JOURNAL OF VIROLOGY, Oct. 1999, p. 8837–8842 Vol. 73, No. 10
0022-538X/99/$04.00+0

Development of Murine Leukemia Virus-Based Self-Activating Vectors That Efficiently Delete the Selectable Drug Resistance Gene during Reverse Transcription

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Received 18 March 1999/Accepted 6 July 1999

Expression of the selectable drug resistance gene in retroviral vectors used for gene therapy can lead to a decreased expression of the gene of interest and may induce a host immune response, resulting in a decreased efficiency of gene therapy. In this study, we demonstrate that high-frequency deletion of direct repeats, an inherent property of reverse transcriptases, can be used to efficiently excise the drug resistance gene during reverse transcription. One retroviral vector containing a direct repeat deleted the neomycin resistance expression cassette during a single replication cycle at >99% efficiency.

Most retroviral vectors used for gene therapy are based on murine leukemia virus (MLV) and express a gene of interest as well as a drug resistance gene. A drug resistance gene is typically necessary for selection of virus-producing helper cells or cell clones so that high virus titers can be achieved. The bacterial neomycin phosphotransferase gene (*neo*) is commonly used as a selectable marker in retroviral vectors (35). The expression of two genes can be achieved by using the viral long terminal repeat (LTR) promoter to express the gene of interest and an internal promoter to express the drug resistance gene. Expression of the gene of interest and the drug resistance gene from different promoters often results in promoter interference and reduction of the expression of either gene in comparison to the levels of expression from vectors bearing only one gene (1, 4). These differences have been attributed to promoter interference between the 5′ LTR promoter and the internal promoter and to the downregulation of the LTR promoter in certain cell types (1, 2, 4, 11, 12, 32). Usually, the nonselected gene of interest is expressed at a level lower than that of the selected drug resistance gene. Alternatively, an internal ribosomal entry site (IRES) can be used instead of an internal promoter to express two gene products from one RNA transcript (18–20). In one study, however, expression of the IRES-*neo* cassette from a retroviral vector reduced the expression of the nonselected genes of interest (5). Therefore, removal of the drug resistance gene along with its promoter or translational control regions from the vector during infection may lead to an enhanced and sustained expression of the gene of interest.

It is also desirable to remove the drug resistance gene from target cells during gene therapy so that unnecessary expression of a foreign protein can be avoided. Expression of the drug resistance gene in cells can lead to development of a host immune response against the transduced cells, which may reduce the long-term efficacy of gene therapy (25, 33). Specific T-cell responses to components of the retroviral vector, such as the product of the selectable marker gene hygromycin phosphotransferase B, have been observed (22).

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The Cre/*loxP* recombination system from bacteriophage P1 was recently used to delete a neomycin resistance expression unit from proviruses in transduced hematopoietic cells (13). In this system, it is necessary that the infected target cells express the Cre recombinase protein and that the *loxP* sites flank the drug resistance expression unit. The Cre recombinase can be expressed either from the retroviral vector bearing the gene of interest or from a separate expression vector. The effects of long-term expression of the Cre recombinase in target cells on the stability of the human genome are unclear. While this system may be applicable to ex vivo gene therapy approaches, its usefulness in the deletion of the drug resistance gene in the context of in vivo gene therapy may be limited because of the need for expression of the Cre recombinase in the target cells. Additionally, the frequency of deletion of the *neo* resistance expression unit was observed to be 74% (13). It was hypothesized that frequent rearrangements in the retroviral vectors resulted in the loss of one or both *loxP* sites, which reduced the efficiency of excision.

Directly repeated sequences have been shown to be deleted accurately and at high frequencies in both spleen necrosis virus- and MLV-based retroviral vector systems (9, 21, 30). It has long been observed that directly repeated sequences found within retroviral genomes are unstable (8, 17, 28, 31, 36). Deletion of direct repeats occurs during reverse transcription and involves the viral reverse transcriptase dissociating from one copy of the direct repeat and reassociating with the homologous sequence in the second copy of the direct repeat (10, 21).

