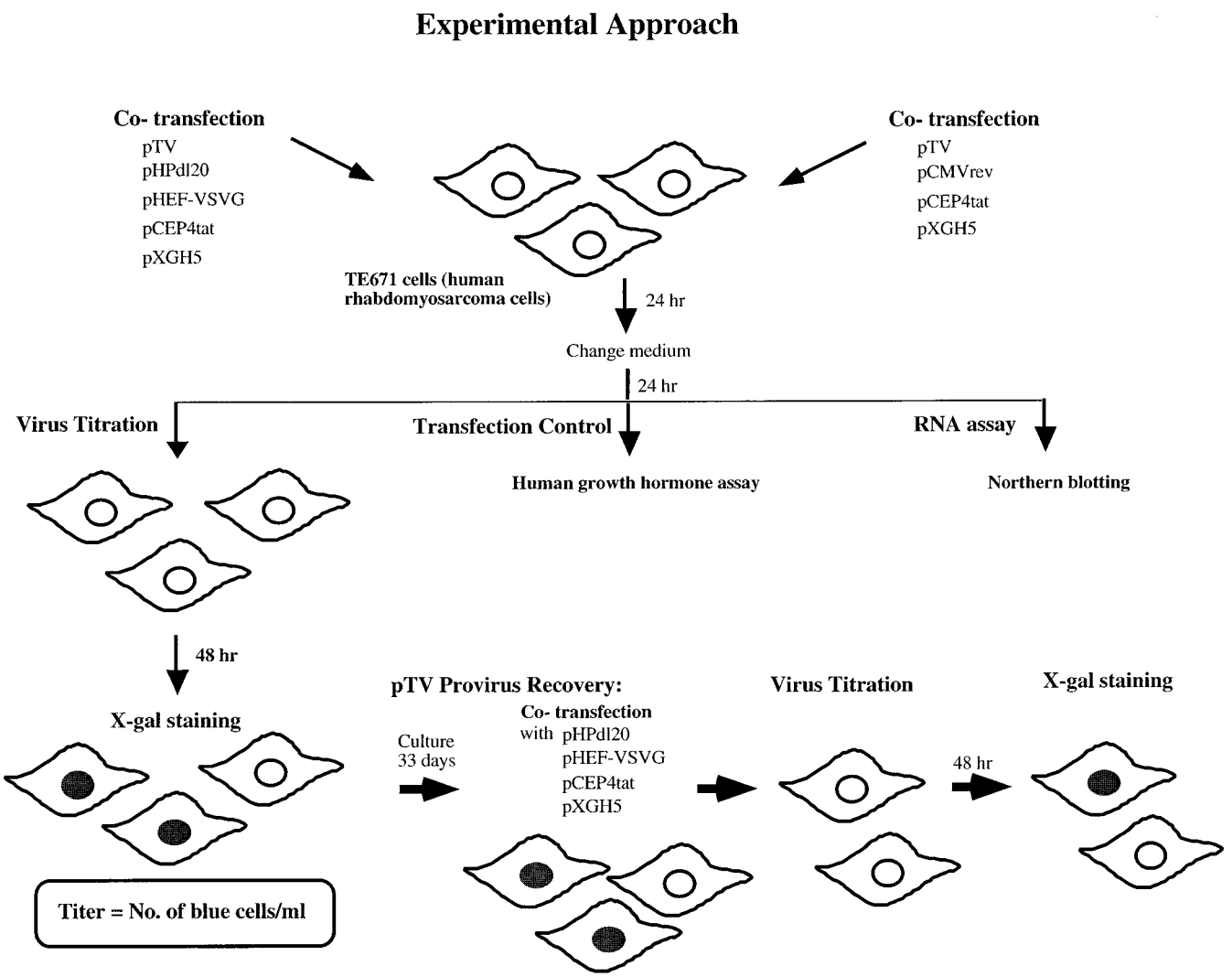


FIG. 2. Experimental approach diagram. For vector production and titration, five plasmids were cotransfected into TE671 cells as shown on the left. Human growth hormone plasmid pXGH5 was used as transfection control. To examine RNA expression of pTV vectors, pCMVrev, pCEP4tat, and pXGH5 were cotransfected.

encoding plasmid pHEF-VSVG is used for pseudotype virus production (Burns *et al.,* 1993; Chang *et al.,* 1999) (Figs. 1A and 1B). Although pHP expresses *tat* and *rev* functions of HIV-1, a tat expression plasmid, pCEP4tat, is supplemented in DNA cotransfection to enhance virus production. Cotransfection of the above four plasmids into human rhabdomyosarcoma TE671 cells routinely produces 10^5–10^6 infectious vectors per milliliter as determined by titration assay as illustrated in Fig. 2, which also shows the experimental approaches for analyses of different pTV LTR mutants.

5′ U3 replacement with a heterologous enhancer/promoter

Based on previous studies of chimeric HIV-1 LTRs (Chang *et al.,* 1993), we replaced the U3 of the 5′ LTR with a truncated CMV-IE enhancer/TATA promoter and generated pTVΔCT. This replacement eliminates the entire 5′ U3 of HIV-1 except for 25 nucleotides upstream of the NFκB binding sites. When assayed for vector efficiency and compared with that of the wild-type construct pTVΔ, pTVΔCT exhibited a vector titer close to wild-type level (Fig. 3A). To examine the chimeric promoter activity, poly(A)$^+$ cytoplasmic RNA was purified 42 h after cotransfection of the pTV DNA with a Rev expression vector, pCMVrev, in the presence or absence of pCEP4tat, followed by Northern blotting and hybridization using a lacZ probe. The results showed that both wt and mutant constructs expressed unspliced, full-length mRNA in the absence of Tat and both were Tat-responsive (Fig. 3B).

3′ U3 deletion to generate a SIN vector

Retroviral vectors with 3′ U3 mutations are SIN after reverse transcription (Bishop, 1983; Hwang *et al.,* 1997;

