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Ψ⁻ Vectors: Murine Leukemia Virus-Based Self-Inactivating and Self-Activating Retroviral Vectors

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We have developed murine leukemia virus (MLV)-based self-inactivating and self-activating vectors to show that the previously demonstrated high-frequency direct repeat deletions are not unique to spleen necrosis virus (SNV) or the neomycin drug resistance gene. Retroviral vectors pKD-HTTK and pKD-HTpTK containing direct repeats composed of segments of the herpes simplex virus type 1 thymidine kinase (HTK) gene were constructed; in pKD-HTpTK, the direct repeat flanked the MLV packaging signal. The generation of hypoxanthine-aminopterin-thymidine-resistant colonies after one cycle of retroviral replication demonstrated functional reconstitution of the HTK gene. Quantitative Southern analysis indicated that direct repeat deletions occurred in 57 and 91% of the KD-HTTK and KD-HTpTK proviruses, respectively. These results demonstrate that (i) deletion of direct repeats occurs at similar high frequencies in SNV and MLV vectors, (ii) MLV Ψ can be efficiently deleted by using direct repeats, (iii) suicide genes can be functionally reconstituted during reverse transcription, and (iv) the Ψ region may be a hot spot for reverse transcriptase template switching events.

Innovative gene therapy approaches to cancer treatment have become feasible within the last few years. One promising approach involves the utilization of metabolic suicide genes in gene therapy (12, 33, 34). Various drug-dependent suicide genes, such as the herpes simplex virus type 1 thymidine kinase (HTK) gene, the cytosine deaminase gene, and the purine nucleotide phosphorylase gene, are currently being investigated for their effectiveness in tumor eradication (12, 34, 35, 42).

The potential of toxic gene therapy with the HTK gene was first demonstrated by retrovirus-mediated delivery of the HTK gene to rat glioblastomas (12). Administration of the prodrug ganciclovir (GCV) to treated rats resulted in eradication of the glioblastomas. GCV is a nucleoside analog that, upon phosphorylation, can be incorporated into a growing DNA chain. Only glioblastoma cells expressing the HTK gene can phosphorylate GCV, and subsequent incorporation of the phosphorylated GCV into DNA leads to chain termination and cell death. Clinical trials based on these and other studies are in progress (11, 16, 25–27). Initial reports indicate substantial tumor reductions (9).

Approximately 63% of the currently approved gene therapy clinical trials utilize retroviral delivery vectors (2). Even though the retroviral vectors currently used are replication defective and do not express viral proteins, safety concerns remain (3, 7, 8, 18, 38, 43, 44). One primary concern is that viral packaging cells used to make infectious viral stocks of therapeutic vectors may harbor a replication-competent retrovirus (RCR) (6, 29). Recent reports indicate that even second-generation packaging cell lines, which are designed to suppress recombination events by expressing viral proteins from different constructs, generate RCRs (6, 29). The pathogenic potential of RCRs was recently heightened when a preparation of bone marrow stem cells that had been transduced with retroviral vectors was contaminated with RCRs. After autologous transplantation of the stem cells, 3 of 10 rhesus monkeys developed rapid, progressive T-cell lymphomas (39, 45). These observations raise safety concerns. Some of these concerns can be addressed by the development of safer retroviral vectors which will reduce the risk of RCR generation and spread of the therapeutic vector to nontargeted cells.

Self-inactivating U3⁻ retroviral vectors were previously developed to address this concern (15, 36). Removing the U3 sequence from the 3′ long terminal repeat (LTR) in the original retroviral vector resulted in a final integrated provirus lacking the U3 sequence in both the 5′ and 3′ LTRs after completion of reverse transcription. Without a 5′ U3 promoter, the expression of full-length viral RNA was inhibited, allowing the gene of interest to be expressed from an internal promoter. Unfortunately, these vectors were found to have a high rate of recombination during transfection and cell propagation, which regenerated the U3 sequence (37).