We previously used the high frequency of direct-repeat deletion to develop self-inactivating and self-activating vectors based on both MLV and spleen necrosis virus (9, 21). It was shown that directly repeated sequences could be used to efficiently delete the viral packaging signal and functionally reconstitute *neo* or the herpes simplex virus thymidine kinase gene (HTK) during reverse transcription. A 701-bp direct repeat composed of overlapping fragments of HTK was deleted at a rate of 57% and functionally reconstituted HTK in one replication cycle (9). When the same direct repeat flanked the MLV encapsidation sequence (Ψ), the deletion frequency increased to 91%. The provirus in the infected target cells lacked Ψ and expressed a functional HTK.

In this study, we sought to establish whether direct repeats

could be used to efficiently delete drug resistance genes and their control regions. Previous studies indicated that direct repeats could be used to delete at least 818 bp of viral sequence (length of Ψ) during reverse transcription (9). Most of the drug resistance genes and their control regions range in length from approximately 1.5 kb (for example, the simian virus 40 promoter plus the hygromycin B phosphotransferase gene [14]) to 4 kb (for example, the simian virus 40 promoter plus the Na^+-K^+ ATPase gene that confers resistance to ouabain [23]). The effect of increasing linear distance between direct repeats on the frequency of deletion was unknown. Therefore, it was not clear whether direct repeats could be used to delete longer sequences encoding drug resistance genes and their control regions. In this report, we demonstrate that direct repeats can be used to delete the *neo* selectable marker and its translational control region at >99% efficiency.

Construction of MLV-based retroviral vectors. To determine the efficiency of using direct repeats for deletion of selectable markers, the vectors pKD-HTneoTK and pKD-HTΨneoTK were constructed by standard procedures (see Fig. 1A and 2A) (34). Viruses derived from these vectors are named KD-HTneoTK and KD-HTΨneoTK, respectively. A detailed description of all cloning steps is available upon request.

Protocol to determine the deletion frequency of KD-HTneoTK and KD-HTΨneoTK after one round of viral replication. Vectors pKD-HTneoTK and pKD-HTΨneoTK (10 μg each per 60-mm-diameter dish) were transfected into PG13 helper cells by calcium phosphate precipitation as previously described (34). PG13 cells were subjected to G418 selection (an analog of neomycin) at a final concentration of 600 μg/ml (0.87 mM; Gibco). Approximately 2,500 G418-resistant colonies were separately pooled and expanded from each transfection. For each vector, virus was harvested from transfected G418-resistant cells and used to infect 143B target cells plated at a density of 2×10^5 cells per 60-mm-diameter dish in the presence of Polybrene (50 μg/ml) as previously described (16). The target 143B cells are a TK-deficient human osteosarcoma cell line (obtained from the American Type Culture Collection). Infected 143B cells were subjected to either G418 (400 μg/ml, 0.58 mM) or hypoxanthine-aminopterin-thymidine (HAT; as specified by Boehringer Mannheim) selection 1 day after infection. Two weeks later, drug-resistant colonies were counted and viral titers were determined from results of four to eight independent experiments. All 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).

Frequency of direct-repeat deletion determined by viral titers obtained from pools of G418-resistant packaging cells. The results of infections with viruses derived from pools of G418-resistant packaging cells are summarized in Table 1. The G418 titers represent the population of viruses that did not undergo a direct-repeat deletion, whereas the HAT titers represent the population of viruses that underwent direct-repeat deletion and reconstituted a functional HTK. Therefore, the frequency of direct-repeat deletion and reconstitution of HTK was determined by dividing the HAT titers by the sum of the G418 and HAT titers. KD-HTneoTK and KD-HTΨneoTK underwent direct-repeat deletion and excised the IRES-*neo* cassette with average deletion frequencies of 91% ± 0.8% and 93% ± 1.8%, respectively. Thus, the frequencies of deletion observed were consistent between independent experiments. The high frequency of deletion was consistent with our recent observation that increasing distance between direct repeats generally increases the frequency of deletion (10).

