Chapter 2, High-level gene expression in T cells

Retroviral vectors for high level transgene expression in T lymphocytes

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Retroviral Vectors for High-Level Transgene Expression in T Lymphocytes

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ABSTRACT

Efficient expression of genes transferred by retroviral vectors is a prerequisite for gene therapy, especially when the biological effect depends on the amount of transgene product. High-level gene expression is desirable for several gene therapy approaches involving T lymphocytes. We evaluated standard retroviral vectors with *cis*-regulatory control elements of the Moloney murine leukemia virus (Mo-MLV) with or without the human T cell-specific CD2 enhancer. For comparison, vectors containing the long terminal repeat (LTR) of myeloproliferative sarcoma virus (MPSV) and an improved 5′ untranslated region were used (MP71 vectors), with or without the woodchuck hepatitis virus posttranscriptional regulatory element (PRE). All vectors expressed the enhanced green fluorescent protein (GFP) to measure transgene expression. In mouse T cells MP71 vectors with and without the PRE yielded an up to 10-fold higher expression level compared with the Mo-MLV-based vectors currently used for gene transfer into T lymphocytes. A high multiplicity of infection (MOI) of standard Mo-MLV vectors could not reach expression levels obtained with a low MOI of MP71 vector. *Ex vivo*-transduced mouse T lymphocytes maintained the vector-dependent differences in level of transgene expression in Rag-1-deficient mice when adoptively transferred. In four human T cell lines and human primary T lymphocytes MP71 vectors yielded an up to 75-fold higher GFP expression level in comparison with the standard Mo-MLV vector. In contrast to mouse T cells, the integration of the PRE into MP71 vectors induced in human T cells a further significant increase in transgene expression level. Southern blot analysis of CEM T cells revealed that the superior performance of MP71 vectors was not due to a higher rate of viral integration. In summary, MP71 vectors are useful tools for stable, high-level gene expression in T lymphocytes, for example, in the expression of T cell receptor genes.

OVERVIEW SUMMARY

Sufficient levels of transgene expression are one important requirement in the genetic manipulation of T lymphocytes for therapeutic purposes. The present study shows that vectors with the LTR of MPSV and an improved 5′ untranslated leader region (MP71 vectors) mediate up to 75-fold higher transgene expression in T cells compared with standard Mo-MLV-based vectors. The difference in favor of the MP71 vectors was most pronounced in T cells and less distinct in fibroblasts, arguing for a differentiation-specific effect. This observation may have broader consequences for gene therapy targeting inherited disorders of T lymphocytes, infection with the human immunodeficiency virus (HIV), and various modes of immunotherapy.

INTRODUCTION

A NUMBER OF gene therapy approaches involve gene transfer into T lymphocytes. Examples are correction of inherited gene deficiencies such as adenosine deaminase, intracellular

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immunization against infection with human immunodeficiency virus, the transfer of safety genes such as thymidine kinase, and, more recently, genetic engineering of T lymphocytes to transfer antigen specificity. Examples of the latter approach are antibody fusion genes to obtain tumor specificity (Eshhar *et al.*, 1993) or T cell receptor (TCR) genes to obtain virus or tumor specificity (Clay *et al.*, 1999; Cooper *et al.*, 2000; Willemsen *et al.*, 2000; Kessels *et al.*, 2001; Stanislawski *et al.*, 2001). In the latter case, the number of TCR molecules on the T cell is important for their function. T lymphocytes expressing a few TCR molecules function aberrantly, for example, fail to produce cytokines. The same T cells, after TCR upregulation by the tetracycline-inducible system, function normally *in vivo* (Labrecque *et al.*, 2001). Moloney murine leukemia virus (Mo-MLV)-derived vectors are commonly used to express genes in T lymphocytes; however, the expression level of the transferred genes was often unsatisfactory, at least if expression of the transgene did not result in a growth advantage for the transduced cells (Blaese *et al.*, 1995; Bordignon *et al.*, 1995; Weijtens *et al.*, 1998; Cavazzana-Calvo *et al.*, 2000). In some retroviral vectors internal promoters were used for transgene expression but, because of promoter interference, downregulation of transgene expression may occur (Emerman and Temin, 1984).

Retroviral *cis* elements regulating transcriptional activity are located in the U3 region of the long terminal repeat (LTR) and in the retroviral leader sequence. Replacing the Mo-MLV LTR with that of the murine myeloproliferative sarcoma virus (MPSV) led to increased transgene expression in myeloid stem cells, T cell lines (Baum *et al.*, 1995), embryonic carcinoma cells (Challita *et al.*, 1995), and mouse and human lymphohematopoietic cells (Onodera *et al.*, 1998; Halene *et al.*, 1999; Hawley, 2001). Locus control regions (LCRs) are *cis*-regulatory elements that confer high-level, tissue-specific transgene expression in a position-independent, copy number-dependent fashion (Graeves *et al.*, 1989; Kowolik *et al.*, 2001). Insertion of the LCR of the human *CD2* gene into a Mo-MLV-based vector resulted in T cell-specific gene expression (Indraccolo *et al.*, 2002). Other *cis* elements modulate transgene expression through posttranscriptional processing and transport of RNA. Removal of aberrant AUGs and insertion of an intron in the 5′ untranslated region of transgenic RNA may promote mRNA export and translation (Hildinger *et al.*, 1999; Schambach *et al.*, 2000). A further *cis* element that can enhance transgene expression is the posttranscriptional regulatory element (PRE) of hepadna viruses (e.g., hepatitis B virus and woodchuck hepatitis virus) (Huang and Yen, 1994; Donello *et al.*, 1998). PREs seem to act independently of transcription and splicing and may improve gene expression by modification of polyadenylation or translation (Donello *et al.*, 1998; Schambach *et al.*, 2000).

To analyze the efficacy of different *cis*-regulatory elements with respect to the level of transgene expression in T lymphocytes, we compared the Mo-MLV-LTR-based vector pLXSN (Miller and Rosman, 1989) with a variant containing a T cell-specific transcriptional enhancer from the human *CD2* gene. In parallel, we evaluated MP71 retroviral vectors, which combine MPSV-LTR promoter-enhancer sequences and improved 5′ untranslated sequences derived from the murine embryonic stem cell virus (MESV) (Hildinger *et al.*, 1999; Schambach *et al.*, 2000). One of the MP71 vectors contained, in addition, the PRE of woodchuck hepatitis virus in the 3′ untranslated region.

MATERIALS AND METHODS

Cell lines

NIH 3T3 cells (CCL92; American Type Culture Collection [ATCC], Manassas, VA), ecotropic packaging cells GP+E86 (Markowitz *et al.*, 1988), and amphotropic packaging cells PA317 (Miller and Buttimore, 1986) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Amphotropic packaging cells PT67 (Miller and Chen, 1996) and HT1080 human fibrosarcoma cells (CCL121; ATCC) were grown in DMEM supplemented with 10% FCS and 2 m*M* glutamine. J558L mouse plasmacytoma cells (TIP6; ATCC) were grown in RPMI 1640 medium with 10% FCS. Human T cell lines CEM (CCL119; ATCC), H9 (HTB176; ATCC), HUT78 (TIB161; ATCC), and Jurkat (TIB152; ATCC) were grown in RPMI 1640 medium with 10% FCS, 10 m*M* HEPES, and 2 m*M* glutamine. The mouse T cell line TG40 (Sussman *et al.*, 1988) was grown in RPMI 1640 medium with 10% FCS, 2 m*M* glutamine, penicillin and streptomycin (100 U/ml), and 50 μM 2-mercaptoethanol. The mouse T cell line T58 (Letourneur and Malissen, 1989) was grown in RPMI 1640 medium with 10% FCS, 2 m*M* glutamine, penicillin and streptomycin (100 U/ml), 50 μM 2-mercaptoethanol, 1 μM HEPES, and 13 μM $NaHCO_3$. All tissue culture media were purchased from GIBCO (Karlsruhe, Germany); FCS was purchased from Greiner (Frickenhausen, Germany).