We have developed self-inactivating and self-activating spleen necrosis virus (SNV)-based retroviral vectors that utilize the high frequency of direct repeat deletion during reverse transcription (23). Large direct repeats of 1,333, 788, and 383 bp from the neomycin phosphotransferase (*neo*) gene were placed in a retroviral vector flanking the encapsidation sequence (E). Deletion of one copy of the direct repeat, along with the intervening sequence E, resulted in deletion frequencies of 93, 85, and 40%, respectively. Deletion of the 383-bp direct repeat resulted in reconstitution of a functional *neo* gene and deletion of E. Mobilization of this E-minus (E⁻) vector by a replication-competent reticuloendotheliosis virus was reduced 267,000-fold (23). These studies indicated that self-inactivating direct repeat vectors may be useful for delivering toxic genes more safely to targeted cells.

Murine leukemia virus (MLV)-based vectors are widely used in human gene therapy clinical trials (2, 9, 10). Before self-inactivating vectors can be used to improve the safety and effectiveness of human gene therapy, it is necessary to demonstrate that the high frequency deletion of direct repeats is not a unique property of SNV reverse transcriptase or specific to *neo*. In this report, we describe the development of MLV-based self-inactivating and self-activating retroviral vectors for

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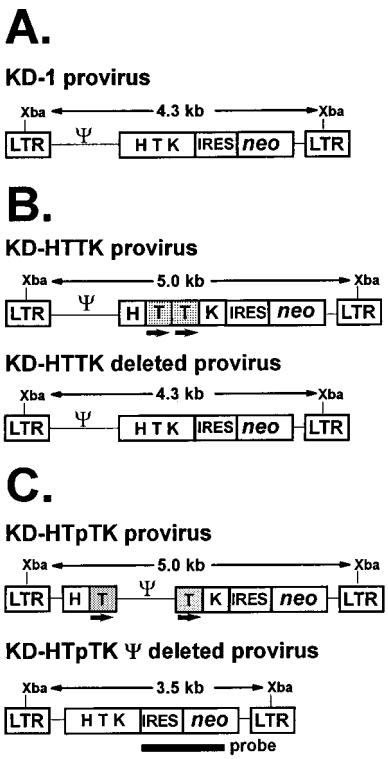

FIG. 1. Structures of MLV-based retroviral vectors. (A) pKD-1 contains the HTK gene and *neo* expressed from an IRES. Restriction digestion of genomic DNAs with *Xba*I, which cuts in the LTRs, is expected to generate a 4.3-kb band from the KD-1 proviruses. (B) pKD-HTTK was derived by inserting two overlapping fragments of the HTK gene, creating a 701-bp direct repeat (shaded boxes underlined with arrows). Restriction digestion of genomic DNAs with *Xba*I is expected to generate a 5.0-kb band from the proviruses without deletions and a 4.3-kb band from the proviruses with deletions. (C) pKD-HTpTK contains the same overlapping fragments, except that the HT fragment was placed 5′ of the MLV packaging sequence (Ψ). Restriction digestion of genomic DNAs with *Xba*I is expected to generate a 5.0-kb band from the proviruses without deletions and a 3.2-kb band from the proviruses with deletions. The black bar below the provirus with the deletion indicates the 1.2-kb IRES-*neo* fragment used as a probe for Southern analysis.

suicide gene therapy utilizing direct repeats derived from the HTK gene. Interestingly, the results suggest that MLV Ψ may play a role in increasing template switching events.

Construction of retroviral vectors with direct repeats. Three MLV-based retroviral vectors were constructed to determine the efficiency of direct repeat deletion (Fig. 1). All of the vectors were constructed by using standard molecular cloning procedures (41). pKD-1, pKD-HTTK, and pKD-HTpTK are plasmids, whereas KD-1, KD-HTTK, and KD-HTpTK are the viruses derived from these plasmids. To generate pKD-1, a 1,343-bp fragment containing the HTK gene was excised from a derivative of plasmid pME123 (17) and inserted into pWH390, a derivative of pLAEN (1), 5′ of the encephalomyocarditis virus internal ribosomal entry site (IRES) fused to *neo* from Tn*5* (20–22). To construct pKD-HTTK, a 994-bp fragment containing the 5′ two-thirds of the HTK gene (the HT fragment) was isolated and inserted into pWH390 5′ of the IRES to generate pKD-HT. Next, a 1,044-bp fragment containing the 3′ two-thirds of HTK (the TK fragment) was isolated and inserted between the HT fragment and the IRES of pKD-HT to generate pKD-HTTK. pKD-HTTK contains a 701-bp direct repeat composed of the middle one-third of HTK (the T portion). To construct pKD-HTpTK, the same HT fragment used to generate pKD-HTTK was inserted 5′ of Ψ in pWH390 to generate pKD-HTp. The same TK fragment used to generate pKD-HTTK was inserted between MLV Ψ and the IRES to generate pKD-HTpTK. pKD-HTpTK contains the same 701-bp direct repeat present in pKD-HTTK, but the direct repeat flanks MLV Ψ. In all three vectors, *neo* was expressed from a bicistronic mRNA derived from the 5′ LTR promoter through internal translational initiation at the IRES.