TABLE 1. Virus titers after infection with KD-HTneoTK or KD-HTΨneoTK pools

Expt	Drug selection	KD-HTneoTK		KD-HTΨneoTK	
		Virus titer (CFU/ml)	Deletion frequency (%)[a]	Virus titer (CFU/ml)	Deletion frequency (%)
1	G418	8.6×10^2		23	
	HAT	8.3×10^3	91	3.9×10^2	94
2	G418	9.2×10^2		22	
	HAT	1.1×10^4	92	3.9×10^2	95
3	G418	9.9×10^2		34	
	HAT	9.2×10^3	90	5.9×10^2	95
4	G418	9.4×10^2		33	
	HAT	9.2×10^3	91	4.1×10^2	93
5	G418	ND[b]		130	
	HAT	ND		1.2×10^3	90
6	G418	ND		47	
	HAT	ND		1.0×10^3	96
7	G418	ND		34	
	HAT	ND		4.4×10^2	93
8	G418	ND		25	
	HAT	ND		3.8×10^2	94
Avg	G418	9.3×10^2		44	
	HAT	9.4×10^3	91[c]	6.0×10^2	94[c]

[a] The deletion frequency was determined by dividing the HAT titer by the sum of the HAT titer plus the G418 titer.
[b] ND, not determined.
[c] The mean ± standard deviation for the KD-HTneoTK deletion frequency was 91% ± 0.8%. The mean ± standard deviation for the KD-HTΨneoTK deletion frequency was 94% ± 1.8%.

Structures of deleted and undeleted KD-HTneoTK proviruses determined by Southern blot analysis. Genomic DNAs were isolated from pools of infected 143B cells that were selected for either G418 or HAT resistance and analyzed by Southern hybridization by standard procedures (34). The pools of cells were derived from infections with low dilutions of virus (infected with undiluted or 10-fold-diluted virus) and contained at least 2,000 G418- or HAT-resistant 143B cell colonies. A 1.3-kb DNA fragment encoding the HTK gene was used to generate a probe with [α-^{32}P]dCTP (specific activity, $>10^9$ cpm/μg; ICN Biomedicals) with a Random Priming DNA-Labeling Kit (Boehringer Mannheim). The expected structures of the deleted and undeleted proviruses derived from KD-HTneoTK are shown in Fig. 1A. Results of a representative Southern blot analysis of genomic DNAs derived from pools of cells infected with KD-HTneoTK and digested with *Xba*I are shown in Fig. 1B. The expected 5.0-kb undeleted band was detected in genomic DNAs from two independent pools of G418-resistant cells (G418 lanes A and B). The expected 2.9-kb deleted band was also detected in genomic DNAs from two independent pools of HAT-resistant cells (HAT lanes A and B). As expected, the 2.9-kb deleted band was not detectable in G418-resistant cells and the 5.0-kb undeleted band was not detectable in HAT-resistant cells. Faint additional bands that ranged in size from 3 to 5 kb were detected in G418-resistant cells (G418 lanes A and B). The process of transfection is mutagenic, and these bands may have resulted from deletions and rearrangements during transfection of the packaging cells (6). The results of these experiments indicated that the IRES-*neo* cassette was efficiently deleted from proviruses that were selected for resistance to HAT. However, it was possible that some of the HAT-resistant cells contained two proviruses, one conferring resistance to HAT

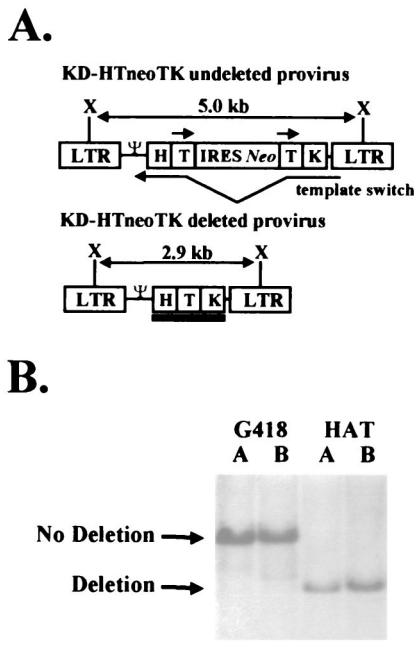

FIG. 1. Structures and results of Southern blot analysis of KD-HTneoTK proviruses. (A) KD-HTneoTK contains overlapping fragments of HTK (labeled "HT" and "TK") which create a 701-bp direct repeat (arrows above boxes labeled "T") flanking the IRES-*neo* expression cassette (1.4 kb). Genomic DNA digested with *Xba*I (X) is expected to generate a 5.0-kb band from undeleted proviruses and a 2.9-kb band from deleted proviruses. The black bar below the deleted provirus indicates the 1.3-kb HTK probe used for Southern blot analysis. The arrow below the undeleted provirus depicts the direct-repeat deletion event during reverse transcription that leads to functional reconstitution of the HTK gene. (B) Southern blot analysis of KD-HTneoTK from two independent pools of G418- and HAT-resistant 143B cells (lanes A and B). Restriction digestion of proviral DNAs with *Xba*I is expected to generate a 5.0-kb undeleted band from the G418-resistant pools (labeled "No Deletion") and a 2.9-kb deleted band from the HAT-resistant pools (labeled "Deletion").