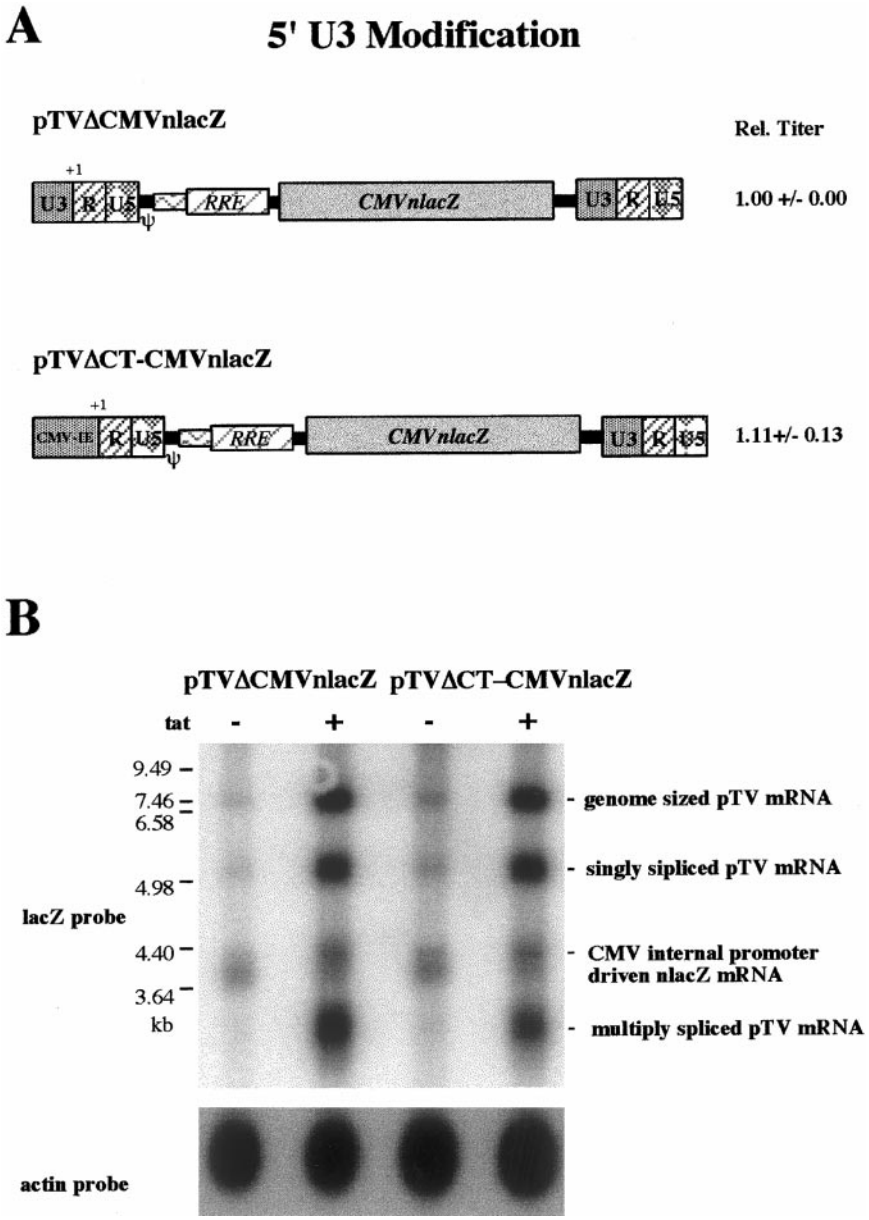


FIG. 3. Replacement of 5′ U3 in pTV and study of vector function. (A) pTV constructs and relative vector titers. The 5′ U3 of pTVΔCMVnlacZ was replaced with CMV-IE enhancer/TATA promoter to generate pTVΔCT-CMVnlacZ. Vector titer was determined as described in Fig. 2 and presented relative to the titer of wild-type pTVΔCMVnlacZ which was arbitrarily set at 1.00 with standard errors ($n = 4$). The actual titer value of the wild-type pTV construct is $7.3 \pm 0.2 \times 10^5$. (B) Northern analysis of pTVΔCMVnlacZ and pTVΔCT-CMVnlacZ with or without Tat. TE671 cells in a six-well culture dish were cotransfected with 20 μg of pTV, 4 μg of pCMVrev, 3 μg of pCEP4tat (tat+) or control pcDNAZeo/3.1(+) (tat−) DNA, and 0.1 μg of pXGH5. Cytoplasmic poly(A)$^+$ RNA was harvested 42 h later as described under Materials and methods. The blot was hybridized with either a lacZ probe or an actin probe as indicated on the left. The four major vector RNA species are denoted on the right: genome-sized pTV RNA; singly spliced pTV RNA, generated by using the 3′ splice site in HIV env; CMV internal promoter-driven nlacZ mRNA, including a comigrating spliced RNA using a cryptic 3′ splice site in the CMV promoter; and multiply spliced pTV RNA, generated by using a cryptic 3′ splice site in the nlacZ gene (see Cui *et al.,* 1999). RNA molecular weight markers are shown on the left.

Miyoshi *et al.,* 1998; Olson *et al.,* 1994; Temin, 1990; Zufferey *et al.,* 1997). To see if the 3′ U3 region in pTV can be deleted, a series of 3′ U3 deletions were made by PCR mutagenesis using primers shown in Fig. 5. We first deleted NFκB and Sp1 binding sequences in the 3′ U3 as described under Materials and Methods. This mutation did not alter virus titer when compared with the wild-type pTV (Δ97 bp, pTVΔCMVnlacZdl3′U3kB/Sp1, Fig. 4). This encouraged us to delete more of the 3′ U3 sequence. Further deletions were made to include most of the U3 sequence with or without the flanking 9098–9154 and 9512–9528 regions (according to the numeric system of HIV-1 plasmid pNL$_{4-3}$). These mutations were generated by PCR mutagenesis using primers c–f listed

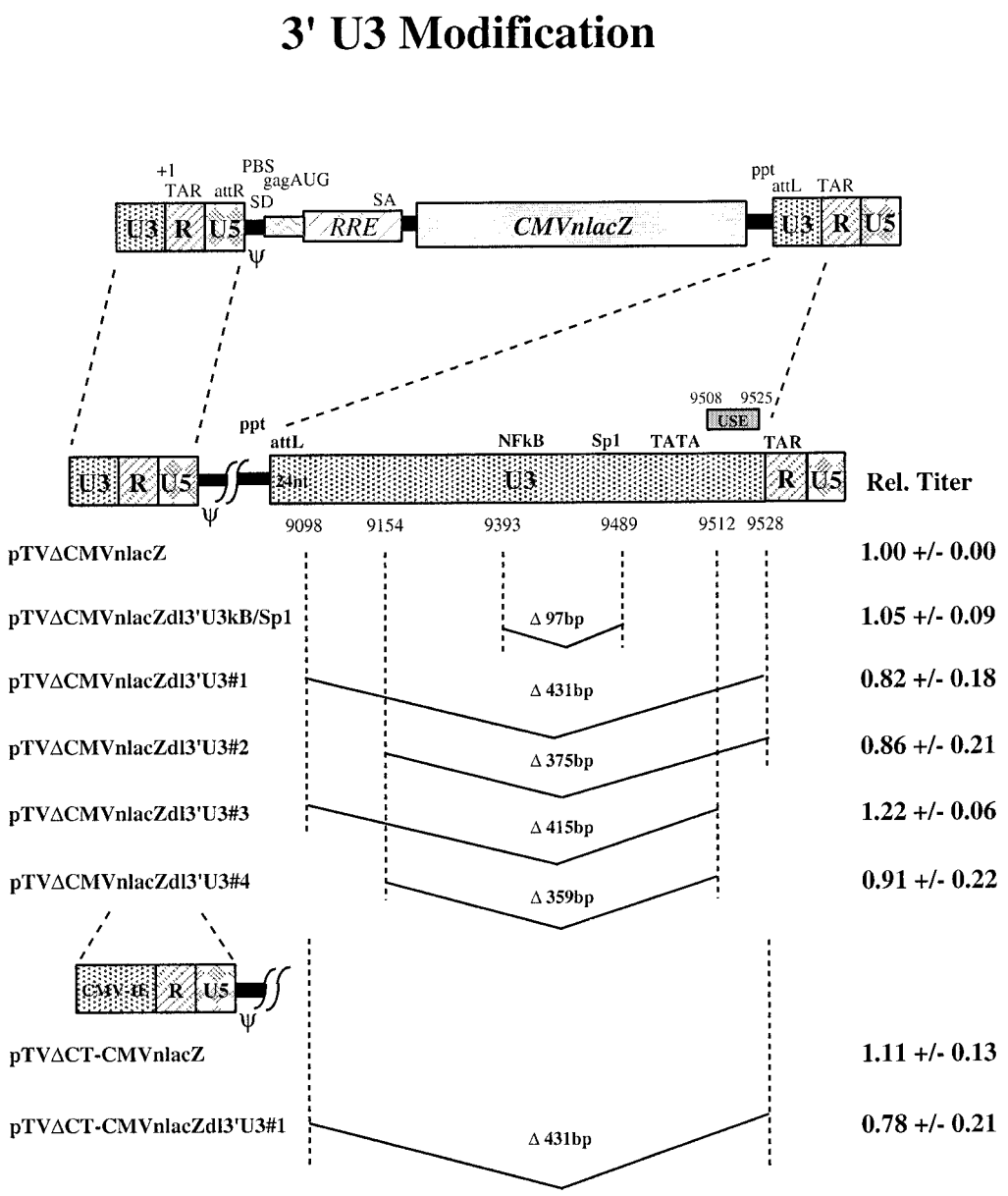
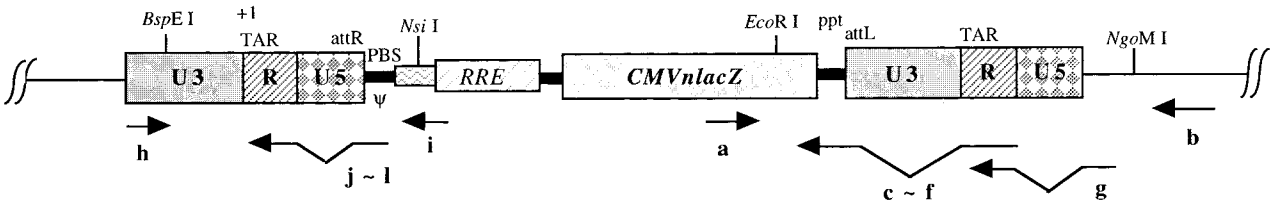