Protocol used to determine the frequency of direct repeat deletion by comparison of virus titers. Retroviral constructs pKD-1, pKD-HTTK, and pKD-HTpTK were separately transfected into PG13 helper cells (obtained from the American Type Culture Collection). PG13 is an MLV-based helper cell line expressing MLV *gag-pol* and the gibbon ape leukemia virus envelope (31). Since the gibbon ape leukemia virus receptor is not present in murine cells, reinfection of the helper cells is not possible. These transfected cells were then selected for resistance to G418, an analog of neomycin (600 μg/ml; 0.87 mM). The transfections were carried out by the previously described dimethyl sulfoxide-Polybrene method (24).

To determine the relative titers of KD-1, KD-HTTK, and KD-HTpTK, approximately 500 G418-resistant colonies containing plasmids encoding each vector were separately pooled and expanded. For each construct, 2.5×10^6 G418-resistant, transfected helper cells were plated on 100-mm-diameter dishes and the culture medium was changed 24 h later. Virus was then harvested 24 h later, serially diluted, and, in the presence of Polybrene (50 μg/ml), used to infect 143B, a thymidine kinase-deficient human osteosarcoma cell line (obtained from the American Type Culture Collection). Infected 143B cells were subjected to selection with either G418 (400 μg/ml; 0.58 mM) or hypoxanthine-aminopterin-thymidine (HAT; as specified by Boehringer Mannheim Biochemicals), and drug-resistant colonies were counted to determine virus titers. Approximately 2,000 HAT- and G418-resistant colonies were separately pooled. The PG13 and 143B cells were maintained in Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with penicillin (50 U/ml; Gibco), streptomycin (50 μg/ml; Gibco), and bovine calf serum (10% for PG13 and 6% for 143B; HyClone Laboratories).

Comparison of G418- and HAT-resistant colonies after virus infections. Virus titers were determined by quantitation of G418-resistant, as well as HAT-resistant, 143B colonies after infection (the data are summarized in Table 1). Six independent infections of 143B cells were performed with KD-1, nine independent infections were performed with KD-HTTK, and three independent infections were performed with KD-HTpTK. All of the KD-1, KD-HTTK, or KD-HTpTK proviruses are expected to confer resistance to G418, regardless of whether or not they underwent direct repeat deletion. All of the KD-1 proviruses are also expected to confer resistance to HAT. On the other hand, only the KD-HTTK and KD-HTpTK proviruses that underwent direct repeat deletion are expected to reconstitute a functional HTK gene and confer resistance to HAT. Therefore, the ratio of HAT-resistant colonies to G418-resistant colonies provides a measure of the frequency of direct repeat deletion.

A comparison of the HAT- and G418-resistant colony titers obtained from KD-1 indicated that the HAT-resistant colony titers were consistently higher than the G418-resistant colony titers. The average HAT-resistant colony titer of KD-1 was 370×10^3 CFU/ml, whereas the average G418-resistant colony titer was 220×10^3 CFU/ml; the average ratio of HAT-resistant to G418-resistant colony titers was approximately 1.8. Since all of the KD-1 proviruses should confer resistance to both HAT and G418, this difference in the titers most likely

