

High Efficiency Retroviral Vectors with Improved Safety

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ABSTRACT: Almost all currently available retroviral vectors based on murine leukemia virus (MLV) contain one or more viral coding sequences. Because these sequences are also present in the packaging genome, it has been suggested that homologous recombination may occur between the same nucleotide sequence in the packaging genome and the vector, resulting in the production of replication competent retrovirus (RCR). Up until now, it has been difficult to completely remove viral coding sequences since some were thought to be involved in the optimum function of the retroviral vector. For example, the gag coding sequence present in almost all available retroviral vectors has been believed to be necessary for efficient viral packaging, while the pol coding sequence present in the highly efficient vector MFG has been thought to be involved in achieving the high levels of gene expression. However, we have now developed a series of retroviral vectors that are absent of any retroviral coding sequences but produce even higher levels of gene expression without compromising viral titer. In these vectors, the intron and exon sequences from heterologous cellular or viral genes are present. When compared to the well known MLV-based vectors, some of these newly developed vectors have been shown to produce significantly higher levels of gene expression for a longer period. In an experimental system that can maximize the production of RCR, our newly constructed vectors produced an absence of RCR. These vectors should prove to be safer than other currently available retroviral vectors containing one or more viral coding sequences.

Key Words: MLV, Retroviral vector, Splicing, Heterologous intron/exon

I. INTRODUCTION

Murine leukemia virus (MLV)-based vectors are the most frequently used gene delivery vehicles, being employed in more than 50% of the approved clinical protocols worldwide (The Journal of Gene Medicine website [http://www.wiley.co.uk/genetherapy/clinical/vectors.html]). However, there are still many problems with retroviral vectors currently in clinical use. In particular, the issue of safety has often been raised, mainly because of the possibility of the generation of replication-competent retrovirus (RCR). The mechanism of RCR generation is not yet completely understood, but it is thought to result from homologous recombination between the packaging genome and the vector (Miller *et al.*, 1986; Muenchau *et al.*, 1990; Otto *et al.*, 1994). Indeed, almost all currently available retroviral vectors contain significantly long viral coding sequences corresponding to *gag*, *pol* and *env*

(Fig. 1). For example, the LN series vectors, that are often considered the first generation retroviral vectors, contain the 420 bp *gag* coding sequence (Miller and Roseman, 1989). Although this region has previously been thought to contain the information necessary for viral packaging (Armentano *et al.*, 1987; Bender *et al.*, 1987), our recent data suggests that the N-terminal *gag* sequence could be deleted without any significant effects on viral titer and gene expression in certain environments (Kim *et al.*, 1989). Another type of vector called MFG expresses a gene of interest as a spliced message at high levels (Fig. 1). Although MFG has been shown to drive stable, high levels of gene expression *in vivo* as well as *in vitro* and produce high viral titers (Byun *et al.*, 1996; Kral *et al.*, 1995), this vector contains even more viral coding sequences, 420 bp for *gag*, 377 bp for *pol* and 99 bp for *env* (Dranoff *et al.*, 1993; Ohashi *et al.*, 1992), raising the possibility of an even higher frequency of producing RCR than the LN series vector.

We recently reported construction of retroviral vectors

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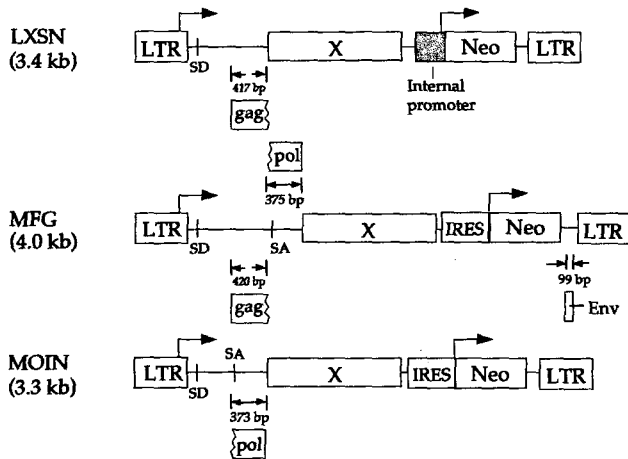


Fig. 1. Viral coding sequences present in currently available MLV-based retroviral vectors. LN series vectors contain the 420 bp *gag* coding sequence (Miller and Roseman, 1989). MFG contains all viral coding sequences; 420 bp for *gag*, 377 for *pol* and 99 for *env* (Dranoff *et al.*, 1993, Ohashi *et al.*, 1992). In MOIN, the 377 bp *pol* coding region is included (Kim *et al.*, 1998). Retroviral vectors and coding sequences were drawn in approximate scale only.

with improved safety, gene expression and versatility (Kim *et al.*, 1998). The unique feature of these vectors was the total absence of the *gag* coding sequence as well as the *env* coding sequence without compromising viral titers, and therefore increasing the safety of the vector (Fig. 1). However, this vector still contains the 377 bp *pol* coding sequence which harbors the splice acceptor (SA) sequence as well as its downstream sequence containing the 284 bp leader (transcribed but untranslated) sequence for *env* (Fig. 1). This *pol* coding sequence is also present in all available packaging lines, again increasing the frequency of homologous recombination which could result in RCR. Now we can report on the successful construction of retroviral vectors that contain no viral coding sequences but still manage to drive high levels of gene expression in transduced target cells. These vectors should prove to be safer than other currently available retroviral vectors that all contain one or more viral coding sequences.