Retroviral vectors

Retroviral vectors containing the enhanced green fluorescent protein (GFP) gene and used in this study are shown in Fig. 1.

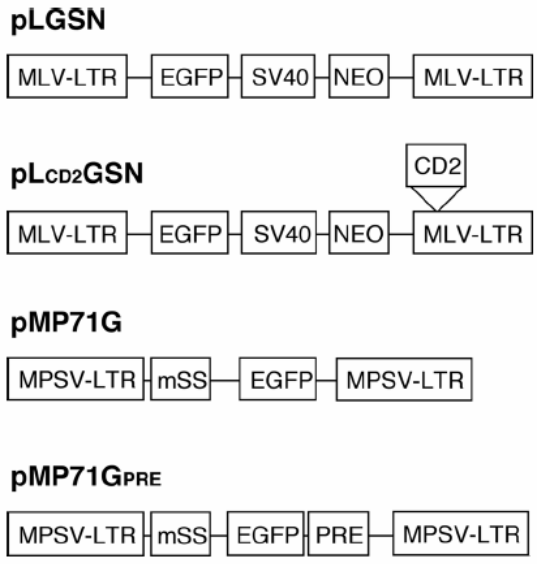


FIG. 1. Schematic representation of vector constructs. CD2, T cell-specific CD2 enhancer; EGFP, enhanced green fluorescent protein gene; LTR, long terminal repeat; MLV, Moloney murine leukemia virus; MPSV, myeloproliferative sarcoma virus; mSS, modified mRNA splice site; NEO, neomycin resistance gene; PRE, woodchuck hepatitis virus posttranscriptional regulatory element; SV40, simian virus 40 promoter.

The vector pLGSN is a derivative of pLXSN (Miller and Rosman, 1989) and carries the Mo-MLV-LTR promoter-enhancer elements to drive GFP (G) gene expression and the simian virus 40 (S, SV40) promoter to regulate neomycin resistance (N, *neo*) gene expression (Klein *et al.*, 1997). The Mo-MLV enhancer element located between bases 2976 and 3186 in the original vector pLXSN was deleted in vector pLGSN and replaced by a 1.0-kbp sequence containing a T cell-specific enhancer from the human *CD2* locus control region (LCR), to generate pLCD2GSN (Indraccolo *et al.*, 2002). This 1-kbp *CD2* sequence is a truncated LCR that has lost LCR activity and retains a transcriptional enhancer as previously reported (Lake *et al.*, 1990; Indraccolo *et al.*, 2001). The vector pMP71G contains the MPSV LTR and an improved leader sequence derived from MESV to drive GFP gene expression (Hildinger *et al.*, 1999; Schambach *et al.*, 2000). The leader sequence of MP71 vectors is devoid of *gag* sequences and aberrant AUGs that match the Kozak consensus for initiation of translation; this leader contains a designer splice acceptor oligonucleotide that interacts with the retroviral splice donor to create an alternatively spliced intron in the 5′ untranslated region (Hildinger *et al.*, 1999). To generate pMP71G, the GFP gene cloned into pLGSN was amplified by polymerase chain reaction (PCR) (forward primer, 5′-GAGCGGCCGCGCCACCATG-3′; reverse primer, 5′-CGGAATTCTTACTTGTACAGCTC-3′) and cloned into the single *Not*I and *Eco*RI sites of pMP71. The vector pMP71GPRE is a derivative of pMP71G and was generated by introduction of the 856-bp woodchuck hepatitis virus posttranscriptionalregulatory element (PRE) (Huang and Yen, 1994) into the single *Eco*RI site of pMP71G. The sense orientation of the PRE fragment was proven by *Not*I–*Spe*I digestion. Retrovirally transduced transgenes derived from pLGSN, pLCD2GSN, pMP71G, and pMP71GPRE are referred to as LGSN, LCD2GSN, MP71G, and MP71GPRE, respectively.

Transfection of packaging cells and generation of GFP retrovirus-containing supernatant

Packaging cells were transfected with retroviral vector plasmids by calcium phosphate precipitation (Amersham Pharmacia Biotech, Freiburg, Germany) as described (Uckert *et al.*, 2000a). Briefly, packaging cells (5×10^5) were plated into a 100-mm tissue culture dish (Costar, Bodenheim, Germany) and after 24 hr were transfected with 10 μg of vector DNA (pLGSN or pLCD2GSN) or cotransfected with 1 μg of plasmid pWLneo (Stratagene, Heidelberg, Germany) and 10 μg of vector DNA (pPM71G or pMP71GPRE). After 24 hr, cells were washed twice with phosphate-buffered saline (PBS: 1% NaCl, 0.025% KCl, 0.14% Na_2HPO_4, 0.025% KH_2PO_4, pH 7.3), medium was replaced with fresh medium, and selection was performed in the presence of G418 (0.8 mg/ml; Sigma, Deisenhofen, Germany). After approximately 12 days, when nontransfected control cells died, bulk cultures were expanded and used to generate high-titer retrovirus producer cells by fluorescence-activated cell sorting (FACS) enrichment of high-fluorescence producer cells (FACSVantage; BD Biosciences Immunocytometry Systems, San Jose, CA) (Uckert *et al.*, 2000b). High-titer virus vector particle-containing supernatant (virus supernatant) was produced at 37°C in tissue culture roller bottles (Costar) (Uckert *et al.*, 2000b).

Virus titration

The titer of GFP retroviruses was determined by infecting NIH 3T3 cells with serial dilutions of virus supernatant in 24-well plates (Costar). After 48 hr, the cell number was determined, the percentage of GFP-transduced cells was determined by flow cytometry (Epics XL2; Coulter, Miami, FL), and green colony-forming units (GFU) per milliliter were calculated (Uckert *et al.*, 2000b). For comparative analysis of GFP expression levels, the titers of various GFP retroviruses (1×10^6 to 5×10^6 GFU/ml) were equilibrated after titration on NIH 3T3 cells to approximately 1×10^6 GFU/ml.

Transduction of cell lines

Mouse T cell lines (2×10^5 cells per well, MOI of 5) and human T cell lines (1×10^5 cells per well, MOI of 10) were transduced once with virus supernatant for 72 hr in non-tissue culture 24-well plates (Falcon; BD Biosciences Discovery Labware, Franklin Lakes, NJ) coated with RetroNectin (recombinant human fibronectin fragment CH-296, 10 μg/cm^2; Takara Shuzo, Otsu, Japan) according to the manufacturer's instruction. Other cell lines (1×10^5 cells per well) were transduced with an MOI of 10 in 24-well tissue culture plates in the presence of Polybrene (4 μg/ml; Sigma). After 72 hr, the level of transgene expression (measured as mean fluorescence intensity, MFI) and the transduction efficiency (measured as percentage of GFP$^+$ cells) were determined by FACS analysis. All experiments were performed twice and one representative result is shown.