TABLE 1. Virus titers after infection with KD-1, KD-HTTK, or KD-HTpTK

Expt no. and drug selection	Virus titer (10^3 CFU/ml)		
	KD-1	KD-HTTK	KD-HTpTK
1			
HAT	380	40	7.4
G418	180	30	3.4
2			
HAT	240	160	22
G418	110	160	10
3			
HAT	380	71	2.5
G418	280	69	5.2
4			
HAT	400	ND[a]	ND
G418	190	ND	ND
5			
HAT	380	66	ND
G418	300	86	ND
6			
HAT	410	97	ND
G418	230	68	ND
7			
HAT	ND	250	ND
G418	ND	260	ND
8			
HAT	ND	82	ND
G418	ND	95	ND
9			
HAT	ND	72	ND
G418	ND	25	ND
Avg titer			
HAT	370	110	11
G418	220	100	6
Avg $HAT^r/G418^r$ ratio[b]	1.8	1.3	1.6
% with deletions		72	89

[a] ND, not determined.
[b] Average ratios of HAT- and G418-resistant colonies were determined for each experiment, and then the ratios were averaged.

reflects the different sensitivities of 143B cells to the HAT and G418 drug selections. Similarly, the HAT- and G418-resistant colony titers obtained from KD-HTTK were compared. The average HAT-resistant colony titer of KD-HTTK was 110×10^3 CFU/ml, and the average G418-resistant colony titer was 100×10^3 CFU/ml; the average ratio of HAT-resistant to G418-resistant colony titers was approximately 1.3. Based on the results obtained with KD-1, we expected the HAT-resistant colony titer to be 1.8-fold higher than the G418-resistant colony titer if 100% of the KD-HTTK proviruses had undergone direct repeat deletion and reconstitution of the HTK gene. Therefore, the 1.3-fold higher HAT-resistant colony titer for KD-HTTK suggested that approximately 72% of the KD-HTTK proviruses ($1.3 \div 1.8 \times 100\%$) had undergone direct repeat deletion and reconstitution of the HTK gene. Finally, the HAT- and G418-resistant colony titers of KD-HTpTK were also compared. The average HAT-resistant colony titer of KD-HTpTK was 11×10^3 CFU/ml, and the average G418-resistant colony titer was 6×10^3 CFU/ml. The KD-HTpTK titers were approximately 36-fold lower than the KD-1 titers (34-fold and 37-fold for the HAT- and G418-resistant colony titers, respectively). The lower titers of KD-HTpTK suggested that perhaps insertion of the HT fragment upstream of Ψ interfered with viral RNA packaging or initiation of reverse transcription. The average ratio of HAT- to G418-resistant colony titers of KD-HTpTK was 1.6, suggesting that approximately 89% of the KD-HTpTK proviruses ($1.6 \div 1.8 \times 100\%$) had undergone direct repeat deletion and reconstitution of the HTK gene. These results also suggested that the Ψ region may be a hot spot for template switching events since the presence of Ψ between the direct repeats consistently yielded a higher deletion frequency.

Determination of KD-HTTK direct repeat deletion frequency by Southern analysis. Deletion frequencies in KD-HTTK were also determined by Southern blot hybridization (Fig. 2A). Genomic DNAs from pools of G418- and HAT-resistant cells were separately isolated and digested with *Xba*I by standard procedures (41). Proviral structures were characterized by Southern analysis. *Xba*I cuts in each LTR and is expected to generate a 5.0-kb band from proviruses with no direct repeat deletion and a 4.3-kb band from proviruses with direct repeat deletions (Fig. 1B). A 1.2-kb IRES-*neo* DNA fragment was used to generate a probe by the Random Priming DNA-labeling Kit (Boehringer Mannheim Biomedicals) with [α^{32}P]CTP (specific activity, $>10^9$ cpm/μg; ICN Biomedicals). The membrane was exposed to a PhosphorImager cassette (Molecular Dynamics) and X-ray film. Quantitation of bands was performed by using the ImageQuant program (Molecular Dynamics).

Southern hybridization analysis of three independent KD-HTTK infections was performed. Since all of the proviruses used can confer G418 resistance, both the 5.0- and 4.3-kb bands from proviruses without and with direct repeat deletions, respectively, were observed in pools of G418-resistant cells (Fig. 2A, G lanes). The intensities of the 5.0- and 4.3-kb bands were quantitated by PhosphorImager analysis (data not shown); the ratios of the 4.3-kb bands with direct repeat deletions to the 5.0-kb bands without direct repeat deletions from the three independent experiments indicated deletion frequencies of 72, 70, and 73%. Therefore, the KD-HTTK direct repeat deletion frequency determined by Southern analysis (avg 72%) was identical to the deletion frequency determined by comparison of HAT- and G418-resistant colony titers (72%). Only the 4.3-kb band derived from proviruses that underwent direct repeat deletion was detectable in pools of HAT-resistant colonies (Fig. 2A, H lanes). The lack of a detectable 5.0-kb band in the pools of HAT-resistant colonies indicated that HAT resistance required deletion of the direct repeat and reconstitution of a functional HTK gene.