II. MATERIALS AND METHODS

1. Cells

NIH3T3 (CRL1658), and Phoenix Ampho (Kinsella and Nolan, 1996) (SD3443) were obtained from the American Type Culture Collection (Rockville, MD,

U.S.A.). NIH3T3 was grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% calf serum. Phoenix Ampho, and FLYA13 (Cosset *et al.*, 1995) were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). Each medium used in this study was supplemented with 120 µg/ml penicillin G (Sigma P-3032; 1690 U/mg) and 200 µg/ml streptomycin sulfate (Sigma S-9137; 750 U/mg).

2. Plasmids

Many plasmids used in this study were constructed by polymerase chain reaction (PCR) using proof-reading *Pfu* DNA polymerase (Stratagene, USA). The nucleotide sequences of final constructs were always determined to confirm that there were no mutations introduced by this amplification step. The retroviral vector MIN, which does not have any viral coding sequences was constructed as follows. pMLV (Shinnick *et al.*, 1981) was used for amplification of 5 and 3 LTR regions. The nucleotide sequences of primers used in amplifying the 5LTR region of MLV are as follows (the restriction linkers attached to each primer are underlined):

HHIR: AAGCTTATGTGAAAGACCCCTCCTG
HindIII

5LTR3: GGATCCGCGGGCCACGCGTATTTTCA-
BamHI SacII ApaI MluI
GACAAATACAGAAAC

The amplified product covered the 5' LTR and 5' non-coding regions containing packaging signals of MLV. The amplified HindIII-BamHI fragment was cloned into pUC 18, generating p5LTR. To amplify 3LTR region, PCR was performed using primers 3LTR5, and 3LTR3.

3LTR5: GGATCCTCGAGGATAAAATAAAAGATTTT-
BamHI XhoI
ATTTAGTCTCC

3LTR3: GAATTCAATGAAAGACCCCGCTGAC
EcoRI

The amplified product covered the entire 3' untranslated region downstream from the stop codon of *env*, containing polypurine tract and 3'LTR. The amplified

BamHI-EcoRI fragment was then cloned in p5LTR, resulting in pM, retroviral backbone. To insert the IRES/NEO cassette into pM, the BamHI-BamHI EMCV IRES/NEO fragment from pCBIN (Kim *et al.*, 1998) was filled in by the klenow fragment, and then inserted into the filled-in BamHI-XhoI site of pM, resulting in pMIN.

To construct MIN-AI, and MIN-EI which have cellular intron and splice acceptors, PCR was performed with genomic DNA from adult human peripheral blood mononuclear cells using the following primer pairs:

EF5: ACGCGTGGCAATTGAACCGGTGCCTAGAG-
MluI
AAGGTAGG
EF3: GCTAGCTTTGGCTTTTAGGGGTAGTTTTCA-
NheI
CGACAC
Act5: ACGCGTGCCAGCAGCACCCCAAGGCGGC-
MluI
CACCGCCAAA
Act3: GCTAGCGGTGAGCTGCGAGAATAGCCGG-
NheI
GCGCGCTGT

The amplified products covered the promoter, exon1, intron, and partial exon2 of human EF-1 α gene, and human β -actin gene respectively. The amplified MluI-NheI fragments were used to replace MluI-NheI fragment of pC3.1 (Invitrogen, USA) resulting in pEF, and pbact, respectively. The Klenow treated-XhoI-BamHI fragment of pEF (from +772 to +1008 of human EF-1a gene) was inserted into the T4 polymerase treated-ApaI-BamHI site of MIN, generating MIN-EI. The Klenow-treated BglI-BamHI fragment of pbact (from +717 to +849 of human β actin gene) was inserted into the T4 polymerase treated-ApaI-BamHI site of MIN, resulting in MIN-AI.

To construct MIN-2, HCMV major IE promoter region was amplified using pEQ276 (Biegalka *et al.*, 1991) as a template with CMV5 and CMV3 primers.