Isolation and transduction of mouse primary T lymphocytes

Total mouse splenocytes were isolated from 10- to 12-week-old female P14 TCR-transgenic C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). CD8$^+$ T cells of P14 mice recognize the H2-D^b-restricted peptide gp33–41 (amino acid sequence KAVYNFATM) derived from the glycoprotein of lymphocyte choriomeningitis virus (LCMV) (Pircher *et al.*, 1989). A single-cell suspension of splenocytes was prepared and red blood cells were lysed by NH_4Cl treatment. Splenocytes (2×10^6/ml) were cultured for 24 hr in the presence of peptide at 1 μg/ml. Stimulated cells (2×10^5/well) were transduced once with virus supernatant (MOI of 5) in RetroNectin-coated 24-well non-tissue culture plates in the presence of peptide (1 μg/ml) and interleukin 2 (IL-2, 10 U/ml; Boehringer, Mannheim, Germany). The level of GFP expression and the transduction efficiency were determined by FACS analysis. The experiments were performed twice and one representative result is shown.

Isolation and transduction of human primary T lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by centrifugation on Ficoll-Hypaque (Seromed-Biochrom, Berlin, Germany) and stimulated for 72 hr with IL-2 (100 U/ml; Boehringer) and immobilized anti-CD3 monoclonal antibody (MAb) (2.5 μg/ml) and anti-CD28 MAb (0.5 μg/ml; both from Pharmingen, Hamburg, Germany) as described (Uckert *et al.*, 2000a). Peripheral blood lymphocytes (PBLs; 2×10^5/well) were transduced three times within 72 hr with virus supernatant (MOI of 10) in RetroNectin-coated

24-well non-tissue culture plates in the presence of IL-2 (100 U/ml). Three days after transduction, PBLs were labeled with phycoerythrin (PE)-conjugated MAb directed against T cell markers (CD3, CD4, and CD8; Pharmingen) and the level of GFP expression and the transduction efficiency were measured by flow cytometry (FACSCalibur; BD Biosciences Immunocytometry Systems). The experiments were performed three times and one representative result is shown.

Adoptive transfer of mouse T lymphocytes

P14 TCR-transgenic T lymphocytes were seeded at a density of 1×10^6 cells per well in RetroNectin-coated 24-well non-tissue culture plates, stimulated as described above, and transduced with GFP retrovirus supernatant at a titer of 4×10^6 GFU/ml. For adoptive T cell transfer, 5×10^7 P14 TCR-transgenic T cells transduced with LGSN (10% GFP^+ cells) and MP71GPRE (30% GFP^+ cells) were injected into the tail vein of 10-week-old female Rag-1-deficient mice (Jackson Laboratory). On days 10 and 30 after adoptive transfer, mice were killed. Peripheral blood and spleen were isolated and T lymphocytes were stained with a PE-labeled MAb (Pharmingen) directed against the TCR β chain (Vβ8.1) of P14 TCR-transgenic T cells. The percentage of GFP^+/Vβ8.1^+ T cells was determined by flow cytometry.

Reverse transcription PCR

Total RNA was isolated from CEM T cells on day 57 after transduction with the various GFP retroviruses, using TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically. Reverse transcription PCR (RT-PCR) was carried out with the Titan One Tube RT-PCR system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. A 589-bp GFP product was amplified with the forward primer 5′-CTGACCCTGAAGTTCATCTG-3′ and the reverse primer 5′-TGTACAGCTCGTCCATGC-3′. As a control for cDNA amounts, an RT-PCR was performed with standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. RT-PCRs were carried out in a Biometra (Göttingen, Germany) T-Personal thermocycler under the following conditions: RNA denaturation at 68°C for 5 min and incubation at 55°C for 30 min after addition of the enzyme mix, followed by DNA denaturation at 94°C for 2 min, 40 cycles of amplification (94°C for 40 sec, 55°C for 40 sec, 68°C for 55 sec), and a single extension at 68°C for 7 min. From cycle 10 onward, the elongation time was prolonged by 5 sec for each cycle. Aliquots were taken out at cycles 30 and 35. As negative controls, a no-template control and a no-RT control (preincubation of the enzyme mix at 94°C for 2 min) were included. PCR products were then analyzed by 1.5% agarose gel electrophoreses.

Southern blot analysis of copy number integration

CEM T cells were transduced at MOIs of 0.5, 1, and 10 with MP71GPRE as a standard and with the various GFP retroviruses at an MOI of 10 as described above. After 72 hr, genomic DNA was isolated by a sodium dodecyl sulfate (SDS)–proteinase K method (Krause *et al.*, 1989). After extraction, 20 μg of DNA was digested with *Nhe*I (LGSN) and *Xba*I (MP71GPRE), which cut once in each of the corresponding LTRs. DNA was electrophoresed through a 1% agarose gel and transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech). A cDNA probe representing the GFP fragment was radioactively labeled to a specific activity of 4×10^8 dpm/μg DNA with [α-^{32}P]dCTP (specific radioactivity, 370 MBq/ml; Amersham Pharmacia Biotech), using a random priming kit (Amersham Pharmacia Biotech). The hybridized membrane was washed twice under high-stringency conditions and exposed to X-ray film (Amersham Pharmacia Biotech) (Uckert *et al.*, 1986).

RESULTS

Pseudotyping of GFP retrovirus determines transduction efficiency of mouse and human T cells

To evaluate the influence of various *cis*-regulatory elements on the level of GFP expression in various mouse and human T cells, an efficient transduction of these cells is a prerequisite. Therefore, the retroviral vector LGSN was pseudotyped with the envelope of an ecotropic MLV (Mo-MLV) and of amphotropic MLVs (MLV-4070A and MLV-10A1) and the transduction efficiency of murine and human T cell lines was analyzed by flow cytometry, using virus supernatants equilibrated in titer to 1×10^6 GFU/ml on NIH 3T3 cells. Table 1 shows that the murine T cell lines TG40 and T58 were most efficiently transduced by the GFP retrovirus carrying the ecotropic Mo-MLV envelope (TG40, 92%; T58, 90%), whereas viruses with amphotropic envelopes were less effective. The MLV-4070A-pseudotyped vector transduced between 10% (T58) and 20% (TG40) of the cells, whereas MLV-10A1-pseudotyped vector transduced 30% of TG40 and 15% of T58 cells. In contrast, both MLV-4070A-pseudotyped and especially MLV-10A1-pseudotyped GFP retroviruses efficiently transduced human T cell lines. The highest transduction efficacy was achieved when the MLV-10A1 pseudotype was used to transduce CEM (60%) and H9 cells (75%). On the basis of these results, GFP retroviruses used for the transduction of murine T cells were pseudotyped with the ecotropic MLV envelope, whereas for the transduction of human T cells the MLV-10A1 envelope was selected.