Additional bands smaller than 4.3 kb were also detected in pools of G418-resistant cells (Fig. 2A, G lanes). These bands resulted from inactivating deletions from the HTK gene, since none of these bands were detectable in pools of HAT-resistant cells (Fig. 2A, H lanes). Transfection of mammalian cells is a mutagenic process (5), and it was hypothesized that these deletions occurred during transfection of the PG13 cells with pKD-HTTK DNA.

Deletion of the direct repeat and reconstitution of the HTK gene may also have occurred during transfection of the PG13 helper cells with pKD-HTTK DNA. To determine the proportion of direct repeat deletions that occurred during transfection, genomic DNA from a pool of transfected PG13 helper

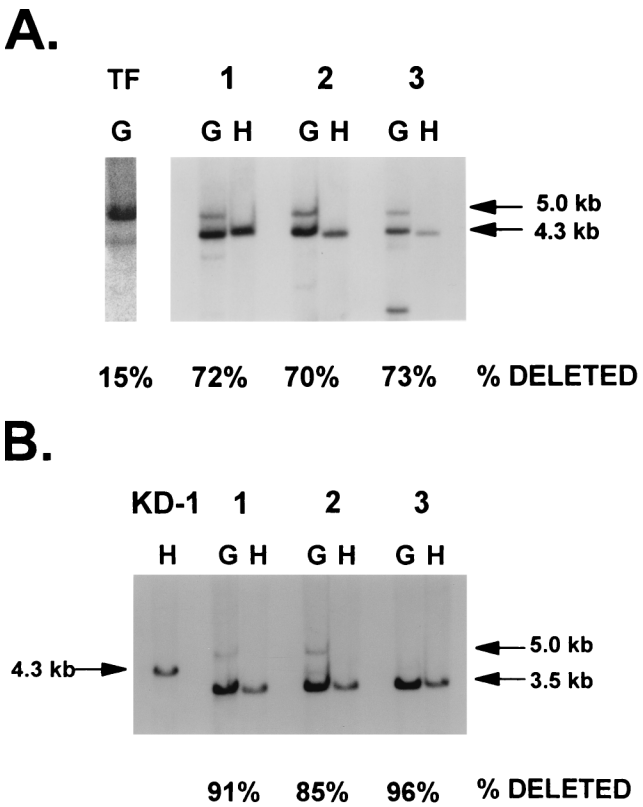

FIG. 2. Southern analysis of proviral DNAs from KD-HTTK- and KD-HTpTK-infected pools of G418- and HAT-resistant colonies. (A) Analysis of three independent sets of KD-HTTK-infected pools of 143B cells. In each experiment (sets of lanes numbered 1 to 3), pools of G418- and HAT-resistant colonies were generated (lanes labelled G and H, respectively). Restriction digestion with *Xba*I of the genomic DNAs from the G418-resistant pools generated a 5.0-kb band from proviruses without deletions and the 4.3-kb band from proviruses with deletions (Fig. 1B). The intensities of the 5.0-kb band from the provirus with deletions and the 4.3-kb band from the provirus with deletions were quantified by PhosphorImager analysis (ImageQuant program). The percentage below each lane refers to the fraction of the proviruses which generated the 4.3-kb band. The genomic DNAs from the HAT-resistant pools generated only the 4.3-kb band from the proviruses with deletions. Southern analysis of pooled PG13 helper cell clones transfected with KD-HTTK (lane TF) indicated that direct repeat deletion occurred in 15% of the KD-HTTK vectors during transfection and selection for G418 resistance. (B) Analysis of three independent sets of KD-HTpTK-infected pools of 143B cells. In each experiment (sets of lanes numbered 1 to 3), pools of G418- or HAT-resistant colonies were generated (lane G or H, respectively). Restriction digestion of the genomic DNAs from the G418-resistant pools with *Xba*I generated a 5.0-kb band from proviruses without deletions and a 3.5-kb band from proviruses with deletions (Fig. 1C). The intensities of the 5.0-kb band from proviruses without deletions and the 3.5-kb band from proviruses with deletions were quantified by PhosphorImager analysis (ImageQuant program). The percentage below each lane refers to the fraction of proviruses that generated the 3.5-kb band. The genomic DNAs from the HAT-resistant pools generated only the 3.5-kb band from the proviruses with deletions. Southern analysis of KD-1-infected cells generated a 4.3-kb band (lane KD-1). The KD-1 structure shown in Fig. 1 will generate the same proviral band as the KD-HTTK provirus with deletions.