CMV5: ACGCGTTGACATTGATTGATTATTG
MluI
CMV3: AAGCTTCGTGTCAAGGACGGT
HindIII

The amplified fragment containing HCMV major

immediate early promoter, exon1, intronA, and exon2 region was used to replace MluI-HindIII fragment of pC3.1, resulting in pCN. The HpaI-BamHI fragment of pCN, which contained 3' region of intron A and 5' region of exon2 (from +837 to +964, just before start codon) was cloned into T4 polymerase treated-ApaI-BamHI site of MIN, resulting in MIN-2.

To construct MPIN, splice acceptor region of MLV was amplified by PCR using pMLV as a template with BGSA, and SABam3 primers.

BGSA: AGATCTTATATGGGGCACC
BglII
SABam3: GGATCCTGTAAGTGAGCTT
BamHI

The amplified BglII-BamHI fragment was cloned into BamHI site of MIN, resulting in MPIN.

To construct the retroviral vectors expressing CAT, the BamHI CAT fragment from PCRII-CAT (Kim *et al.*, 1998) was inserted BamHI site of each MIN-derived vectors, so that the CAT gene was linked with NEO through EMCV IRES.

To construct retroviral vectors expressing human IRAP, IRAP cDNA was cloned from the total RNA of human peripheral blood lymphocytes by RT-PCR. The nucleotide sequences of primers used in this step are as follows:

IRAP5: AAGCTTATGGAAATCTGCAGAGGCCTCC-
HindIII
GCAGTCAC
IRAP3: GTCGACCTACTCGTCCTCCTGGAAGTAG-
SalI
AATTTGGT

The HindIII-SalI fragment of IRAP cDNA was filled in by the Klenow fragment, and then inserted into the filled BamHI site of MIN, and MIN-EI, resulting in MIN-IRAP, and MIN-EI-IRAP respectively.

3. Transfection and Transduction

Phoenix Ampho, or FLYA13 cells were transfected using the FuGene6 (Boehringer Mannheim, USA) according to the manufacturer's instructions. Transfection efficiency was measured in most experiments by

X-gal staining using the same culture plates or duplicate dishes, if necessary. Supernatants from the transfected packaging cells were collected, usually 48 hrs after transfection, filtered through a 0.45 μ m filter, and used for transduction of target cells. To analyze transiently transduced cells, NIH3T3 was transduced with 0.5 ml of viral supernatant in the presence of 8 μ g/ml polybrene for 5 hrs followed by the addition of fresh medium. To determine viral titer, NIH3T3 was also transduced using same method with serially diluted viral supernatant. The following day, the cells were re-fed with a fresh medium. Containing G418, and viral titer was determined as described previously (Kim *et al.*, 1998).

4. Enzyme and Cytokine Assays

CAT assays were performed by standard procedures as previously described by Byun *et al.*⁹ Two days after transfection or transduction, cells were harvested, washed once with PBS, and resuspended in 0.25 M Tris-HCl (pH 7.5). Total proteins were prepared by 4 to 5 cycles of freeze-thaw followed by incubation at 65°C for 7 min. Equivalent amounts of protein were assayed for CAT activity. The percent conversion of ¹⁴C-chloramphenicol to its acetylated forms was determined by quantitating the intensity of each spot with a phosphoimager (FUJIX BAS 1000). The levels of human IRAP were determined by enzyme linked immunoadsorbent assay using the commercially available kits from R and D Systems Inc. (Minneapolis, Minnesota, U.S.A.; DRA00). The activity of β -galactosidase expressed in cells containing the LacZ gene was measured by the ONPG assay. The cells with an introduced LacZ gene were stained with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as described previously (Kim *et al.*, 1998).

5. RNA Blot Hybridization

Total cellular RNAs were prepared by the guanidine thiocyanate-caesium chloride method. The DNA probe used for hybridization was the BamHI CAT fragment of MIN-CAT. As a control for variation in the amount of RNA loaded, the same filter was hybridized with EcoRI fragment of actin cDNA.

6. PCR of Genomic DNA

To test whether retroviral sequence was preserved in transduced target cells, total DNA was prepared by lysing transduced and selected-NIH3T3 cell line using TES (10 mM Tris-HCl; 1 mM EDTA; 0.7% SDS) followed by treatment with 400 μ g/ml proteinase K at 50°C for 1 hour, phenol:chloroform extraction, and ethanol precipitation. The polymerase chain reaction (PCR) was performed using 5 μ g of total genomic DNA and oligonucleotide primers specific to various regions of the retroviral vector as indicated in Fig. 5. The samples were amplified through 30 cycles that included the following parameters; denaturation at 94°C 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min 30 sec. The amplified DNA fragments were analyzed by agarose gel electrophoresis.

7. Helper Virus Assay

To assay the possible presence of RCR, BAG mobilization assay (Pear *et al.*, 1993) was carried out. MIN vector was used for the test. 293T cells were transfected with 15 μ g of retroviral vector and the 30 μ g of packaging construct, pkat2ampac. After 48 hr transfection, viral supernatants from transfected cells were taken and used to infect BAG cells, and the cells were passed three times. Cell free supernatants from passages 3 of infected cells were used to infect NIH3T3 cells, and the infected cells were assayed for β -galactosidase or G418.