and the other conferring resistance to G418. Based on the sensitivity of Southern blot analysis, <10% of the HAT-resistant cells were infected with another virus that retained the IRES-*neo* cassette.

Most KD-HTneoTK-infected cells that are resistant to HAT are sensitive to G418. To more accurately determine the frequency of proviruses that retained the IRES-*neo* cassette in cells selected for resistance to HAT, HAT-resistant cells were plated at low densities and subjected to G418 selection (Table 2). HAT-resistant 143B cells obtained from infections with low dilutions of virus (undiluted and 10-fold-diluted virus; multiplicity of infection [MOI], <0.005) were seeded at low densities of 20, 40, or 80 cells per 60-mm-diameter dish (experiment 4 with KD-HTneoTK [Table 1]). Sixty dishes were plated for each cell density, and 30 dishes each were subjected to either HAT or G418 selection. A total of 3,896 HAT-resistant colonies and 157 G418-resistant colonies were obtained from cells infected with low dilutions of virus. The results indicated that approximately 4% of the HAT-resistant cells were also resistant to G418. In addition, HAT-resistant 143B cells obtained from infections with high dilutions of virus (100- and 1,000-fold-diluted virus; MOI, <0.00005) were seeded at a density of 10 cells per 60-mm-diameter dish. Sixty dishes were plated, and 30 dishes each were subjected to either HAT or G418 selection. A total of 303 HAT-resistant colonies were obtained from cells infected with high dilutions of virus. In contrast to the results obtained with cells that were infected with low dilutions of virus, G418-resistant colonies were undetectable in pools

TABLE 2. Selection of HAT-resistant KD-HTneoTK 143B cells for resistance to G418

MOI[a]	No. of cells plated/dish[b]	No. of HAT resistant cell clones/30 dishes	No. of G418 resistant cell clones/30 dishes	% of G418 resistant cell clones
<0.005	20	453	16	3.5
	40	1,130	29	2.6
	80	2,313	112	4.8
	Total	3,896	157	4.0
<0.00005	10	303	0	<0.3[c]

[a] The MOI for cells selected for resistance to G418. The MOI for low dilutions of virus (undiluted or 10-fold-diluted virus) was calculated by dividing the G418 titer with the number of infected cells. For undiluted virus, the MOI was determined by the formula 9.4×10^2 CFU/ml $\div$ 2×10^5 cells (i.e., <0.005). The MOI of high dilutions of virus (100- and 1,000-fold-diluted virus) was determined by the formula 9.4 CFU/ml $\div$ 2×10^5 cells (i.e., <0.00005).

[b] HAT-resistant cells were counted, diluted, and plated at the indicated cell densities. The numbers of HAT-resistant colonies obtained were lower than expected because the plating efficiency was less than 100%.

[c] Fewer than 1 of 303 cell clones (<0.3%) were resistant to G418.

that were infected with high dilutions of virus. Therefore, <0.3% (<1/303) of the HAT-resistant cells that were infected with high dilutions of virus were resistant to G418.

The HAT-resistant cells that were also G418 resistant most likely contained two proviruses, one conferring resistance to HAT and one conferring resistance to G418. Cells infected with low dilutions of virus are expected to contain a higher proportion of doubly infected cells than cells infected with high dilutions of virus. The frequencies of G418-resistant cells obtained were consistent with the expected frequencies of double infection.