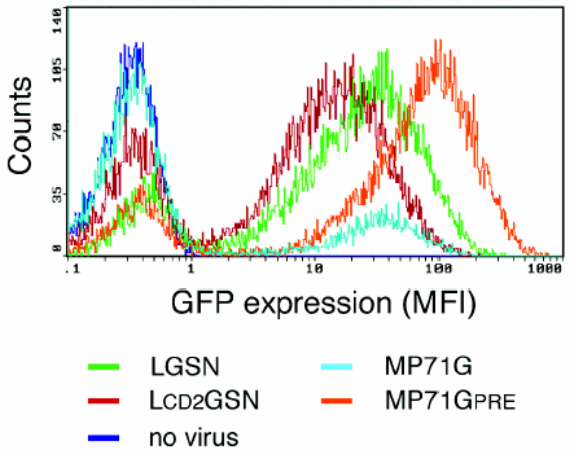
Modest increase in transgene expression obtained with MP71 vectors in mouse non-T cell lines

To elucidate whether more recently developed retroviral vectors could increase transgene expression in comparison with standard pLXSN vectors, a series of retrovirus vectors was constructed, all containing the same EGFP cDNA (Fig. 1). First,

TABLE 1. TRANSDUCTION EFFICIENCY OF MURINE AND HUMAN T CELL LINES WITH VARIOUS LGSN VECTOR PSEUDOTYPES

	LGSN vector pseudotype		
Cell line	*E-MLV*	*A-MLV 4070A*	*A-MLV 10A1*
Mouse			
TG40	92	20	30
T58	90	10	15
Human			
CEM	0	34	60
H9	0	46	75

A

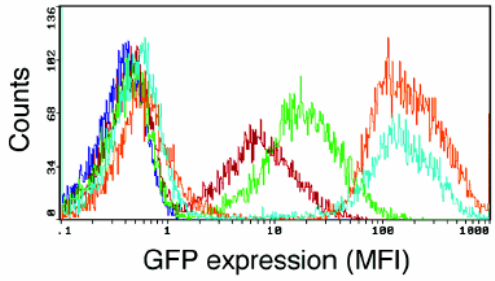


B

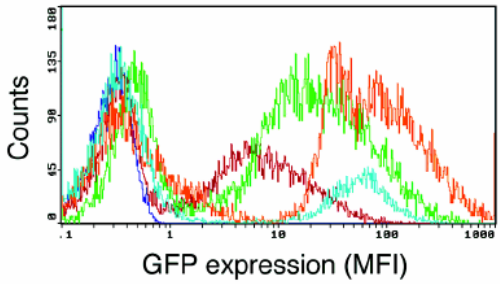
Cell line	MFI	% GFP+ cells
NIH3T3	0.2	0
+ LGSN	25.8	83
+ LCD2GSN	15.2	71
+ MP71G	30.8	24
+ MP71GPRE	74.8	83

FIG. 2. Improved transgene expression by MP71 vectors in NIH 3T3 cells. (**A**) NIH 3T3 (1×10^5 cells per well) were transduced with different GFP retroviruses (MOI of 10) in 24-well plates. After 72 hr, cells were harvested and GFP expression levels (MFI) and transduction efficiencies (percent GFP^+ cells) were determined by flow cytometric analysis. (**B**) Quantitative values of MFI and percentage of transduced cells.

A



B



C

Cell line	MFI	% GFP+ cells
TG40	0.3	0
+ LGSN	18.4	54
+ LCD2GSN	7.8	42
+ MP71G	126.8	42
+ MP71GPRE	149.4	62
T58	0.2	0
+ LGSN	24.0	70
+ LCD2GSN	7.4	52
+ MP71G	54.0	25
+ MP71GPRE	79.0	62

D

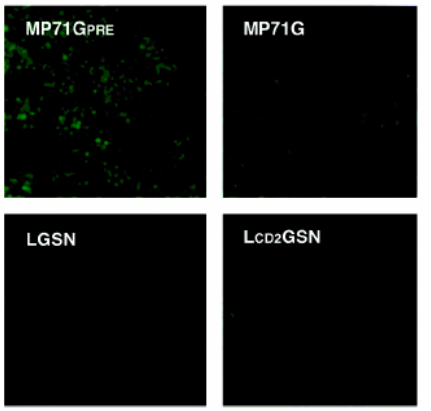


FIG. 3. High GFP expression by MP71 vectors compared with Mo-MLV vectors in mouse T cell lines. T cell lines (**A**) TG40 and (**B**) T58 (each 2×10^5 cells per well) were transduced with different GFP retroviruses (MOI of 5) in RetroNectin-coated 24-well plates. After 72 hr, cells were harvested and GFP expression levels (MFI) and transduction efficiencies (percent GFP^+ cells) were determined by flow cytometric analysis. (**C**) Quantitative values of MFI and percentage of transduced cells. The experiment was performed two times. (**D**) TG40 cells transduced with the indicated GFP retroviruses were visualized by fluorescence microscopy (original magnification, 20-fold).

NIH 3T3 cells were exposed to GFP retrovirus supernatants equilibrated to a titer of 1×10^6 GFU/ml. After 72 hr, the expression level of GFP and the transduction efficiency were determined by FACS analysis. The highest GFP expression level in NIH 3T3 cells was achieved with the vector MP71GPRE (MFI, 74.8) followed by MP71G (MFI, 30.8), LGSN (MFI, 25.8), and LCD2GSN (MFI, 15.2) (Fig. 2). Although Mo-MLV-LTR vectors yielded relatively low GFP expression levels, the transduction efficiency was comparable to that obtained with vector MP71GPRE and even higher in comparison with MP71G. A similar result was obtained when the plasmacytoma cell line J558L was transduced with the various GFP retroviruses (data not shown). Thus, MP71-based vectors mediated a reproducible, although moderate (approximately 2-fold) increase in transgene expression when compared with standard Mo-MLV vectors in these non-T cells.

High increase in transgene expression by MP71 vectors compared with standard Mo-MLV vectors in mouse T cell lines

Next, the GFP expression level of the different vectors was analyzed in mouse T cell lines. Both in TG40 cells (Fig. 3A) and T58 cells (Fig. 3B), MP71-based GFP vectors mediated a strong increase in transgene expression in comparison with those vectors containing Mo-MLV-LTR regulatory elements. Compared with LGSN as a standard, the highest GFP expression in TG40 cells was found for vectors MP71GPRE (8.1-fold increase) and MP71G (6.9-fold). In contrast, LCD2GSN (Fig. 3A and C) yielded a significantly lower level of GFP expression (0.42-fold). The advantage of MP71 vectors was less pronounced in T58 cells, although MP71GPRE (3.3-fold) and MP71G (2.3-fold) were still superior to LGSN. T58 cells showed a higher transduction efficiency with the LGSN vector, possibly resulting in a higher mean fluorescence due to accumulation of integration events (Fig. 3C). Again, LCD2GSN (0.3-fold) was even weaker than LGSN (Fig. 3B and C).

These results were confirmed by fluorescence microscopy. TG40 cells transduced with the vector MP71GPRE showed a bright, intensive fluorescence that was less intensive when cells were transduced with the vector MP71G. Only weak fluorescence was visible when TG40 cells were transduced with the vectors LGSN and LCD2GSN (Fig. 3D).

Low MOI of MP71 vectors yields higher transgene expression than high MOI of standard Mo-MLV vectors

A high multiplicity of infection (MOI) increases the vector copy number in transduced cells and thereby can lead to higher expression levels (Wahlers *et al.*, 2001). To analyze whether an increasing MOI correlated with enhanced GFP expression, TG40 cells were transduced with vector LGSN at MOIs of 50, 10, and 1. Figure 4A and C shows that there is a correlation between virus titer and transduction efficiency. However, a 50-fold increase in the MOI resulted only in an approximately 6-fold increase in the level of transgene expression. At an MOI of 50, nearly all TG40 cells were transduced (98%) and the GFP expression was comparably high (MFI, 67.3). At an MOI of 10, 63% of the cells were transduced and the MFI decreased to 20.9. At an MOI of 1, GFP expression further decreased (MFI, 11.0) and 18% of TG40 cells were transduced (Fig. 4A and C). Similar results were obtained with the vector MP71GPRE (data not shown). In contrast, transduction of TG40 cells with a low MOI of the vector MP71G (MOI of 5) yielded a high GFP expression level (MFI, 126.8) but a relatively low transduction efficiency of 42%, whereas transduction of TG40 cells with a high MOI of a vector carrying weak regulatory elements (LCD2GSN) (MOI of 20) resulted in a low GFP expression (MFI, 30.0) but a high transduction efficiency of 80% (Fig. 4B and C). This result indicates that a high MOI of a vector with suboptimal control elements cannot compete with a vector containing improved elements.