III. RESULTS

Among the retroviral vectors shown in Fig. 1, we chose to use MOIN (Kim *et al.*, 1998) as a starting molecule because we previously demonstrated that *gag* and *env* sequences present in LN and/or MFG are not needed for optimum function of the retroviral vector, while the role of the *pol* coding region was not yet clear. We first derived the retroviral vector MPIN from MOIN (Fig. 2), and tested whether the residual *pol* coding region was necessary in the context of the retroviral vector, and also whether intron and untranslated exon sequences from heterologous genes

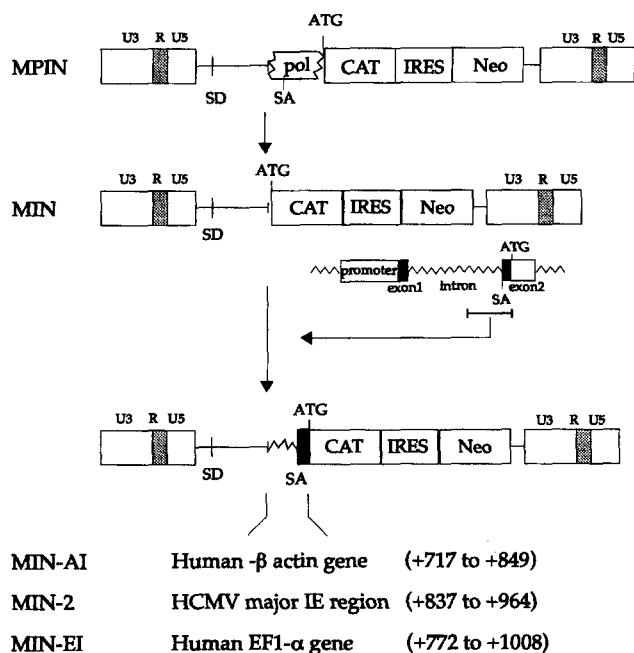


Fig. 2. Construction of retroviral vectors containing heterologous exon and intron sequences. MPIN is a derivative of MOIN. The former contains the entire 5' untranslated leader sequence of *gag*, and extra 17 bp downstream from the stop codon of the *env* gene from MLV. Instead of 373 bp of *pol* sequence of MOIN, MPIN has only 110 bp of *pol* sequence containing SA. In MIN, the entire *pol* coding sequence was removed from MPIN. Various heterologous intron and untranslated exon sequences were isolated from the human β -actin gene, the major immediate early region of HCMV and the human EF-1 α gene as indicated. Coordinates are based on a RNA start site (+1) of each gene. All of these sequences are a part of a 5' untranslated region of the respective genes. They were all isolated by polymerase chain reaction and their nucleotide sequences confirmed by dideoxynucleotide and/or automatic sequencing. A detailed construction procedure is available upon request.

can be used to increase the performance of retroviral vectors, especially viral titer and the level of gene expression. MPIN is a derivative of MOIN. The former contains the entire 5' untranslated leader sequence of *gag*, and extra 17 bp downstream from the stop codon of the *env* gene from MLV. Instead of 373 bp of *pol* sequence of MOIN, MPIN has only 110 bp of *pol* sequence containing SA.

For simplicity of assay, we initially used the bacterial CAT sequence as a reporter gene. All our vectors were designed in such a way that the reporter gene is co-expressed with the bacterial selectable marker *neo* as a bicistronic message using the internal ribosome entry site from EMCV (Byun *et al.*, 1996; Ghattas *et al.*, 1991). The entire nucleotide sequence of MPIN is available from our homepage (<http://plaza.snu.ac.kr/>

~sunyoung/index.htm).

Four retroviral constructs were derived from MPIN. In the retroviral vector MIN, the entire residual *pol* coding sequence was deleted and therefore does not have any viral coding sequences. MIN-AI, MIN-2, and MIN-EI contain, using MIN as a backbone, parts of intron and 5' untranslated exon sequences derived from the +717 to +849 region of the human β -actin gene, the +837 to +964 region of the major immediate early (IE) region of human cytomegalovirus (HCMV), and the +772 to +1008 region of the human elongation factor-1 α (EF-1 α) gene, respectively (Fig. 2). As additional controls, well known retroviral vectors MFG and LXS were also used (Miller and Roseman, 1989; Byun *et al.*, 1996; Dranoff *et al.*, 1993; Ohashi *et al.*, 1992). In the case of MFG, the reporter gene is co-expressed with *neo* as a bicistronic message, similar to the case of the MIN series vectors.