Structures of deleted and undeleted KD-HTΨneoTK proviruses determined by Southern blot analysis. Genomic DNAs were isolated from pools of KD-HTΨneoTK-infected 143B cells that were selected for either G418 or HAT resistance and analyzed by Southern hybridization (34). Each pool of HAT-resistant cells contained at least 4,000 independent colonies. However, because of the low number of G418-resistant colonies obtained, each pool of G418-resistant cells contained approximately 300 to 400 independent colonies. The genomic DNAs were digested with *Xba*I and analyzed by Southern blot analysis. The expected structures of the deleted and undeleted proviruses derived from KD-HTΨneoTK are shown in Fig. 2A. The results of a representative Southern blot analysis of genomic DNAs derived from pools of cells infected with KD-HTΨneoTK are shown in Fig. 2B. The expected 5.0-kb undeleted band was detected in genomic DNAs from two pools of G418-resistant cells (G418 lanes A and B). The expected 2.1-kb deleted band was also detected in genomic DNAs from two pools of HAT-resistant cells (HAT lanes A and B). As expected, the 2.1-kb deleted band was not detectable in G418-resistant cells and the 5.0-kb undeleted band was not detectable in HAT-resistant cells. These results indicated that the IRES-*neo* cassette was efficiently deleted from proviruses that were selected for resistance to HAT. Based on the sensitivity of Southern blot analysis, >90% of the proviruses had deleted the IRES-*neo* cassette.

In addition to the expected 5.0-kb undeleted band, the pools of G418-resistant cells also contained two other bands of high intensity that were 4.0 and 4.3 kb in length (Fig. 2B, G418 lanes A and B). There were two possible explanations for these

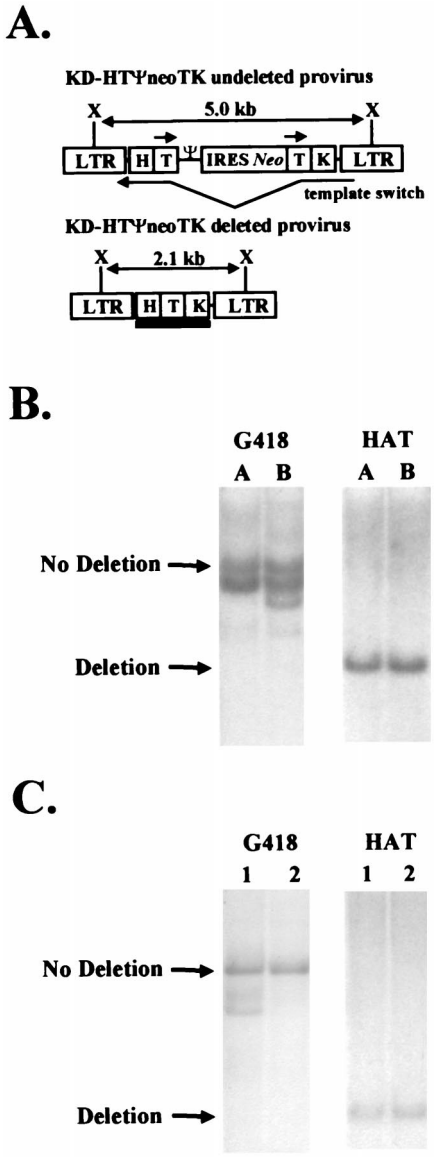

FIG. 2. Structures and results of Southern blot analysis of KD-HTΨneoTK proviruses. (A) KD-HTΨneoTK contains overlapping fragments of HTK (labeled "HT" and "TK") which create a 701-bp direct repeat (arrows above boxes labeled "T") flanking the MLV packaging sequence (Ψ) and the IRES-*neo* expression cassette (2.2 kb). Genomic DNA digested with *Xba*I (X) is expected to generate a 5.0-kb band from undeleted proviruses and a 2.1-kb band from deleted proviruses. The black bar below each deleted provirus indicates the 1.3-kb HTK probe used for Southern blot analysis. The arrow below the undeleted provirus depicts the direct-repeat deletion event during reverse transcription that leads to functional reconstitution of the HTK gene. (B) Southern blot analysis of KD-HTΨneoTK from two independent pools of G418- and HAT-resistant 143B cells (lanes A and B). Restriction digestion of proviral DNAs with *Xba*I is expected to generate a 5.0-kb undeleted band (labeled "No Deletion") from the G418-resistant pools and a 2.1-kb deleted band (labeled "Deletion") from the HAT-resistant pools. (C) Southern blot analysis of pools of 143B cells infected with virus derived from single-cell clones of PG13 cells containing a KD-HTΨneoTK provirus (PG13 clones 1 and 2). Restriction digestion of proviral DNAs with *Xba*I is expected to generate a 5.0-kb undeleted band (labeled "No Deletion") from the G418-resistant 143B pools and a 2.1-kb deleted band (labeled "Deletion") from the HAT-resistant 143B pools.