GFP expression is stable in MPSV vectors

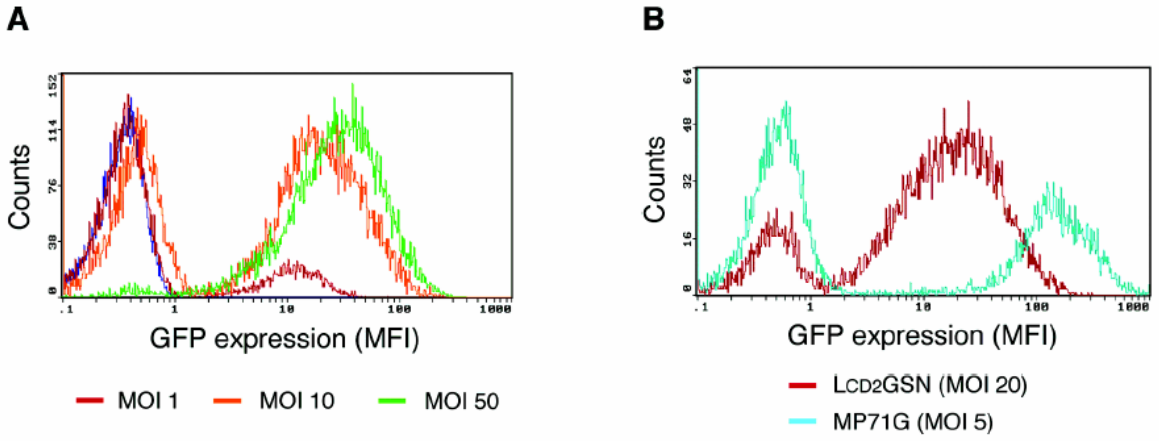
To analyze whether the transgene expression is stable, TG40 cells were transduced with virus supernatants of vectors MP71GPRE, LGSN, and LCD2GSN and cultured without G418 selection. The level of GFP expression and the percentage of transduced cells were compared on days 3 and 100. Figure 5 shows that the MFI decreased only slightly, from 139.0 to 128.0, in MP71GPRE-transduced TG40 cells. GFP expression decreased more when either LGSN or LCD2GSN was used for transduction of TG40 cells. The percentage of GFP^+ cells was stable over a period of 100 days with all GFP retroviruses (Fig. 5).

High transgene expression by MP71 vectors compared with standard Mo-MLV vectors in mouse primary T lymphocytes

To analyze whether MP71 vectors are superior to Mo-MLV-based vectors in driving transgene expression in primary mouse T lymphocytes, spleen cells were isolated from TCR-transgenic P14 mice. After 24 hr of stimulation with the cognate peptide and IL-2, $CD8^+$ T lymphocytes were transduced with the various retroviral vectors. Figure 6A and B shows that MP71 vectors with or without the PRE were superior to the Mo-MLV-derived vectors for GFP expression in T lymphocytes. As observed in TG40 cells, MP71 vectors yielded a higher trans-

FIG. 4. Higher transduction efficiency cannot substitute for promoter strength. (**A**) TG40 cells (1×10^5 per well) were transduced with GFP retrovirus LGSN in RetroNectin-coated 24-well plates at MOIs of 1, 10, and 50 or (**B**) using GFP vector MP71G at an MOI of 5 (taken from Fig. 3A) and LCD2GSN at an MOI of 20 (taken from Fig. 5A). After 72 hr, cells were harvested and GFP expression levels (MFI) and transduction efficiencies (percent GFP^+ cells) were analyzed by flow cytometry. (**C**) Quantitative values of MFI and percentage of transduced cells.

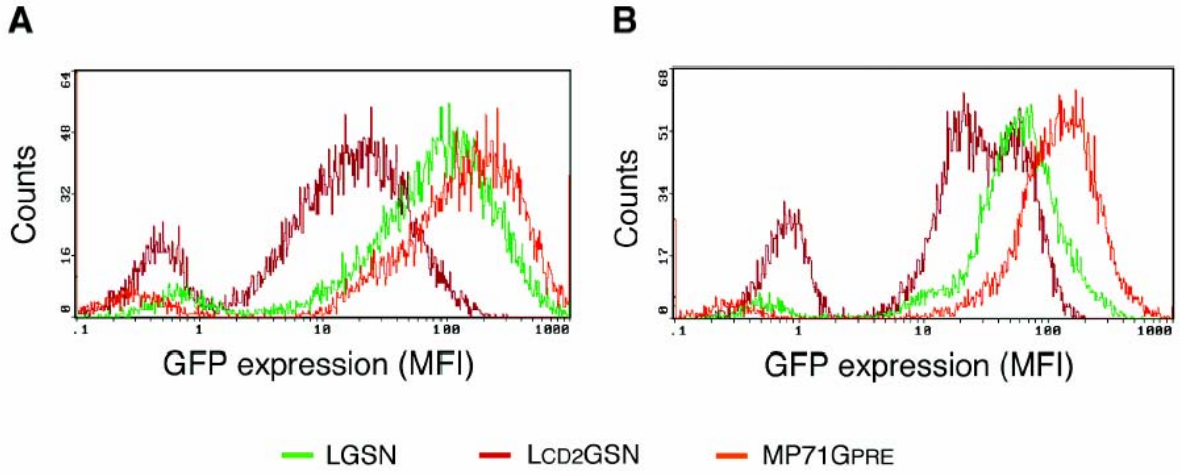
FIG. 5. Transgene expression is stable. (**A**) TG40 cells (1×10^5 per well) were transduced with GFP retroviruses (LGSN, MOI of 50; LCD2GSN, MOI of 20; MP71GPRE, MOI of 40) in RetroNectin-coated 24-well plates. After 72 hr, cells were harvested and some of the cells were analyzed by flow cytometry for GFP expression level and transduction efficiency. (**B**) The cells were grown for 100 days without G418 selection and reanalyzed by FACS. (**C**) Quantification of GFP expression level (MFI) and transduction efficiency (percent GFP^+ cells).



C

Cell line	MOI	MFI	% GFP+ cells
TG40		0.3	0
+ LGSN	1	11.0	18
	10	20.9	63
	50	67.3	98
TG40			
+ MP71G	5	126.8	42
+ LCD2GSN	20	30.0	80

FIG. 4.



C

Cell line	MFI		% GFP+ cells	
	3 d	100 d	3 d	100 d
TG40				
+ LGSN	80.0	60.0	93	93
+ LCD2GSN	30.0	20.0	80	79
+ MP71GPRE	139.0	128.0	91	92

FIG. 5.

gene expression level compared with the LGSN vector (MP71GPRE, 9.4-fold; MP71G, 6.8-fold). In primary T lymphocytes, as previously observed in T cell lines, the addition of the T cell-specific *CD2* enhancer reduced transgene expression (LCD2GSN, 0.65-fold). Similar results were obtained with anti-CD3/anti-CD28 MAb-stimulated T lymphocytes of C57BL/6 mice (data not shown).