cells was also analyzed by Southern blot hybridization (Fig. 2A, TF lane). A ratio of the 5.0-kb band without direct repeat deletions and the 4.3-kb band with direct repeat deletions indicated that during transfection and selection for G418 resistance, direct repeat deletion occurred in 15% of the pKD-HTTK DNAs. Assuming that the vectors with and without deletions are transmitted with equal efficiencies, the frequency of direct repeat deletion during one cycle of reverse transcription was 57% (72% total minus 15% during transfection). It is of note that the structure of the KD-HTTK vector after deletion of the direct repeat is identical to that of KD-1. Comparison of the virus titers obtained with the KD-1 and KD-HTTK vectors (Table 1) indicates that the two vectors are transmitted at nearly equal efficiencies and the average titers are within twofold of each other. Therefore, the KD-HTTK vectors with and without deletions should also be transmitted at similar efficiencies. A few additional faint bands were also obtained with the PG13 cells (Fig. 3B, TF lane); these bands probably resulted from deletions and recombination events occurring during transfection (5).

Determination of KD-HTpTK deletion frequency by Southern analysis. The KD-HTpTK deletion frequency was also determined by Southern blot hybridization (Fig. 2B). Again, the genomic DNAs isolated from the cell pools were digested with *Xba*I. A 5.0-kb band was expected from proviruses without direct repeat deletions, and a 3.5-kb band was expected from proviruses with deletions of the direct repeat and MLV Ψ (Fig. 1C).

Southern hybridization analysis of three independent KD-HTpTK infections was performed by using the IRES-*neo* fragment as a probe. As expected, 5.0-kb bands without deletions and 3.5-kb bands with deletions were obtained with pools of G418-resistant cells (Fig. 2B, G lanes). Quantitative analysis (data not shown) of the 5.0-kb band without deletions and the 3.5-kb bands with deletions from three independent experiments indicated deletion frequencies of 91, 85, and 96%. Therefore, the KD-HTpTK deletion frequency determined by Southern analysis (average, 91%) was very close to the deletion frequency determined by comparison of the HAT- and G418-resistant colony titers (89%). Only the 3.5-kb band derived from proviruses with direct repeat deletions was detectable in pools of HAT-resistant colonies (Fig. 2B, H lanes). The lack of a detectable 5.0-kb band in the pools of HAT-resistant colonies indicated that HAT resistance requires direct repeat and Ψ deletions, as well as HTK gene reconstitution.

Direct repeat deletion of 15% of the KD-HTpTK plasmids also occurred during transfection, as determined by quantitative Southern analysis of transfected helper cells (data not shown). However, direct repeat deletion during transfection is expected to generate KD-HTpTK plasmids that lack Ψ; therefore, any mRNAs transcribed from the plasmids with deletions are not expected to be packaged into virions. Since the plasmids with deletions are not expected to be packaged and reverse transcribed into proviruses, they are not expected to contribute to the 3.5-kb band with deletions. Therefore, the frequency of deletion determined by Southern analysis was not adjusted by the deletions that occurred during transfection. Again, we observed additional bands in pools of G418-resistant cells (Fig. 2B, G lanes), which we hypothesize were generated during the transfection process (5).

Proviruses with deletions of Ψ are inefficiently mobilized by a helper virus. The efficiency of mobilization of proviruses with deletions of Ψ was determined as outlined in Fig. 3. Pools of HAT-resistant 143B cells generated by infection with KD-HTpTK or KD-1 were plated separately at 2×10^5 cells per 60-mm-diameter dish (five dishes per pool) and transfected with 10 μg of wild-type, replication-competent MLV DNA (pAMS; kindly provided by A. D. Miller), and the virus was allowed to spread in the culture for 5 days. The transfected cells were then pooled, and the virus was harvested and used to infect fresh 143B cells, which were then subjected to HAT selection. The virus titer was determined by the number of HAT-resistant colonies.