FIG. 4. Deletion of 3′ U3 in pTV and study of vector function. 3′ U3 deleted pTV constructs and their relative titers. The size of U3 deletion is indicated and the nucleotide numbers on the map are based on pNL4-3; for example, NFκB and Sp1 binding sequences are from nt 9393 to 9489; USE, upstream element. The vector titer with standard error ($n = 4$) relative to pTVΔCMVnlacZ was determined as described in Fig. 2 and presented on the right. The actual titer value of the wild-type pTV construct was $7.3 \pm 0.2 \times 10^5$.

in Fig. 5. In all of these mutants, the left integrase attachment site (attL, 24 nt) was retained which is essential for the integration function of the pTV vector (Reicin *et al.,* 1995). These mutants were compared with wt pTV by cotransfection with pHP in TE671 cells, and the relative vector efficiency was determined. The results are summarized in Fig. 4. The longest 3′ U3 deletion did not appear to have any notable effects on vector titer (pTVΔCMVnlacZdl3′U3#1). To verify this, the 3′ U3 deletion #1 (Δ431 bp) was introduced into the chimeric construct pTVΔCT-CMVnlacZ to generate pTVΔCT-CMVnlacZdl3′U3#1. The titer assay again confirmed that this large U3 deletion did not have a notable effect on vector efficiency. Interestingly, we observed that the two deletion constructs (pTVΔCMVnlacZdl3′U3#1 and #2), which lack an "upstream element" (USE), always produced lower titers than those with USE (pTVΔCMVnlacZdl3′U3#3 and #4), which suggests a positive role of USE on vector function (Fig. 4).

To see if the U3-deleted pTV vectors could be properly reverse transcribed and maintain the U3 deletion in the provirus, we examined the provirus formation in the transduced cells. TE671 cells were transduced with 3′ or both 5′ and 3′ U3-deleted or wild-type U3 vectors, and extrachromosomal DNA was purified. Proviral intermediates in the Hirt preparation were amplified by PCR using

pTVΔCMVnlacZ



Primers used for PCR in Figs. 4, 7A, and 8

	primer sequence	amplification direction and location
a.	GGCGGAATTCCAGCTGAG	5' to 3' in nlacZ
b.	ATAGAACTCCGTTCTCC	3' to 5' in plasmid backbone downstream of 3' LTR
c.	GTCTAACCAGAGAGACCCTGGGAGTGAATTAGCCCTTC	3' to 5' in U3 ~ R
d.	GTCTAACCAGAGAGACCCAGGGAAGTAGCCTTGTG	3' to 5' in U3 ~ R
e.	CCAGTACAGGCAAAAAGCTGGGAGTGAATTAGCCCTTC	3' to 5' in U3 ~ R
f.	CCAGTACAGGCAAAAAGCCAGGGAAGTAGCCTTGTG	3' to 5' in U3 ~ R
g.	CTCTACCTCCTGGGGGGTTGAAGCACTCAAGGCAAG	3' to 5' in R ~ plasmid backbone
h.	AGGCTACTTCCCTGATTGGCAG	5' to 3' in U3
i.	TGTGTTGAATTACAGTAGAAAAATTCCCCTC	3' to 5' in gag
j.	CTAGAGATTTTCCACACTGATGAGCACTCAAGGCAAGC	3' to 5' in R-U5 ~ PBS
k.	CTAGAGATTTTCCACACTGACACACACTACTTTGAGCACTC	3' to 5' in R-U5 ~ PBS
l.	CTAGAGATTTTCCACACTGACACACAACAGACGGGCACAC ACTACTTTGAGCACTC	3' to 5' in R-U5 ~ PBS

FIG. 5. Mutagenesis and PCR primers and their relative locations on pTV. Various primers for PCR mutagenesis are set on the pTV map as shown at the top. The location and direction of these primers are also depicted.

a nested primer set as described under Materials and Methods. The PCR primers were designed to specifically amplify unintegrated intermediates (circular proviral DNA) but not the cotransfected plasmid DNA or the linear preintegration intermediates (Fig. 6A). The results showed that a distinct band of 984 bp representing the product of unmodified one-LTR proviral DNA circles was detected for both pTVΔCMVnlacZ and pTVΔCT-CMVnlacZ (Fig. 6B, lanes 3 and 5). In contrast, the size of the U3-deleted proviral DNAs of pTVΔCMVnlacZdI3′U3#1 (3′ U3 deleted) and pTVΔCT-CMVnlacZdI3′U3#1 (both 5′ and 3′ U3 deleted) was shifted down to 552 bp (Fig. 6B, lanes 4 and 6), which was consistent with the expected deletion size of U3. Southern analysis of the PCR-amplified products did not detect wild-type U3 signal in provirus of the U3-deleted vector constructs (not shown). This result demonstrates that both the single and the double U3-deleted constructs can be properly reverse transcribed after transduction.

Substitution of 3′ U5 with a heterologous polyadenylation signal increased vector titer

To delete more LTR sequences from pTV, the 3′ U5 was further modified. We first deleted the entire 3′ U5 and generated pTVΔCMVnlacZdI3′U3#1U5 using mutagenesis primer g listed in Fig. 5 (Fig. 7A). This deletion led to a severe loss of vector function as determined by vector titration assay (12% of wild-type level, Fig. 7A). Northern analysis showed marked reduction of genome-sized mRNA to an undetectable level (not shown). These results suggested a possible defect in polyadenylation and/or stability of mRNA. Accordingly, we substituted U5 with a heterologous polyadenylation sequence derived from bovine growth hormone (bGH) gene and generated a new pTV construct, pTVΔCMVnlacZdI3′U3#1U5pA. Analyses of this construct showed an improved vector function almost twice as high as that of the wild type. This was confirmed when the 3′ U5 of the previously