Four retroviral constructs, together with three control vectors, MPIN, MFG and LXS, were transfected to the amphotropic packaging line Phoenix (Kinsella and Nolan *et al.*, 1996). All transfections and subsequent experiments were performed in triplicate per experiment by two different investigators at more than three different times. Culture supernatants were taken 2 days post-transfection and passed through the 0.45 μ m filter. At the same time, the level of CAT activity was measured using transfected packaging lines. This would represent the level of gene expression produced from a given retroviral construct. Cell-free viral supernatants were used to transduce a variety of cell lines including NIH3T3 cells, and the level of CAT activity determined 2 days after transduction. (Because similar results were obtained in most of the cell lines compared, only the results from NIH3T3 cells are shown in this report.) This value would represent both relative viral titer and the level of gene expression from a given construct. Another set of transduced cells were allowed to grow in the presence of G418. In this stable transduction assay, NIH3T3 cells were transduced at an m.o.i. of <0.1, selected with 0.8 mg/ml of G418, and the level of CAT activity was again measured after drug-resistant populations were obtained, using the same amount of protein. Because a low m.o.i. was used causing transduced cells to contain less than a single copy integration of the vector, the level of CAT activity would reflect the

Table 1. Effects of heterologous intron and exon sequences on the performance of MLV-based retroviral vectors

Vector	Relative CAT activity			Viral titer
	Transient transfection	Transduction		
		Transient	Stable	
MIN	1.0	1.0	1.0	1.0
MPIN	1.8±0.3	2.5±0.4	1.5±0.3	0.9±0.1
MIN-AI	0.9±0.2	1.1±0.2	1.1±0.2	0.8±0.2
MIN-2	3.8±0.5	3.7±0.5	4.5±0.5	0.9±0.3
MIN-EI	3.5±0.4	3.3±0.4	3.5±0.4	1.1±0.2
MFG	1.0±0.2	1.1±0.3	0.8±0.2	1.0±0.3
LXSN	0.2±0.1	0.2±0.1	0.2±0.1	0.5±0.2

As described in the text, retroviral vectors were transfected to various packaging lines. (Here only the results from the Phoenix line are summarized). The level of CAT activity and viral titer were determined 2-3 days after transfection. A variety of target cells were transduced with cell-free viral supernatants prepared by passing through 0.45 mm filter. In this figure, only the results from NIH3T3 cells are shown. The level of CAT activity was measured 3 days after transduction ("transient transduction"). In all CAT assays, the same amount of protein extracts were used. All assays were performed in duplicate or triplicate by two different investigators at different times.

actual level of gene expression from a specific construct in transduced target cells and would be less likely to be influenced by differences in gene dosage.

The results from these transfection, transduction, and viral titer assays are summarized in Table 1. All retroviral vectors except for LXSN produced comparable viral titers. (Note that for convenient comparison between vectors containing exon/intron sequences and a vector lacking them, all values were standardized by those from MIN.) However, the introduction of heterologous exon/intron sequences had significant effects on the level of gene expression being produced from retroviral vectors. For example, MIN-2 and MIN-EI, each containing heterologous intron and exon sequences, produced almost 4-fold higher levels of gene expression (in transient transfection assays), while still producing comparable viral titers. High level gene expression from these vectors was evident also when the level of CAT activity was compared between transduced, selected cell lines. Both constructs gave an average of 3-4 fold higher level of CAT activity in G418-resistant cell populations than MIN or MFG.

It appears that exon/intron sequences from various genes work differently in the context of the retroviral vector. For example, MIN-AI, which has the exon/intron sequence from the β -actin gene gave lower lev-

els of gene expression than MIN-2 and MIN-EI, while still being comparable to the control vector MIN and MFG, and averaging 4 times higher than LXSN. Similar results were obtained using 293T cells by the so called 3-plasmid transfection method (Soneoka *et al.*, 1995).

To determine the pattern of RNA expression from new retroviral vectors, we analyzed the quality and quantity of viral RNAs produced in the transduced cells as well as in the transfected packaging cells. First, the packaging line Phoenix Ampho was transfected with retroviral constructs. Total RNA was then prepared and analyzed by Northern hybridization using the CAT sequence as a probe (Fig. 3A). L-CAT-SN showed the genomic transcript alone, while MFG produced two RNA species, a genomic transcript and a spliced RNA (Fig. 3A, lane 1 and 2). In this hybridization analysis, L-CAT-SN showed only the genomic size transcript because a subgenomic RNA would not contain the CAT sequence used as a probe. Similar to MFG, MPIN, which contains the retroviral splice acceptor sequence located in the *pol* gene, generated two RNA species, a genomic transcript and a spliced RNA (Fig. 3A, lane 3). MIN, which does not have a splicing acceptor sequence only produced a genomic transcript (Fig. 3A, lane 4). The intron and splice acceptor sequences from the human EF-1 α gene appeared to work very efficiently, because an extremely large amount of smaller spliced RNAs were produced relative to a longer genomic transcript (Fig. 3A, lane