additional bands. First, these bands may have resulted from deletions or rearrangements during the mutagenic process of transfection (6). The pools of G418-resistant cells were composed of fewer colonies (<400) than the pools of HAT-resistant cells (>4,000), and it was possible that some cells containing aberrant proviral structures were selectively amplified during expansion of the pools. Second, the additional bands may have resulted from aberrant splicing of the viral RNA in the packaging cells.

TABLE 3. Virus titers after infection with KD-HTΨneoTK PG13 helper cell clones

Clone	Drug selection	Virus titer (CFU/ml)	% Deleted[a]
1	G418	9	
	HAT	2.1×10^3	>99
2	G418	19	
	HAT	1.6×10^3	99
3	G418	32	
	HAT	1.3×10^3	98
Avg	G418	20	
	HAT	1.7×10^3	99[b]

[a] Percentages of proviruses in target cells with the IRES-*neo* cassette deleted were determined by dividing the HAT titer by the sum of the G418 titer plus the HAT titer.

[b] The mean ± standard deviation for the deletion frequency of KD-HTΨneoTK was 99% ± 0.6%.

Analysis of PG13 helper cell clones containing KD-HTΨneoTK. To determine whether the aberrant bands observed in the G418-resistant pools were the result of mutagenic transfection or aberrant splicing, single-cell helper clones of KD-HTΨneoTK were isolated. The vector, pKD-HTΨneoTK, was transfected into PA317 helper cells, an amphotropic MLV helper cell line (26). Virus from pools of G418-resistant cells composed of greater than 600 colonies was harvested and used to infect PG13 helper cells. Twelve G418-resistant PG13 cell clones were isolated and screened by Southern blot analysis to verify the vector structure by three different restriction enzyme digestions (data not shown). Additionally, each helper cell clone was shown to contain only one KD-HTΨneoTK provirus by Southern blot analysis (data not shown). Only 3 of the 12 cell clones analyzed contained an intact provirus, and the remaining 9 cell clones contained proviruses that exhibited deletions and rearrangements.

Deletion frequency of KD-HTΨneoTK clones determined by viral titers. Virus was harvested from the three KD-HTΨneoTK PG13 clones containing an intact provirus and used to infect 143B cells as previously described. The infected 143B cells were subjected to either G418 or HAT selection, and the virus titers were determined (Table 3). The high HAT-resistant titers and low G418-resistant titers indicated that direct-repeat deletion and reconstitution of a functional HTK occurred at a high rate. The deletion frequencies ranged from 98 to >99%, indicating that the vast majority of proviruses in target cells had deleted the IRES-*neo* cassette. Therefore, helper cell clones provided a higher frequency of deletion (99%) than pools of helper cells (94%; $P = 0.002$, two-sample t test).

Deletion frequencies of KD-HTΨneoTK clones determined by Southern blot analysis. Pools of 143B cells infected with virus were derived from two of the PG13 cell clones. At least 2,000 HAT-resistant colonies and <400 G418-resistant colonies were separately pooled and expanded. Genomic DNAs isolated from the pools were digested with *Xba*I and analyzed by Southern blotting (Fig. 2C). Similar to the results obtained from infections with pools of helper cells (Fig. 2B), the ex-

pected 5.0-kb undeleted band was detected in genomic DNAs from two pools of G418-resistant cells (Fig. 2C, G418 lanes 1 and 2). The expected 2.1-kb deleted band was also detected in genomic DNAs from two pools of HAT-resistant cells (HAT lanes 1 and 2). As expected, the 2.1-kb deleted band was not detectable in G418-resistant cells and the 5.0-kb undeleted band was not detectable in HAT-resistant cells.

Similar to the results obtained from infections with pools of helper cells, the G418-resistant cells also contained other bands of high intensity that were 4.0 and 4.3 kb in length (G418 lanes 1 and 2). These additional bands were not the result of mutagenic transfection since the KD-HTΨneoTK provirus in the PG13 helper cell clones had the expected structure. Furthermore, since a single intact provirus was present in each cell clone, the additional bands resulted, at least partially, from aberrant splicing of the viral RNA in the helper cell clones.