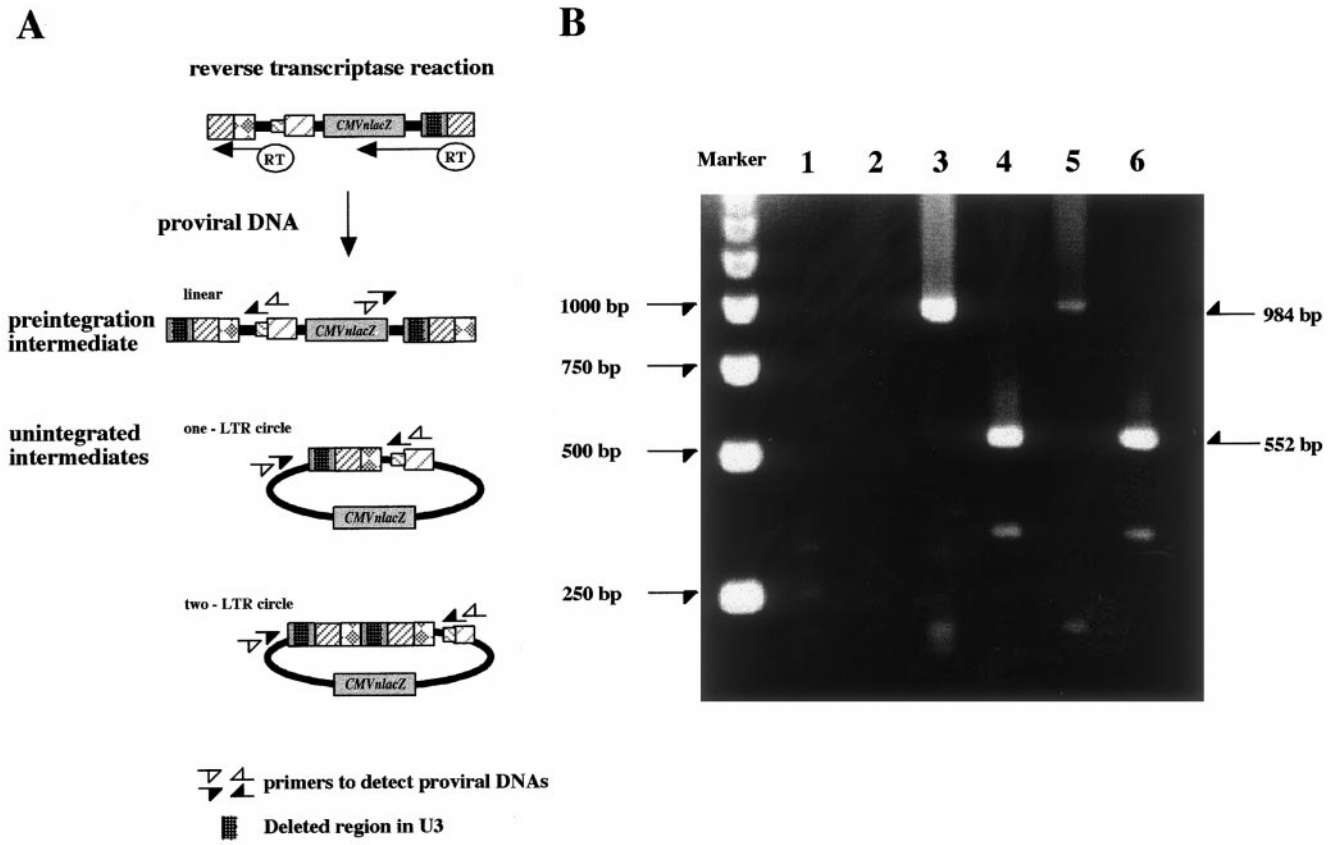


FIG. 6. Detection of proviral DNA after lentiviral vector transduction. Hirt DNA was harvested as described under Materials and methods 12 h after transduction and amplified by PCR using two nested primer pairs. (A) RT integration intermediate diagram. Hatched box shows the 432-bp region deleted in 3′ U3. (B) Detection of proviral DNA. The expected fragment sizes for one-LTR circles of wild-type and U3-deleted pTV constructs are shown on the right. A 250-bp DNA ladder was used as a size marker; lane 1, primers without template; lane 2, mock transduction; lane 3, pTVΔCMVnlacZ; lane 4, pTVΔCMVnlacZdI3′U3#1; lane 5, pTVΔCT-CMVnlacZ; and lane 6, pTVΔCT-CMVnlacZdI3′U3#1.

modified pTVΔCT-dI3′U3#1, which had 5′ and 3′ U3 modifications, was also replaced with the bGHpA and tested (pTVΔCT-CMVnlacZdI3′U3#1U5pA, Fig. 7A).

To see if these modified vectors can efficiently infect nondividing cells, we blocked the cell cycle of TE671 cells by γ-irradiation (20,000 rad) and then transduced these irradiated cells with different LTR-modified vectors (Fig. 7B). The transduction efficiencies of these vectors were studied in both dividing and nondividing cells and compared with that of the wild-type pTV. The titer of wild-type pTV on irradiated cells was determined to be 0.94 ± 0.05 in relation to wild-type pTV on dividing cells which was arbitrarily set at 1 (Fig. 7B, a). In addition, the titers of both the 3′ U3/U5- and 5′U3–3′U3/U5-modified vectors on irradiated cells were comparable to that on nonirradiated cells (Fig. 7B, b and c vs d). These results confirmed that LTR-modified lentiviral vectors transduced both dividing and nondividing cells at similar efficiencies.

To examine the SIN phenotype of the pTV vectors with 3′ U3/U5 deletion, a provirus recovery experiment, as illustrated in Fig. 2, was performed. TE671 cells were transduced with wild-type pTV and the modified dI3′U3#1U5pA vectors and continuously cultured for 33 days until passage 10. The long-term propagation of the transduced cells would reduce the level of contaminating carryover transfection DNA and the extrachromosomal proviral DNA. The percentages of transduced cells in these passage 10 cultures were determined by X-gal staining to be 50 and 55% for pTVΔCMVnlacZ and pTVΔCMVnlacZdI3′U3#1U5pA, respectively (not shown). To recover packageable proviral RNA, these cultures were transfected with the lentiviral helper construct pHPdI20, the pseudotype env construct pHEF-VSVG, and pCEP4tat to enhance LTR transactivation, and 48 h later the culture supernatants were harvested and assayed on TE671 cells. The average of four repeated assays is shown in Table 1. The cells transduced by pTVΔCMVnlacZ released more than 10^4 per milliliter of infectious vectors, whereas cells transduced by pTVΔCMVnlacZdI3′U3#1U5pA released zero infectious units of viral vector. This result suggests that there is no LTR-derived full-length RNA present in cells transduced with pTVΔCMVnlacZdI3′U3#1U5pA and that the provirus derived from the SIN vector does not have a transcriptionally active LTR after integration.