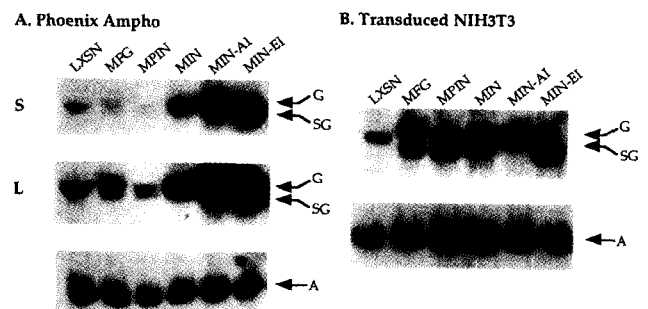


Fig. 3. RNA blot analysis of Phoenix packaging cells transfected with newly constructed retroviral vectors (a) and NIH3T3 cells transduced with cell free viral supernatants from transfected Phoenix cells (b). The Total RNA (20 μ g) was subjected to 1% formaldehyde-agarose gel electrophoresis, blotted to nitrocellulose membrane (Hybond-C; Amersham, RPN303W, US), and hybridized with a 32 P-labeled CAT probe. G, genomic transcript; SG, subgenomic RNA; A, cellular actin RNA; S, short exposed autoradiogram; L, long exposed autoradiogram.

6). In contrast, the intron and splice acceptor sequences from the human β -actin gene did not work efficiently, because in MIN-AI, only a genomic transcript was detectable as in the case of the MIN vector (Fig. 3A, lane 5). Similar results were obtained when the pattern of RNA expression was analyzed in transduced NIH3T3 cells (Fig. 3B). Similar to the RNA pattern in transfected packaging cells, L-CAT-SN, MIN, and MIN-AI showed genomic transcripts alone, while MFG, MPIN, and MIN-EI produced two RNA species, a genomic transcript and a spliced RNA. MIN-2, which contains the intron and splice acceptor from the major IE region of HCMV, produced two RNA species, a genomic transcript and an extremely large amount of smaller spliced RNAs as in the case of the MIN-EI vector (data not shown). It appears that the vectors which could produce larger amounts of a spliced RNA resulted in higher levels of CAT activity relative to other vectors as exemplified by MIN-EI and MIN-2.

To demonstrate that our observation was not restricted to a specific reporter gene, we also introduced the human interleukin 1 receptor antagonist protein (IRAP) (Thompson *et al.*, 1991) cDNA into some of the MIN series retroviral vectors. We chose MIN as a no splicing vector and MIN-EI as a vector producing a large amount of a spliced transcripts. MFG was used as a control. Three retroviral constructs were transfected to the Phoenix Amphi cells and cell free supernatants were taken to transduce NIH3T3 cells. All transductions were performed at a low m.o.i. to ensure less than a single copy of integra-

Table 2. Comparison of expression levels of IRAP

Vector	Relative IRAP activity			Viral titer
	Transient transfection	Transduction		
		Transient	Stable	
MIN	1.0	1.0	1.0	1.0
MIN-EO	15.9±1.9	7.9±3.5	12.5±4.8	0.6±0.1
MFG	4.1±1.4	3.0±2.5	2.2±0.6	1.3±0.2

The experimental conditions were identical to those described in Table 1 except that the level of IRAP activity was measured from cell culture supernatant. G418-resistant cell populations were obtained by transducing NIH3T3 cells at an m.o.i. around 0.1 and the same number of drug-resistant cells were plated on 6 well dishes, grown for another 2 days, and subjected to enzyme-linked immunosorbent assay. Expression from MIN-IRAP was set to 1. All assays are performed in duplicated or triplicate by two different investigators at many different times. The results were similar in all cases. Here, the result from only one representative set of experiments is shown.

Table 3. Comparison of various vectors in FLYA13 packaging line

Vector	Relative CAT activity			Viral titer
	Transient transfection	Transduction		
		Transient	Stable	
MIN	1.0	1.0	1.0	1.0
MIN-AI	0.6±0.2	0.6±0.1	0.7±0.2	1.0±0.2
MIN-2	5.2±0.5	1.0±0.2	2.8±0.3	0.5±0.1
MIN-EI	4.5±0.7	0.6±0.1	2.2±0.5	0.3±0.1
MFG	1.7±0.3	0.8±0.2	0.5±0.1	1.1±0.2

Retroviral vectors were transfected to FLYA13 packaging line. The levels of CAT activity and viral titer were determined 2 days after transfection. NIH3T3 cells were transduced with cell-free viral supernatant. The level of CAT activity was measured 2 days after transduction ("transient transduction") and also after drug-resistant populations were obtained ("stable transduction"). In all CAT assays, the same amounts of protein extracts were used.

tions in a majority of the cells. As shown in Table 2, MIN-EI gave significantly higher levels of IRAP activity than MIN and MFG in both transduced and transfected cells, but produced about 2-fold lower viral titer. This result confirmed the above observation that the efficient splicing was important for high level expression of the transgene. However, unlike in the case of CAT, MIN produced an average of 3 fold lower levels of IRAP than MFG, while viral titers were comparable. It appears that the level of gene expression and viral titer can vary, though not significantly, depending on the nature of nucleotide sequence of the inserted gene.