Survival of GFP gene-modified CD8$^+$ T lymphocytes is not impaired in vivo

Next, it was evaluated whether the higher GFP expression level in primary T lymphocytes transduced by MP71 vectors compared with cells transduced with Mo-MLV vectors was maintained *in vivo*. P14 TCR-transgenic T lymphocytes were transduced with virus supernatant of MP71GPRE and LGSN, respectively, and 5×10^7 T cells (35% GFP$^+$ after transduction with MP71GPRE, 10% GFP$^+$ after transduction with LGSN) were adoptively transferred into Rag-1-deficient mice. On days 10 and 30 after transfer, Vβ8.1$^+$ T lymphocytes in pheripheral blood and spleen of Rag-1-deficient mice were analyzed for GFP expression. The vector-dependent differences in the expression level of GFP were still valid on day 10 after adoptive T cell transfer (Fig. 7). GFP was expressed at a significantly higher level in those T lymphocytes in which the vector MP71GPRE was used for *in vitro* generation of GFP$^+$ cells. This did not change when T cells were isolated 30 days after transfer, although lower numbers of T cells were recovered (data not shown). The ratio of GFP$^+$ to GFP$^-$ T cells remained approximately the same on days 10 and 30 as before adoptive transfer. This indicates that the GFP expression in T lymphocytes is stable *in vivo*, at least for the time period monitored, and that GFP$^+$ T lymphocytes survive equally as well as GFP$^-$ cells.

Slight increase in transgene expression obtained with MP71 vectors in a human non-T cell line

To analyze whether MP71-derived vectors are able to induce a higher level of transgene expression in human cells in comparison with Mo-MLV vectors, all vector constructs were pseudotyped with the envelope of MLV-10A1. First, HT1080 fibroblast cells were transduced with GFP retrovirus supernatants at an MOI of 10. After 72 hr, the GFP expression level and the transduction efficiency were determined. The highest level of GFP expression was achieved with the vector MP71GPRE (MFI, 195.8), whereas the others induced a lower MFI (MP71G, 76.2; LGSN, 63.8; and LCD2GSN, 74.2) (Fig. 8). Compared with LGSN as a standard, the increase for vector MP71GPRE was 3-fold. In contrast to the results obtained in mouse non-T cell lines, only the MP71 vector containing the PRE mediated a moderate increase in transgene expression, whereas MP71 without PRE was in the range of LGSN and LCD2GSN. As shown in Fig. 8B, all vectors efficiently transduced HT1080 cells (62 to 98%).

MP71 vectors are superior in comparison with standard Mo-MLV vectors in human T cell lines

Next, the GFP expression level of the various vectors was evaluated in various human T cell lines. T cell lines were transduced with GFP retrovirus supernatants at an MOI of 10 and GFP expression level and transduction efficiency were determined 72 hr later. Both in CEM and HUT78 cells, MP71-based vectors mediated a strong increase in GFP transgene expression in comparison with Mo-MLV-based vectors. Using LGSN as a standard, the highest GFP expression in CEM cells was obtained for vector MP71GPRE (9.9-fold) and MP71G (4.3-fold). In contrast, a lower level of GFP expression was achieved with vector LCD2GSN (0.3-fold) (Fig. 9A and C). The advantage of MP71 vectors was even more pronounced in HUT78 T cells, where MP71GPRE (75.5-fold) and MP71G (16.8-fold) were clearly superior to LGSN (Fig. 9B and C). Again, LCD2GSN (0.6-fold) yielded a lower GFP expression level in comparison with LGSN (Fig. 9B and C). These results were confirmed in two other human T cell lines. In H9 cells MP71GPRE and MP71G yielded a 37.3-fold and a 13.7-fold increase in GFP expression, respectively, whereas in Jurkat cells MP71GPRE and MP71G yielded a 26.7-fold and a 14.0-fold higher level of transgene expression in comparison with the standard vector LGSN (data not shown). GFP gene expression with all GFP retroviruses was stable over a period of 100 days (data not shown).

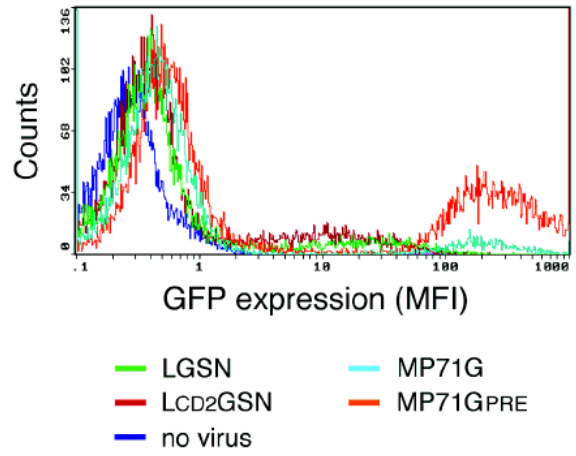
High increase in transgene expression by MP71 vectors compared with standard Mo-MLV vectors in human primary T lymphocytes

We then used the various GFP retroviruses to transduce human primary T lymphocytes with virus supernatants at an MOI of 10, and analyzed GFP expression level by flow cytometry. In comparison with LGSN, the GFP expression achieved with MP71GPRE was significantly higher (22.1-fold) as shown for donor 1 (Fig. 10A). This result was confirmed when PBLs of two other donors were transduced and mean values were calculated. Overall, MP71GPRE showed a 20-fold increased transgene expression level in comparison with the standard vector LGSN (Fig. 10B). Vector MP71G also yielded a significantly higher GFP expression level (15-fold increase) when compared with LGSN, but was, as already seen in T cell lines, less efficient than MP71GPRE (data not shown). Vector LCD2GSN yielded a low level of GFP expression (0.2-fold) compared with LGSN (data not shown). The transduction of equal T cell numbers (approximately 20% with all vectors, MP71G and LCD2GSN not shown) indicates proper equilibration of the various virus supernatants.

Cis-regulatory elements of MP71-based vectors are responsible for high level of transgene expression

To investigate whether the higher level of transgene expression achieved with MP71 vectors is based on a higher efficiency of their *cis*-regulatory elements in comparison with Mo-MLV vectors or to varying integration events, genomic DNA samples of transduced cells were analyzed. Increasing the MOI of MP71GPRE from 0.5 to 1 and 10 led to an increasing intensity of GFP-specific bands in Southern blots and, as expected, to an increasing transduction efficiency ranging from 21% (MOI of 0.5), to 32% (MOI of 1), and to 53% (MOI of 10) of GFP$^+$ T cells (Fig. 11A). Compared with the standard MOI of 10, the copy number integration of MP71GPRE was the same, whereas those for LGSN were clearly higher, supporting the conclusion that the latter contained a suboptimal configuration of *cis*-regulatory elements. Furthermore, the various GFP vectors were not rearranged in CEM cells as indicated by DNA bands that correspond to the calculated size of LGSN (3633 bp) and MP71GPRE (2965 bp) fragments.