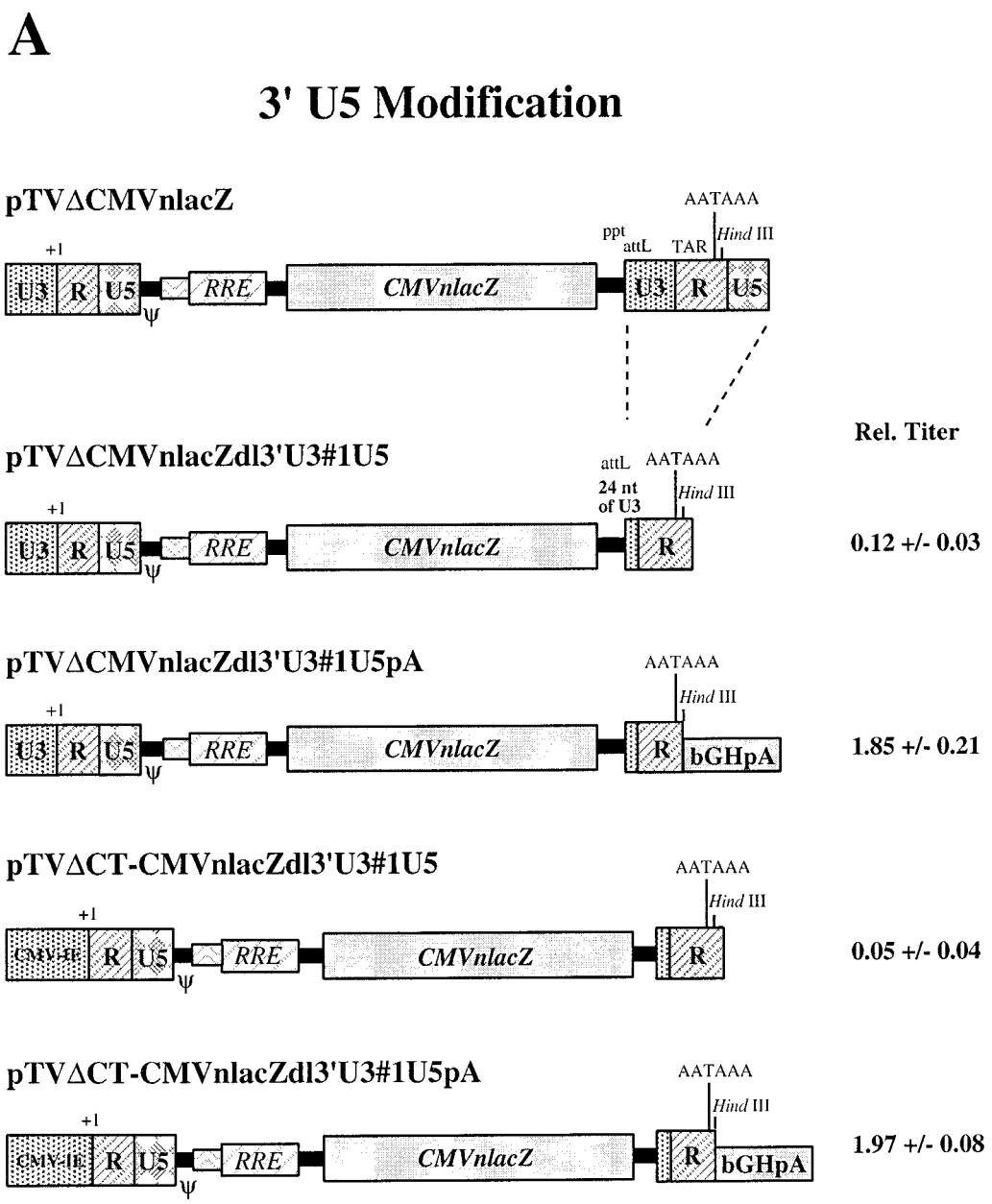


FIG. 7. Generation of 3′ U5-deleted SIN vectors and transduction of nondividing cells. (A) pTV vectors with 3′ U5 modifications. The 3′ U5 was deleted by PCR mutagenesis as described under Materials and Methods (pTVΔCMVnlacZdl3′U3#1U5). In pTVΔCMVnlacZdl3′U3#1U5pA, bGH polyadenylation sequence was inserted at the *Hind*III site in the 3′ R. The same modifications were introduced into the 5′ U3-replaced pTV vectors. Relative titers to the control pTVΔCMVnlacZ were determined and shown on the right with standard errors ($n = 4$). The actual titer value of the wild-type pTV construct was $7.3 \pm 0.2 \times 10^5$. (B) Lentiviral transduction of dividing and nondividing cells. Viral vectors derived from pTVΔCMVnlacZ (a), pTVΔCMVnlacZdl3′U3#1U5pA (b), and pTVΔCT-CMVnlacZdl3′U3#1U5pA (c and d) were used to transduce both irradiated (a to c) and normal TE671 cells (d), and 48 h later, the transduced cells were fixed and stained with X-gal and photographed under a Zeiss Axiovert 25 inverted microscope (10 × 10). The enlarged morphological change of irradiated cells (a to c) was apparently different from normal cells (d). The transduction efficiency is presented under each photo as relative vector titers ($n = 4$). The actual titer value of the wild-type pTV construct was $6.9 \pm 0.1 \times 10^5$.

5′ U5 is critical to optimal vector function

To further improve this vector system, we attempted to delete the 5′ U5 sequence by PCR mutagenesis using primers j–l as listed in Fig. 5. A pTV mutant with the entire 5′ U5 deleted except for the 24-nt attR was first made and examined. The titration assay showed that this U5 deletion construct, pTVΔCMVnlacZdl5′U5Δ62-3′U3U5pA, exhibited only 30% of the wild-type vector function (Δ62, Fig. 8). When the 5′ 12 and 27 nt of U5 were added back to the Δ62 construct to generate the Δ50 and Δ35 pTV mutants, respectively, the titration assay showed that the Δ50 construct restored 20% of vector function. Interestingly, the Δ35 con-

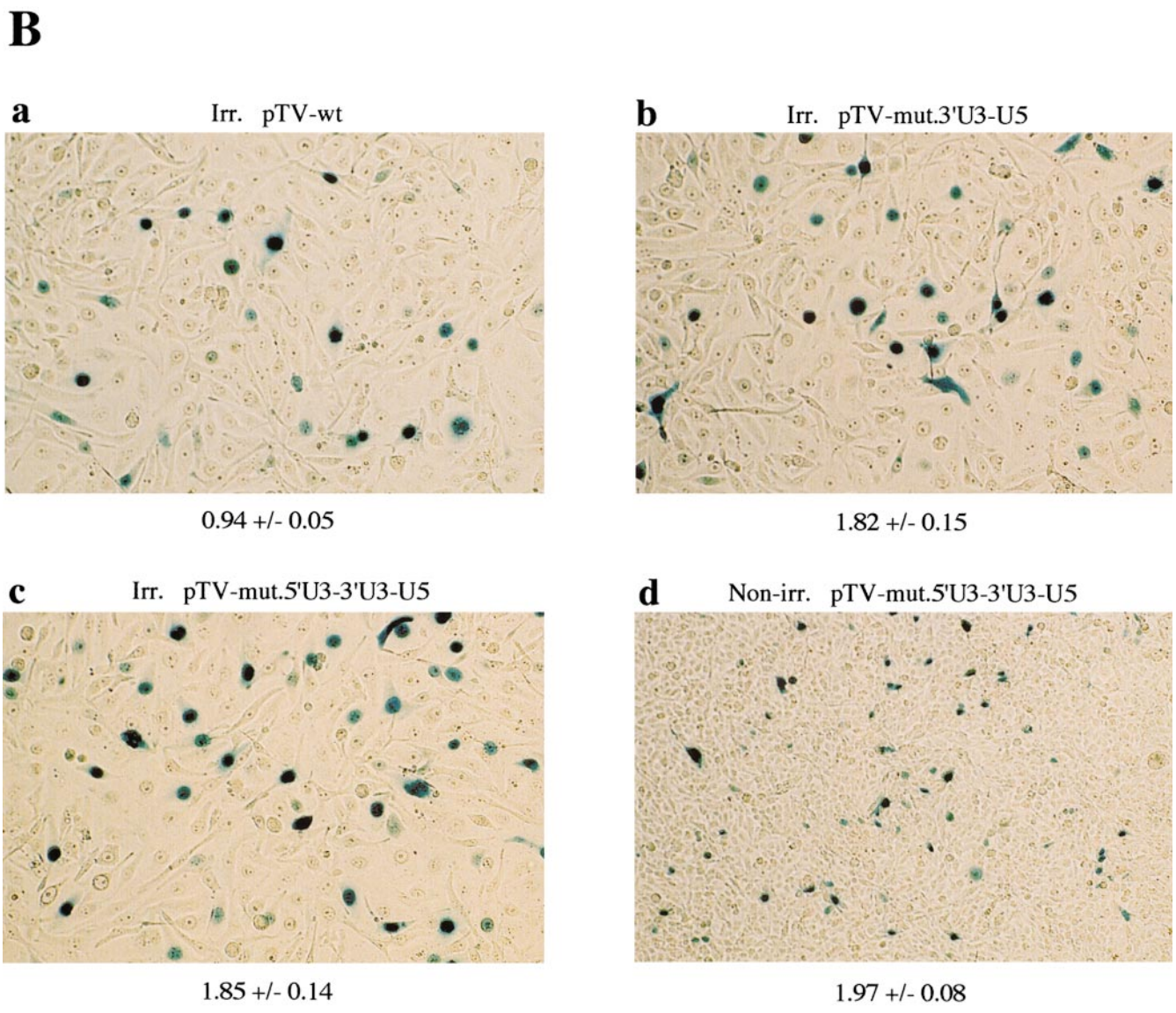


FIG. 7—*Continued*

struct restored vector efficiency close to the level of wild-type pTVΔCMVnlacZ. Nevertheless, compared with its parental construct pTVΔCMVnlacZdl3′U3#1U5pA, the Δ35 vector function was still reduced by 50%.