Effects of heterologous intron/exon sequences on the performance of retroviral vectors varied depending on the packaging lines used. For example, as shown in Table 3, when FLYA13 cells (Cosset *et al.*, 1995) were used, MIN-2 and MIN-EI gave 3-5 times higher levels of gene expression in the transfected packaging line, but produced 2-3 times lower viral titer, thus resulting in little difference in the level of gene expression in transiently transduced cells. However, both MIN-2 and MIN-EI gave almost 3 times higher levels of CAT activity in transduced, selected cell populations. These results suggested that these constructs drove high levels of gene expression due to efficient splicing, which, however, in turn decreased viral titer. Our data from RNA analysis indeed suggests that viral titer may have been decreased because the amount of viral RNA containing the packaging signal sequence ("genomic size" transcript) was too effi-

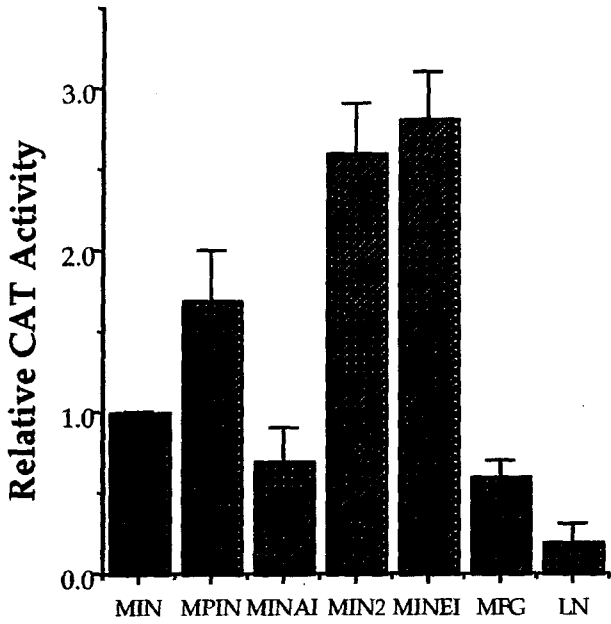


Fig. 4. Comparison of the level of gene expression in long-term passaged transduced lines. NIH3T3 cells were transduced at an m.o.i. of <0.1, and selected with 0.8 mg/ml of G418. After G418-resistant populations were obtained, cells continued to be passed in the absence of G418 by repeated trypsinization and repopulation. After 4 weeks, levels of CAT activity were compared using the same amount of protein extracts. The assays were performed again in triplicate by two investigators using two different origins of drug-resistant populations. Because similar results were obtained, one representative data is shown.

ciently spliced to subgenomic RNA, lowering the amount of packageable RNA (Fig. 3). This result indicates that effects of the splicing efficiency of different intron/exon sequences could vary in different cell lines.

We compared various constructs for the level of gene expression in long-term passaged transduced, selected lines. Once G418-resistant populations were obtained, cells continued to be passed in the absence of drug for another 4 weeks by repeated trypsinization and repopulation. As shown in Fig. 4, the level of CAT activity was significantly higher in cells transduced with MIN-2 and MIN-EI than in those with other retroviral constructs, especially MFG and LXSJ, resulting in an even greater difference between the vectors compared. This result suggested that use of the exon/intron sequence might provide stable gene expression in transduced cells.

We have also tested the stability of the vector in stably transduced cells by PCR. Total DNAs were prepared from transduced cells, which have been cultured more than 10 weeks in the absence of G418,