A



B

Mouse primary T lymphocytes	MFI	% GFP+ cells
P14	0.2	0
+ LGSN	20.1	13
+ LCD2GSN	13.1	16
+ MP71G	137.1	9
+ MP71GPRE	188.0	34

FIG. 6. High transgene expression by MP71 vectors compared with Mo-MLV vectors in mouse primary T lymphocytes. (**A**) Primary splenic T lymphocytes were stimulated with peptide (1 μg/ml) for 24 hr. Cells (2×10^5 per well) were transduced in the presence of peptide (1 μg/ml) and IL-2 (10 U/ml) with various GFP retroviruses (MOI of 5) in RetroNectin-coated 24-well plates. After 72 hr, cells were harvested and GFP expression levels (MFI) and transduction efficiencies (percent GFP$^+$ cells) were determined by flow cytometric analysis. (**B**) Quantitative values of MFI and percentage of transduced cells.

To further analyze whether the higher level of transgene expression obtained with MP71 vectors is based on a higher efficiency of their *cis*-regulatory elements in comparison with Mo-MLV vectors, RT-PCR was performed. For this purpose, RNA was isolated from CEM T cells transduced with MP71GPRE (46.1% GFP$^+$ cells) and LGSN (64.7% GFP$^+$ cells). Different amounts of RNA were used to synthesize complementary DNA, which then was amplified with GFP- and GAPDH-specific primers. Although CEM cells were less efficiently transduced by MP71GPRE, these cells revealed more in-

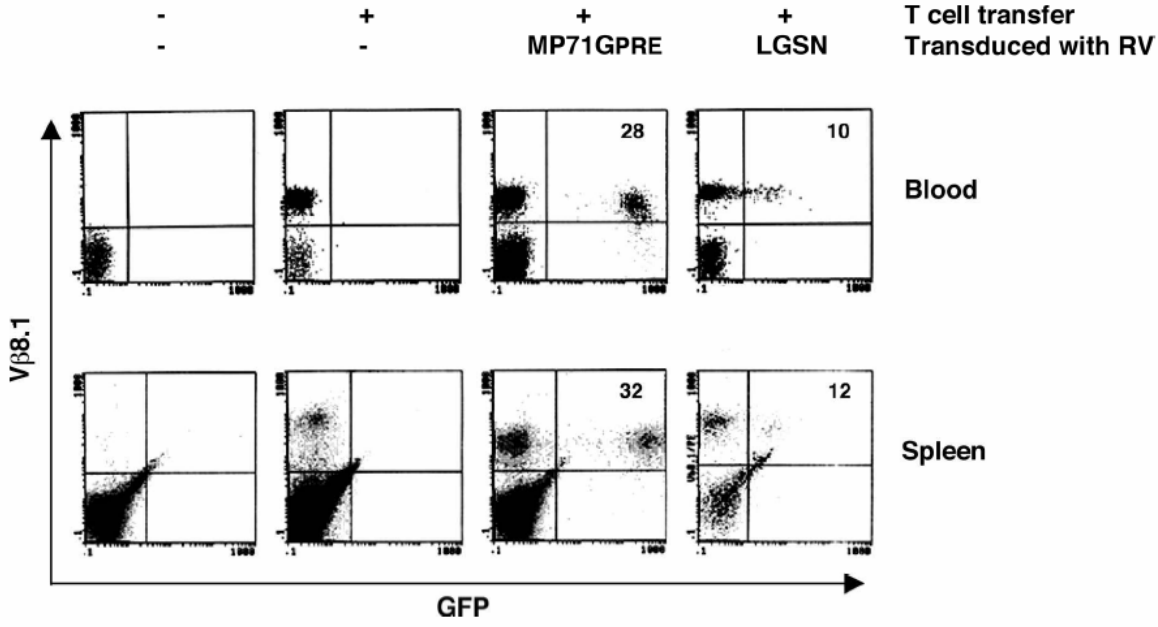
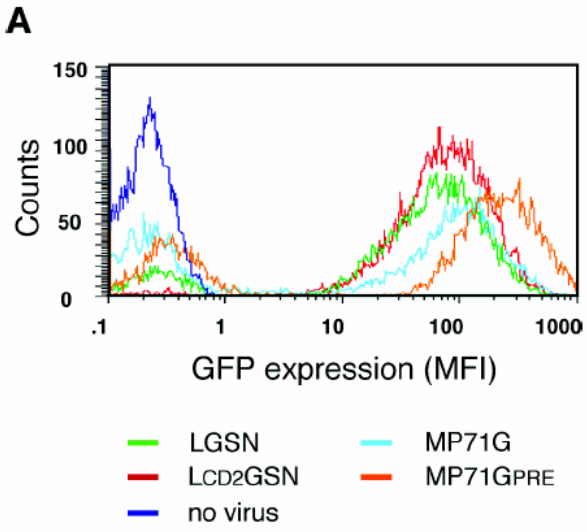


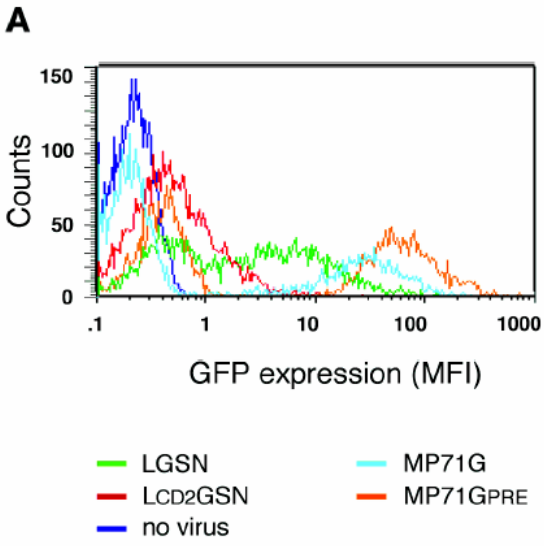
FIG. 7. Survival of GFP gene-modified CD8$^+$ T lymphocytes is not impaired in Rag-1-deficient mice. Primary splenic T lymphocytes (Vβ8.1$^+$) were isolated from P14 TCR-transgenic mice and transduced with GFP vectors LGSN and MP71GPRE in RetroNectin-coated 24-well plates. T cells (5×10^7) were adoptively transferred by tail vein injection into T cell-deficient Rag-1-deficient mice (35% GFP$^+$ after transduction with MP71GPRE, 10% GFP$^+$ after transduction with LGSN). Ten days after T cell transfer, mice were killed and peripheral blood and splenic T lymphocytes were isolated. T cells were stained with a PE-labeled Vβ8.1 MAb directed against the TCR β chain of P14 TCR-transgenic T lymphocytes. The percentage of GFP$^+$/Vβ8.1$^+$ T lymphocytes (indicated by the number in the upper right-hand corner of each panel) recovered from Rag-1-deficient mice after adoptive transfer was analyzed by flow cytometry. Shown is one representative mouse of three for each vector transduction.

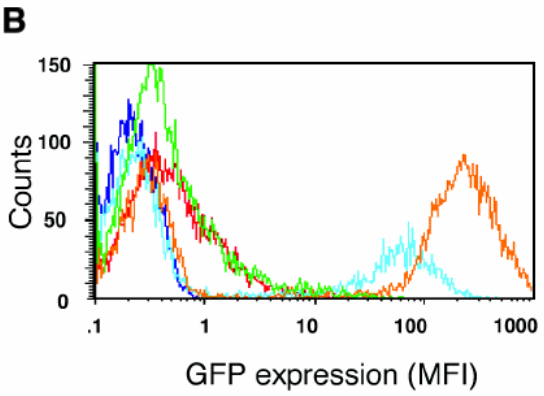


B

Cell line	MFI	% GFP+ cells
HT1080	0.2	0
+ LGSN	63.8	87
+ LCD2GSN	74.2	98
+ MP71G	76.2	62
+ MP71GPRE	195.8	72

FIG. 8. Improved transgene expression by MP71 vectors in HT1080 cells. (**A**) HT1080 cells (1×10^5 per well) were transduced with various GFP retroviruses (MOI 10) in 24-well plates. After 72 hr, cells were harvested and GFP expression levels (MFI) and transduction efficiencies (percent GFP^+ cells) were determined by flow cytometric analysis. (**B**) Quantitative values of MFI and percentage of transduced cells.