Further consideration of the structure of the KD-HTΨneoTK provirus supports the notion that aberrant splicing is responsible for the additional bands observed in pools of G418-resistant cells. During MLV replication, a full-length mRNA used for expression of *gag-pol* and a spliced mRNA used for expression of *env* are generated (7). The full-length viral mRNA is packaged into virions, but the spliced message is not packaged because it lacks the Ψ. The MLV splice donor site that is used to express the viral *env* is located just 5′ to the HT fragment. This splice donor site can utilize any cryptic splice acceptor sites that may be present in the HT fragment to give rise to spliced RNAs. These spliced RNAs are expected to contain Ψ and are therefore expected to be efficiently packaged into virions. Identification of the 4.3- and 4.0-kb proviral bands in Fig. 2C (G418 lanes 1 and 2) suggests that spliced RNAs lacking approximately the first 700 and 1,000 bp of HT, respectively, were generated. Furthermore, reverse transcription of these spliced RNAs should result in proviruses that can confer resistance to G418 but not HAT, since a portion of the HT fragment is deleted. Therefore, the resulting G418-resistant titers for KD-HTΨneoTK do not fully represent the population of viruses that did not undergo direct-repeat deletion. This population also contains spliced RNAs that were unable to undergo direct-repeat deletion but still retained resistance to G418. Thus, the frequency of direct-repeat deletion for KD-HTΨneoTK is likely to be higher than indicated in Table 3 (>99%).

In conclusion, self-activating and self-inactivating retroviral vectors containing directly repeated sequences can be used to efficiently delete drug resistance genes and their control regions from retroviral vectors during the course of reverse transcription. Deletion of the selectable marker gene and its control regions may be used to prevent promoter interference and increase the expression of the therapeutic gene. Deletion of the selectable marker may also decrease the probability of eliciting an immune response against the therapeutically infected cells.

The efficiency of deletion of IRES-*neo* (>99%) with direct repeats was higher than the 74% efficiency observed with the Cre/*loxP* system (13). The advantages of using direct repeats for deletion of selectable markers are that it is not necessary to express potentially harmful proteins in the target cells or include *loxP* sequences in the vector, which are subject to a high rate of mutation. Therefore, direct-repeat vectors can be used to easily delete the selectable markers in the course of in vivo gene therapy.

The directly repeated sequences may be derived from any source, including the therapeutic gene that is being delivered to target cells. We have observed that direct repeats derived from several different sequences, including those of HTK, *neo*, bacterial β-galactosidase, green fluorescent protein, and the M13 bacteriophage, delete at very high rates (references 3, 9, 15, 21, and 30 and data not shown). Therefore, it is very likely that overlapping fragments derived from any gene of interest can be used to delete the selectable marker genes from retroviral vectors. This strategy can be used to ensure that the provirus in the target cell will express only the gene of interest.

It should be noted that it is possible to generate high-titer stocks of retroviral vectors lacking selectable markers by using highly transfectable packaging cell lines and transient transfection (24, 27, 29, 37). Nevertheless, it may be desirable to generate helper cell clones containing an integrated provirus. Since the process of transfection is known to be mutagenic (6), it is possible that a high proportion of the retroviral vectors produced from these systems express mutated gene products. Therefore, construction of stable helper cell clones could be used to verify the integrity of the gene of interest before infection of target cells. In addition, stable packaging cell clones may provide better quality control, since variations between different transfections can be avoided.

Future studies will be aimed at improving the virus titers of these vectors by selection of high-titer-producing cell clones, deletion of the viral splice donor site, and insertion of the HT fragment at different locations upstream of the Ψ. These manipulations should improve the viral titers by at least 100-fold.

We especially thank Wei-Shau Hu for her intellectual input throughout the project. We also thank Jeffrey Anderson, Ben Beasley, Sara Cheslock, Que Dang, Elias Halvas, Carey Hwang, and Wenhui Zhang for helpful comments on the manuscript and for valuable discussion of the results.

This work was supported by Public Health Service grant CA58875 from the National Institutes of Health and American Cancer Society grant VM84706 to V.K.P.

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