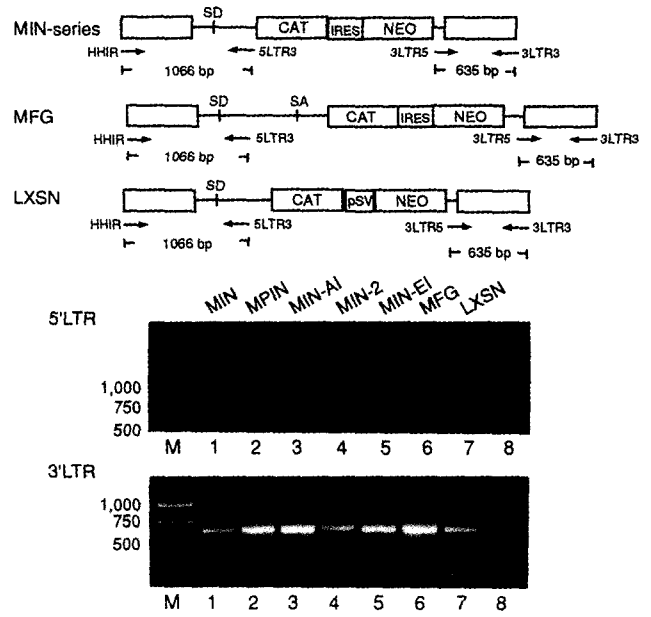


Fig. 5. Test for preservation of retroviral sequences in transduced cells. Total cellular DNAs were prepared from G418-resistant NIH3T3 cells transduced with various retroviral vectors. The two pairs of oligonucleotide primers were used to amplify the regions indicated in the figure. SD, splice donor; SA, splice acceptor. The preservation of the nucleotide sequence in the middle of the 5' and 3' LTRs was evident by biological assays performed for CAT and Neo.

followed by PCR with the oligonucleotide primer as shown in Fig. 5. If the retroviral vectors stably transferred the retroviral sequences to the target cells, these primers would amplify 1066 bp, and 635 bp from the 5' and 3'LTRs of the viral genome, respectively. DNA fragments of the expected lengths were present in all lines. These results suggested that the structure of newly constructed vectors were preserved well in transduced cells for a long period of time. The stability of transgene was obvious as demonstrated by high level expression of the CAT gene.

To test a possible production of RCR, we devised an experimental system in which RCR production could be maximized. 293T cells were transfected with 30 µg of the packaging construct, p_{kat2ampac} (Finer *et al.*, 1994) which contains *gag*, *pol* and *env* coding sequences in one plasmid, and 15 µg of a retroviral vector, MIN. Three days later, cell culture supernatants were taken and used to infect BAG cells. BAG cells contain integrated retrovirus expressing β-galactosidase or Neo that can be rescued by any packaging functions including *gag*, *pol*, and *env*. Infected BAG cells were split every 3 to 4 days, and cell-free culture

supernatants were used to infect NIH3T3 cells after three passages. After 48 h of incubation, cells were assayed for β -galactosidase or selected in the presence of G418. In this experimental system, the MIN vector produced no RCR, suggesting that at a given sensitivity of the assay, no RCR was produced from the newly constructed vector. This result clearly demonstrates that newly constructed retroviral vectors containing no viral coding sequence would have a much lower chance, if any, than other retroviral vectors containing viral coding sequences.

IV. DISCUSSION

Our data shows that entire viral coding sequences could be deleted from the MLV-based retroviral vector without significantly influencing viral titer and the level of gene expression, and also that the addition of some heterologous intron/exon sequences to the vector could lead to increased levels of gene expression in transduced cells while still maintaining high viral titer. The MIN series vectors described in this work should be safer than any other MLV-based retroviral vectors, since the possibility of homologous recombination with the packaging genome is virtually nil.

Our results showed the usefulness of intron and exon, more precisely the nucleotide sequence containing splice acceptor from other genes. Clearly, splice acceptor sequences from the EF-1 α gene and the major IE region of HCMV could significantly increase the level of gene expression, presumably by maintaining high steady state levels of mRNA. In both transiently transfected and stably transduced cells, those constructs generated significantly higher levels of gene expression than other vectors. However, one downside of using these highly efficient splice acceptor sequences appears to be low viral titer. This is probably because the genomic transcript containing the packaging signal sequence is efficiently spliced to the subgenomic RNA.

This problem can probably be overcome in several ways. First, it appears that the splicing efficiency of different splicing acceptors varies depending on packaging cell types. For example, we generally observed similar viral titers between these and other vectors in Phoenix Amphi, but greater differences in FLYA13 or NIH3T3-based packaging lines. Second, the nature of

the inserted gene also seems to play a role. For example, we see little differences between vectors when CAT sequence is used, but 2-5 fold difference in the case of IRAP and other genes such as the gene coding for iduronate-2-sulfatase (IDS) (Wilson *et al.*, 1990) and glucocerebrosidase (GC) (Horowitz *et al.*, 1989) (data not shown). Therefore, the best way appears to be to try several different vectors for each gene of interest and test them for the level of gene expression and viral titer. Another way is to manipulate the splice acceptor in such a way that the maximum possible level of gene expression and the highest possible viral titer are achieved by maintaining a delicate balance between the splicing efficiency (which determines the level of gene expression) and the amount of the genomic transcript (which influences viral titer). We are currently comparing various spliced acceptors and also carrying out mutational analysis on some splice acceptors.

It is generally agreed that viral titer and the level of gene expression in transduced cells are two of the key factors limiting the success of gene therapy trials. We believe that our vectors can serve as prototype vectors that can overcome these problems as well as the issue of safety. A similar concept using heterologous intron/exon sequences can be used not only for other MLV-based vectors but also for vectors based on lentivirus and DNA viruses.

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