TABLE 1

Recovery of Lentiviral Vectors from Transduced TE671 Cells

Proviral cultures	Titer of recovered vectors (±standard error)
pTVΔCMVnlacZ	$1.12 \times 10^4 \pm 0.13$
pTVΔCMVnlacZdl3′U3#1U5pA	0

Note. Provirus-integrated cultures 33 days after transduction were transfected with pHPdl20, pHEF-VSVG, and pCEP4tat, and 48 h later supernatants were used to infect TE671 cells as described in the text. The titer represents averages of three experiments.

DISCUSSION

In this study, we sequentially deleted U3 and U5 sequences of both LTRs of a lentiviral transducing vector construct, pTV, to generate an improved SIN vector. pHP provides helper viral gene functions and pTV produces packageable genome. pHP does not have any LTR sequence because the 5′ LTR had been replaced with a chimeric CMV-IE enhancer/promoter and the 3′ LTR had been replaced with an SV40 polyadenylation signal. The LTRs in pTV, however, have potential risk of generating replication-competent HIV upon recombination with pHP. Thus, deleting LTRs in pTV would eliminate all native HIV LTR sequences in the vector system and greatly improve the safety of the HP/TV vector system. The replacement of both pHP and pTV LTRs with the same CMV-IE enhancer/promoter, however, may not be desirable be-

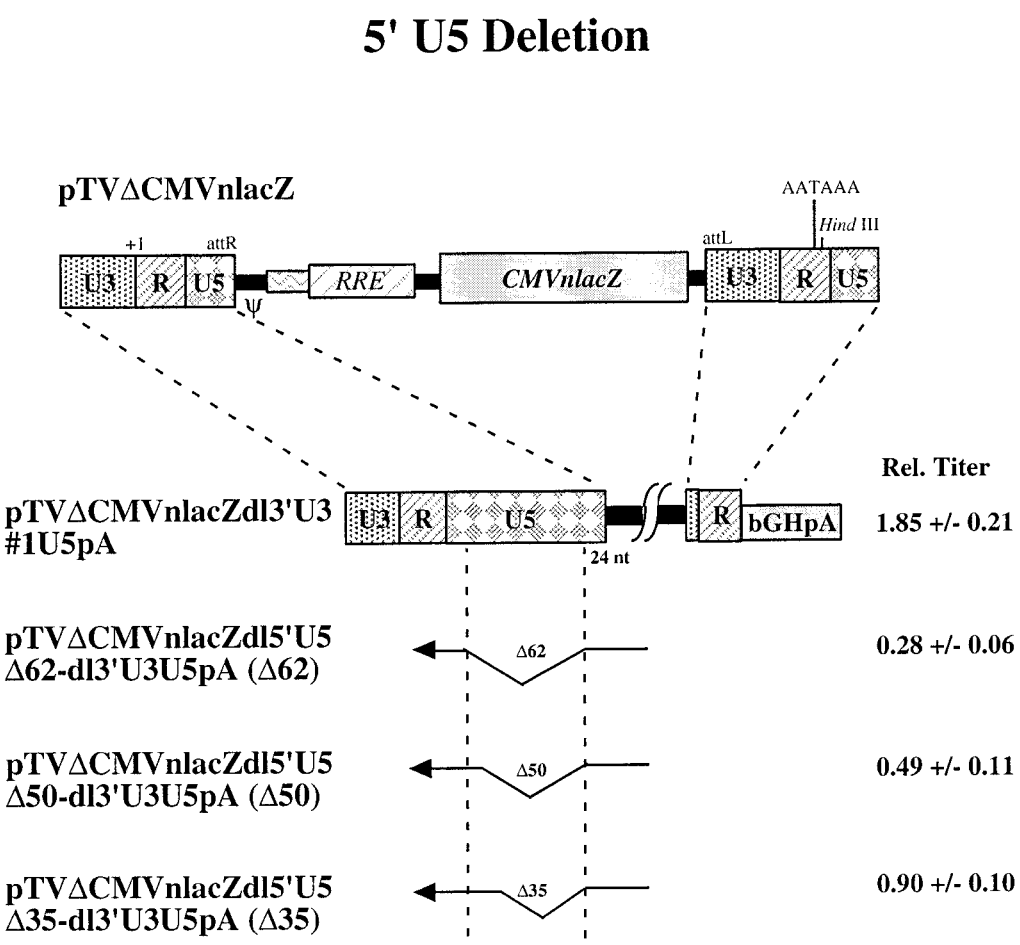


FIG. 8. Analyses of 5′ U5 pTV deletion mutants. 5′ U5 was deleted by PCR mutagenesis as described under Materials and Methods and the sizes of deletions are indicated (Δ62, Δ50, and Δ35). Relative titers to pTVΔCMVnlacZ are presented with standard errors ($n = 4$). The actual titer value of the wild-type pTV construct was $7.3 \pm 0.2 \times 10^5$.

cause it increases the possibility of generating a recombinant virus carrying a heterologous CMV-IE promoter. Nevertheless, this can be avoided by replacing the pHP or the pTV promoter with a heterologous promoter. The replacement of 5′ U3 with a truncated CMV-IE enhancer/TATA promoter did not affect vector efficiency and this chimeric promoter appears to act like the native HIV-1 promoter in TE671 cells (Fig. 3B). Others have reported similar replacement with a full-length CMV-IE enhancer and illustrated a tat-independent promoter function using a different lentiviral vector system (Kim *et al.,* 1998; Miyoshi *et al.,* 1998). Although the truncated CMV-IE enhancer/TATA promoter in pTVΔCT-CMVnlacZ appears to have slightly higher basal promoter activity than that of the native HIV LTR in the absence of Tat as demonstrated in repeated Northern analyses (Fig. 3B), Tat is still required for high vector efficiency in our system.

It appears that except for the first 24 nt of 3′ U3 which contains the left integrase attachment site (attL), all U3 sequences could be deleted without affecting vector function. This result is in agreement with the studies of Miyoshi *et al.* (1998) and Zufferey *et al.* (1998). However, we found that the deletion of USE in the 3′ U3 had a mild effect on vector titer (~20% reduction). This could be due to the ineffectiveness of poly(A) processing, because the interaction of a 160-kDa subunit of cleavage and polyadenylation specificity factor (CPSF) with USE is known to enhance poly(A) processing (Gilmartin *et al.,* 1992, 1995; Valsamakis *et al.,* 1991). The GU-rich or U-rich element in U5 is known to be associated with the cleavage stimulation factor (CstF), which is important for modulating RNA polyadenylation (Keller, 1995; MacDonald *et al.,* 1994). This could explain why deletion of 3′ U5 led to a severe reduction in vector titer and mRNA synthesis. This defect could be overcome by replacing sequence downstream of the polyadenylation signal, AATAAA, with bGH poly(A) sequence (Fig. 7A). The resulting vector produced two times the titer of the parental pTVΔCMVnlacZ, which is more than 1×10^6/ml.