The KD-1 proviruses contain Ψ and should be efficiently mobilized to the target cells by the wild-type MLV. However, KD-HTpTK proviruses in HAT-resistant cells are expected to

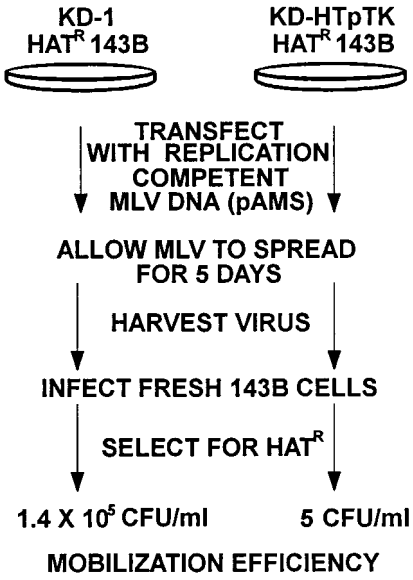

FIG. 3. Mobilization of proviruses with and without Ψ. Pools of KD-1- and KD-HTpTK-infected, HAT-resistant 143B cells were separately transfected with pAMS, a wild-type MLV. The replication-competent MLV was allowed to spread in the culture for 5 days, and then the transfected cells were pooled. The virus was harvested and used to infect fresh 143B cells. The cells were subjected to HAT selection, and virus titers were determined. The KD-1-infected pools generated a HAT-resistant colony titer of 1.4×10^5 CFU/ml, whereas the KD-HTpTK-infected pool generated a titer of only 5 CFU/ml.

lack the Ψ sequence and should not be mobilized. The KD-1 proviruses were mobilized with a titer of 1.4×10^5 CFU/ml, whereas the KD-HTpTK proviruses were mobilized with a titer of 5 CFU/ml. Therefore, the KD-HTpTK proviruses with deletions of Ψ were mobilized at a 28,000-fold lower efficiency than the KD-1 proviruses containing Ψ. The low-level mobilization of KD-HTpTK proviruses suggests that all of the viral sequences necessary for packaging of the viral RNA were not removed by the deletion.

Direct repeats are deleted at high frequencies in MLV-based vectors. The results obtained in this study show that directly repeated sequences are deleted at very high frequencies in MLV-based retroviral vectors. The results are consistent with previous observations that direct repeats were deleted at high frequencies in SNV-based retroviral vectors (23). Since direct repeats composed of the HTK gene were used in this study, the results indicate that high-frequency deletion and reconstitution of a functional gene are not specific to *neo*. Different direct repeats were used in the two studies; therefore, direct comparison of the frequencies of direct repeat deletion is not possible. However, these studies suggest that direct repeats are deleted from MLV- and SNV-based retroviral vectors at similar frequencies. Previously, a 788-bp direct repeat composed of *neo* was deleted 85% of the time in one replication cycle of an SNV vector (11% per 100 bp). In this study, a 701-bp direct repeat composed of the HTK gene was deleted at a frequency of 89% in a single replication cycle of an MLV-based vector (13% of 100 bp). (The results obtained with KD-HTpTK were used for comparison because the direct repeats in both the MLV and SNV vectors flanked the encapsidation regions.) Therefore, directly repeated sequences were deleted from both SNV and MLV vectors at similar frequencies of approximately 11 and 13% per 100 bp during each replication cycle, respectively.

A template misalignment model has been proposed to explain the high frequency of direct repeat deletions (23). This model suggests that during minus-strand synthesis, the growing DNA strand and the associated reverse transcriptase have a high propensity to dissociate from the RNA template. The RNase H activity of reverse transcriptase degrades the template RNA approximately 18 to 20 nucleotides behind the site of polymerization. Therefore, the nascent DNA strand and the template RNA are held together by only a few hydrogen bonds (18 to 20 bp). The reduced base pairing is thought to promote dissociation of the nascent DNA and the reverse transcriptase from the template. Once dissociated, the nascent DNA and reverse transcriptase may reassociate with the point of dissociation or with the homologous point in the 5′ direct repeat. Reassociation with the 5′ direct repeat may be favored by increased base pairing between the nascent DNA and the RNA template, since this portion of the template RNA has not yet been degraded by RNase H. As a result, direct repeat deletion may occur at a high rate and the resulting provirus will lack one copy of the direct repeat and intervening sequences between the direct repeats.