Further deletion of 5′ U5 of pTV is less tolerable (Fig. 8). Since 5′ U5 has been shown to have multiple roles including packaging, reverse transcription, and integration, the defect of the 5′ U5-deleted vector may be multifactorial (Das *et al.,* 1997; Huang *et al.,* 1998; Vicenzi *et*

al., 1994). Vicenzi *et al.* (1994) have reported that the middle part of 5′ U5 could be deleted without affecting HIV-1 replication, while deletions of either 5′ or 3′ one-third impaired virus replication. Results of the three pTV 5′ U5 deletion constructs are consistent with those earlier observations. Further analyses will be necessary to see how 5′ U5 interferes with vector function.

In summary, we have generated a modified lentiviral vector system with most of the native HIV LTR sequences deleted without affecting its function of transducing nondividing cells. The LTR-deleted vector exhibited vector efficiency similar to or better than that of the parental vector. Future steps will be taken to replace the remaining 5′ U5 sequence and the native HIV R sequence so that the pTV vector will contain only minimal native HIV genome sequence.

MATERIALS AND METHODS

Cell culture

TE671 (human rhabdomyosarcoma) cells were obtained from ECACC, England. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 100 units/ml of penicillin–streptomycin (Gibco BRL).

Plasmid constructions

pTVΔCMVnlacZ, pHPdI20, pHEF-VSVG, and pCEP4tat were constructed as described previously (Chang *et al.,* 1999) and are illustrated in Figs. 1A and 1B. Replacement of 5′ U3 with a truncated CMV-IE enhancer/TATA promoter was accomplished by replacing *Stu*I to *Nsi*I of pTVΔCMVnlacZ with *Pme*I to *Nsi*I of CMV-IE (a)/TATA-HIV-1_{NL4-3} (Chang *et al.,* 1993). To generate pTVΔCMVnlacZdI3′U3kB/Sp1, a DNA fragment from dI.kB/Sp1-HIV-1_{NL4-3} (Adachi *et al.,* 1986; Chang *et al.,* 1993; Hunninghake *et al.,* 1989) digested with *Kpn*I and *Ngo*MI was cloned into pTVΔCMVnlacZ. To construct pTV mutants by PCR mutagenesis, the primers listed in Fig. 5 were used. For 3′ U3 deletions, primers a and c–f were used in the first round of PCR, using pTVΔCMVnlacZ as a template. The amplified products were purified by Microcon 50 (AMICON) and used as a mega-primer to pair with 3′ primer b for the second-round PCR using pTVΔCMVnlacZ as template. The amplified fragments were digested with *Eco*RI and *Ngo*MI and cloned into pTVΔCMVnlacZ. To generate pTVΔCMVnlacZdI3′U3#1U5, pTVΔCMVnlacZdI3′U3#1 was used as template and primer g was used as 3′ primer in the first-round PCR. The rest of the cloning steps were similar to that of the 3′ U3 deletion construction. To generate 5′ U5 deletions, primers h and j–l were used for the first-round PCR with pTVΔCMVnlacZ as template. The products were used as mega-primers to pair with 3′ primer i in the second-round PCR. The final PCR products were digested with *Bsp*EI and *Nsi*I and cloned into pTVΔCMVnlacZdI3′U3#1U5pA. All PCR-amplified sequences were verified by DNA sequencing.

pTVΔCMVnlacZdI3′U3#1U5pA was constructed by digesting pcDNA3.1/Zeo(+) (Invitrogen) with *Hin*dIII and *Ngo*MI and a 512-bp DNA fragment containing bGH polyadenylation sequence was isolated and cloned into pTVΔCMVnlacZdI3′U3#1 digested with the same enzymes.

DNA transfection

A modified calcium phosphate DNA transfection protocol was performed as previously described (Chang and Zhang, 1995; Chen and Okayama, 1987). Transfection efficiency was determined by a radioimmunoassay for human growth hormone that was produced by a cotransfected plasmid pXGH5 (Nichols Institute Diagnostics). Supernatants of transfected culture were collected and used for both human growth hormone and virus titration assays.

Vector production and titration

To produce lentiviral vectors, 10 μg of pTV, 10 μg of pHPdI20, and 5 μg of pHEF-VSVG were cotransfected with 1 μg of a tat-encoding plasmid pCEP4tat and 0.1 μg of pXGH5 into TE671 cells (5×10^5) in each well of a 6-well plate the day before transfection. Media were changed 18–24 h after DNA was added, and the following day virus was harvested by filtration using 0.45-μm low-protein binding filters (Millex-HV, Millipore) to remove cell debris. The supernatants were stored at −80°C in aliquots until use. For vector titration, two different dilutions of supernatants were used to infect 4×10^4 TE671 cells plated in 24-well plates in the presence of 8 μg/ml of polybrene. After 3–4 h of infection, fresh media were added, and 48 h after infection, culture was stained with X-gal substrate as previously described (Chang *et al.,* 1999). Virus titer was determined by counting the blue nucleated cells and relative vector titer to the wild-type control was presented after normalization for transfection based on control human growth hormone expression.

Lentiviral transduction of irradiated TE671 cells

TE671 cells were irradiated with 20,000 rad and maintained in a 5% CO_2 incubator for 28 h before transduction. The irradiated cells were transduced with lentiviral vectors at different multiplicity of infection and 48 h later, cells were assayed for lacZ reporter gene expression by X-gal staining, and the transduction efficiency was determined.

Hirt DNA preparation

A modified protocol was used for simultaneous preparation of genomic and Hirt DNA (Motmans *et al.,* 1997). Briefly, cells were resuspended in 250 μl buffer containing 50 mM glucose, 25 mM Tris–HCl (pH 8.0), and 10 mM EDTA after washing with PBS and incubated at room temperature for 5 min. Cells were then lysed with 200 μl lysis buffer containing 200 mM NaOH and 1% SDS on ice for 5 min. The lysate was neutralized by adding 150 μl of 5 M potassium acetate (pH 4.8), and cell debris and chromosomal DNA were pelleted by centrifugation at 10,000 *g* for 5 min. The supernatant containing Hirt DNA was treated with proteinase K, extracted with phenol and chloroform, and followed by ethanol precipitation.

PCR analysis of unintegrated proviral intermediates

Approximately 100 ng of Hirt DNA was used as template to detect proviral intermediates using a nested PCR method. Hirt DNA was purified from cells infected with lentiviral vectors for 12 h. For the first round of PCR, a 5′ primer in lacZ, 5′-ACG ACT CCT GGA GCC CG-3′, and a 3′ primer in gag, 5′-TGT GTT GAA TTA CAG TAG AAA AAT TCC CCT C-3′, were used. For the second-round PCR, another 5′ primer in lacZ downstream of the first lacZ primer, 5′-GGC GGA ATT CCA GCT GAG-3′, and another 3′ primer in gag upstream of the first 3′ primer, 5′-ACT GAC GCT CTC GCA CCC AT-3′, were used. Annealing temperature was 58°C and PCR was performed for 30 cycles for both rounds.

Cytoplasmic poly(A)$^+$ RNA purification and Northern blotting

TE671 cells were seeded into a six-well plate at 5×10^5 cells per well 1 day before transfection. To drive the expression of packageable full-length mRNA from a pTV plasmid, a Rev-expression plasmid, pCMVrev, which is important for the nuclear export of genomic RNA, was cotransfected with the pTV plasmid. Cells were transfected with 20 μg of pTV or its derivatives, 1 μg of pXGH5 for transfection control, and 4 μg of pCMVrev with or without 2 μg of pCEP4tat per well. Media were changed the next day and the following day, cytoplasmic poly(A)$^+$ RNA was harvested for Northern analyses as previously described (Chang and Zhang, 1995; Robinson *et al.,* 1995). The RNA blot was hybridized with a lacZ probe and rehybridized with a chicken β-actin probe to normalize RNA amounts. Before rehybridization, the first probe was stripped off by boiling the blot in ddH_2O containing 0.1% SDS for 5 min.