Suicide genes can be functionally reconstituted during reverse transcription. The results of this study show that the HTK gene could be functionally reconstituted during reverse transcription. These experiments have proven the principle that direct repeats can be utilized to delete any sequence or efficiently reconstitute inactivated genes during reverse transcription. Self-activating retroviral vectors may be useful for development of other suicide genes for cancer therapy. Many suicide genes, such as that for diphtheria toxin or ricin, do not require the presence of a prodrug and may be useful for the treatment of cancers or human immunodeficiency virus type 1 infection (4, 13, 19, 32). However, the toxicity of these genes to the virus producer cells has prevented their development as therapeutic agents. It may be possible to develop self-activating retroviral vectors that prevent expression of these suicide genes in the virus-producing cells and reconstitute them in the target cancer cells during reverse transcription.

MLV Ψ can be efficiently deleted by using direct repeats. The results of this study show that MLV Ψ can be efficiently deleted during reverse transcription. MLV Ψ was deleted in 89% of the proviruses in a single replication cycle when it was flanked by a 701-bp direct repeat. Insertion of the HT fragment upstream of Ψ resulted in a 36-fold reduction in both HAT- and the G418-resistant colony titers. The reduction in virus titers was greater than the previously observed fivefold reduction when Ψ was moved to the 3′ end of a replication-competent MLV (28), suggesting that some sequences have a greater or lesser effect on viral packaging or reverse transcription.

Interestingly, we observed a substantial increase in the deletion frequency when MLV Ψ was present between directly repeated sequences. The results suggest that Ψ may be a hot spot for template switching events. It was recently observed that the dimer linkage site (DLS), the region of Ψ involved in dimerization of the two copackaged RNAs, was the site of intermolecular template switches in 20 of 22 proviruses analyzed (30). However, the template switching events observed in this study occurred within the directly repeated portion of the HTK gene that was several hundred base pairs away from the DLS region. Therefore, the mechanism by which MLV Ψ increased these template switching events is unclear. Presumably, the structure of the viral RNAs at the DLS promoted intramolecular and/or intermolecular template switches within the HTK gene.

Regardless of the mechanism, MLV Ψ was efficiently deleted during a single replication cycle. Deletion of Ψ efficiently inactivated the resulting proviruses and reduced their mobili-

zation to other target cells in the presence of a helper virus 28,000-fold. These self-inactivating retroviral vectors can significantly improve the safety of gene therapy by preventing spread of the therapeutic vector to nontarget tissues. Preventing spread of the therapeutic vector to nontarget tissues may be important when suicide genes are utilized for therapy or when it is important to achieve tissue-specific gene expression.

Other strategies for inactivation of retroviral vectors have been described. Utilization of U3$^-$ vectors, which have the 5′ LTR promoter deleted in the final integrated provirus, has been limited by the fact that a high percentage of these vectors undergo recombination during transfection and cell propagation, regenerating the U3 sequences (36). Recently, the P1 phage site-specific recombinase (Cre) was utilized to inactivate retroviral vectors (40). However, introduction of a recombinase into host cells, which may not be eliminated from some integrated proviruses, has raised other potential safety concerns.

Another potential benefit to utilizing these self-inactivating vectors is that efficient deletion of MLV Ψ suppresses the generation of replication-competent retroviruses in the virus-producing cells. One of the essential events in RCR formation is the acquisition of Ψ by the helper constructs present in the packaging cells. Analysis of the structures of several RCRs has indicated that the Ψ regions are often acquired from the propagated retroviral vectors (3, 14, 39). Therefore, retroviral vectors that efficiently delete Ψ should reduce the probability of the helper constructs capturing Ψ and suppress RCR formation. The effect of these self-inactivating retroviral vectors on the generation of RCRs and the mechanism by which MLV Ψ influences template switching are being investigated.

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