Recovery of integrated lentiviral vectors

The cells infected by virus derived from pTVΔCMVnlacZ and pTVΔCT-CMVnlacZdl3′U3#1U5pA were cultured for 33 days (passage 10). These cells were transfected with 15 μg of pHPdl20, 10 μg of pHEF-VSVG, 2 μg of pCEP4tat, and 0.1 μg of pXGH5 plasmids as described above. After 48 h, 20, 10, and 200 μl of the transfection supernatants, containing 8 μg/ml of polybrene, were used to infect TE671 cells for vector titration as described above.

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REFERENCES

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59,** 284–291.

Bishop, J. M. (1983). Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52,** 301–354.

Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.

Carroll, R., Lin, J. T., Dacquel, E. J., Mosca, J. D., Burke, D. S., and St. Louis, D. C. (1994). A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector system utilizing stable HIV-1 packaging cell lines. *J. Virol.* **68,** 6047–6051.

Chang, L.-J., Urlacher, V., Iwakuma, T., Cui, Y., and Zucali, J. (1999). Efficacy and safety analyses of a recombinant human immunodeficiency virus derived vector system. *Gene Ther.* **6,** 715–728.

Chang, L.-J., McNulty, E., and Martin, M. (1993). Human immunodeficiency viruses containing heterologous enhancer/promoters are replication competent and exhibit different lymphocyte tropisms. *J. Virol.* **67,** 743–752.

Chang, L.-J., and Zhang, C. (1995). Infection and replication of Tat-human immunodeficiency viruses: Genetic analyses of LTR and tat mutations in primary and long-term human lymphoid cells. *Virology* **211,** 157–169.

Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7,** 2745–2752.

Cui, Y., Iwakuma, T., and Chang, L.-J. (1999). The contributions of viral splice sites and *cis*-regulatory elements on lentiviral vector functions. *J. Virol.* **73,** 6171–6176.

Das, A. T., Klaver, B., Klasens, B. I., van Wamel, J. L., and Berkhout, B. (1997). A conserved hairpin motif in the R-U5 region of the human immunodeficiency virus type 1 RNA genome is essential for replication. *J. Virol.* **71,** 2346–2356.

Gilmartin, G. M., Fleming, E. S., and Oetjen, J. (1992). Activation of HIV-1 pre-mRNA 3′ processing *in vitro* requires both an upstream element and TAR. *EMBO J.* **11,** 4419–4428.

Gilmartin, G. M., Fleming, E. S., Oetjen, J., and Graveley, B. R. (1995). CPSF recognition of an HIV-1 mRNA 3′-processing enhancer: Multiple sequence contacts involved in poly(A) site definition. *Genes Dev.* **9,** 72–83.

Gordon, E. M., and Anderson, W. F. (1994). Gene therapy using retroviral vectors. *Curr. Opin. Biotechnol.* **5,** 611–616.

Huang, Y., Khorchid, A., Gabor, J., Wang, J., Li, X., Darlix, J. L., Wainberg, M. A., and Kleiman, L. (1998). The role of nucleocapsid and U5 stem/A-rich loop sequences in tRNA(3Lys) genomic placement and initiation of reverse transcription in human immunodeficiency virus type 1. *J. Virol.* **72,** 3907–3915.

Hunninghake, G. W., Monick, M. M., Liu, B., and Stinski, M. F. (1989). The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. *J. Virol.* **63,** 3026–3033.

Hwang, J. J., Li, L., and Anderson, W. F. (1997). A conditional self-inactivating retrovirus vector that uses a tetracycline-responsive expression system. *J. Virol.* **71,** 7128–7131.

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

Kaul, M., Yu, H., Ron, Y., and Dougherty, J. P. (1998). Regulated lentiviral packaging cell line devoid of most viral *cis*-acting sequences. *Virology* **249,** 167–174.

Keller, W. (1995). No end yet to messenger RNA 3′ processing! *Cell* **81,** 829–832.

Kim, V. N., Mitrophanous, K., Kingsman, S. M., and Kingsman, A. J. (1998). Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J. Virol.* **72,** 811–816.

MacDonald, C. C., Wilusz, J., and Shenk, T. (1994). The 64-kilodalton subunit of the CstF polyadenylation factor binds to pre-mRNAs downstream of the cleavage site and influences cleavage site location. *Mol. Cell. Biol.* **14,** 6647–6654.

Martin, M., Noel, D., and Piechaczyk, M. (1997). Towards efficient cell targeting by recombinant retroviruses. *Mol. Med. Today* **3,** 396–403.

Miller, A. D., Miller, D. G., Garcia, J. V., and Lynch, C. M. (1993). Use of retroviral vectors for gene transfer and expression. *Methods Enzymol.* **217,** 581–599.

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

Miyoshi, H., Takahashi, M., Gage, F. H., and Verma, I. M. (1997). Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94,** 10319–10323.

Motmans, K., Thirion, S., Raus, J., and Vandevyver, C. (1997). Isolation and quantification of episomal expression vectors in human T cells. *Biotechniques* **23,** 1044–1046.

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

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., and Trono, D. (1996b). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.

Olson, P., Nelson, S., and Dornburg, R. (1994). Improved self-inactivating retroviral vectors derived from spleen necrosis virus. *J. Virol.* **68,** 7060–7066.

Poeschla, E., Corbeau, P., and Wong-Staal, F. (1996). Development of HIV vectors for anti-HIV gene therapy. *Proc. Natl. Acad. Sci. USA* **93,** 11395–11399.

Poeschla, E. M., Wong-Staal, F., and Looney, D. J. (1998). Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat. Med.* **4,** 354–357.

Reicin, A. S., Kalpana, G., Paik, S., Marmon, S., and Goff, S. (1995). Sequences in the human immunodeficiency virus type 1 U3 region required for *in vivo* and *in vitro* integration. *J. Virol.* **69,** 5904–5907.

Robinson, D., Elliott, J. F., and Chang, L.-J. (1995). Retroviral vector with a CMV-IE/HIV-TAR hybrid LTR gives high basal expression levels and is up-regulated by HIV-1 Tat. *Gene Ther.* **2,** 269–278.

Srinivasakumar, N., Chazal, N., Helga-Maria, C., Prasad, S., Hammarskjold, M. L., and Rekosh, D. (1997). The effect of viral regulatory protein expression on gene delivery by human immunodeficiency virus type 1 vectors produced in stable packaging cell lines. *J. Virol.* **71,** 5841–5848.

Temin, H. M. (1990). Safety considerations in somatic gene therapy of human disease with retrovirus vectors. *Hum. Gene Ther.* **1,** 111–123.

Valsamakis, A., Zeichner, S., Carswell, S., and Alwine, J. C. (1991). The human immunodeficiency virus type 1 polyadenylation signal: A 3′ long terminal repeat element upstream of the AAUAAA necessary for efficient polyadenylation. *Proc. Natl. Acad. Sci. USA* **88,** 2108–2112.

Verma, I. M., and Somia, N. (1997). Gene therapy—Promises, problems and prospects. *Nature* **389,** 239–242.

Vicenzi, E., Dimitrov, D. S., Engelman, A., Migone, T. S., Purcell, D. F., Leonard, J., Englund, G., and Martin, M. A. (1994). An integration-defective U5 deletion mutant of human immunodeficiency virus type 1 reverts by eliminating additional long terminal repeat sequences. *J. Virol.* **68,** 7879–7890.

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J. Virol.* **72,** 9873–9880.

Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nat. Biotechnol.* **15,** 871–875.