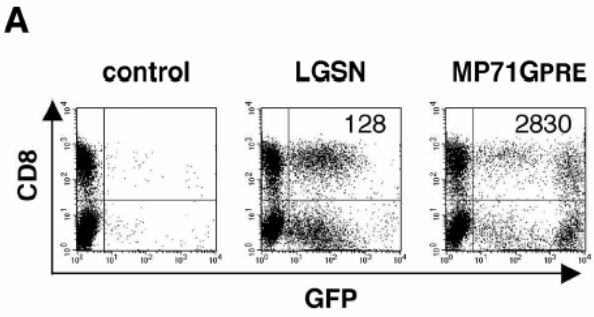




C

Cell line	MFI	% GFP+ cells
CEM	0.3	0
+ LGSN	5.8	59
+ LCD2GSN	1.5	29
+ MP71G	24.8	30
+ MP71GPRE	57.4	47
HUT78	0.2	0
+ LGSN	2.6	17
+ LCD2GSN	1.6	25
+ MP71G	43.6	29
+ MP71GPRE	196.3	57

FIG. 9. High GFP expression by MP71 vectors compared with Mo-MLV vectors in human T cell lines. T cell lines (**A**) CEM and (**B**) HUT78 (each 1×10^5 cells per well) were transduced with various GFP retroviruses (MOI of 10) in RetroNectin-coated 24-well plates. After 72 hr, cells were harvested and GFP expression levels (MFI) and transduction efficiencies (percent GFP^+ cells) were determined by flow cytometric analysis. (**C**) Quantitative values of MFI and percentage of transduced cells.



B

Human primary T lymphocytes	MFI	% GFP⁺CD8⁺
Donor 1	0.3	0
+ LGSN	128	22
+ MP71GPRE	2830	22
Mean of 3 donors	0.2	0
+ LGSN	131	19
+ MP71GPRE	2590	21

FIG. 10. High transgene expression by MP71 vectors compared with Mo-MLV vectors in human primary T lymphocytes. (**A**) Human PBMCs were obtained from healthy donors by Ficoll-Hypaque centrifugation and stimulated with coimmobilized anti-CD3/anti-CD28 MAbs and IL-2 for 72 hr. The cells in each well (2×10^5) were transduced three times within 72 hr with LGSN and MP71GPRE (MOI of 10) in RetroNectin-coated 24-well plates in the presence of IL-2. After 72 hr, GFP expression levels (MFI) and transduction efficiencies (percent GFP^+ cells) were determined by flow cytometric analysis. (**B**) Mean values (three donors) of MFI and percentage of transduced cells.

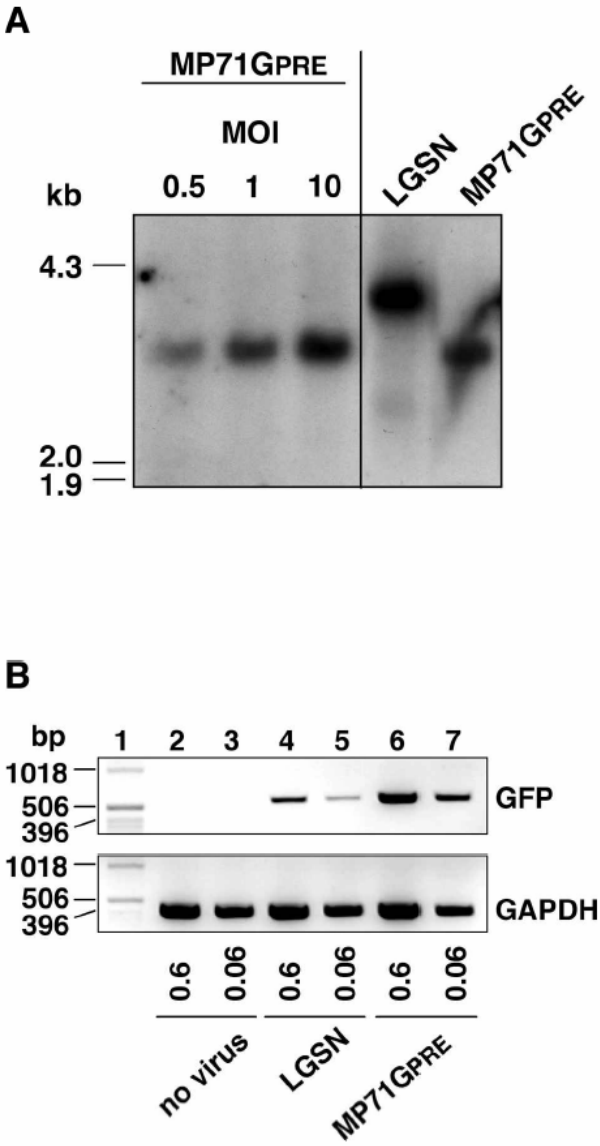


FIG. 11. *Cis*-regulatory elements of MPSV-based vectors are responsible for the high level of transgene expression. (**A**) Genomic DNA was isolated from CEM T cells transduced with the various GFP retroviruses and each 20 μg of DNA was digested with *Nhe*I (LGSN) or *Xba*I (MP71GPRE). Southern blot hybridization for equal copy numbers and the presence of intact proviruses was performed with an α-^{32}P-labeled GFP-specific cDNA probe. (**B**) Expression of GFP in CEM T cells. RT-PCR was performed with 0.6 and 0.06 μg of total RNA isolated from CEM cells on day 57 after transduction with MP71GPRE and LGSN. PCR primers were specific for GFP and GAPDH cDNAs. After 35 cycles of amplification, samples were electrophoresed through a 1.5% agarose gel and stained with ethidium bromide. Lane 1, molecular size markers; lanes 2 and 3, nontransduced CEM cells; lanes 4 and 5, LGSN-transduced CEM cells; lanes 6 and 7, MP71GPRE-transduced CEM cells. Similar results were obtained after 30 cycles of amplification.

tense GFP bands at two different RNA concentrations used for RT-PCR. At an RNA concentration of 0.06 μg in LGSN-transduced CEM cells only a faint band was detectable, whereas a prominent band was visible when CEM cells were transduced by MP71GPRE (Fig. 11B). This result was confirmed at an increased RNA concentration (0.6 μg) used for RT-PCR. Again, MP71GPRE-transduced cells revealed a more intense band in comparison with CEM cells transduced by LGSN. Similar results were obtained when human HUT78 and mouse TG40 T cells were analyzed (data not shown). This indicates that the different GFP expression levels are due to the different *cis*-regulatory elements and not to posttranscriptional regulations.

DISCUSSION

Efficient transduction of target cells and high levels of transgene expression are prerequisites for the application of many

gene therapy protocols. We have analyzed retroviral vectors harboring various *cis*-regulatory elements regarding their capacity to express GFP as a marker gene. We show that MP71 retroviral vectors, containing the MPSV LTR with an improved 5′ untranslated region, yielded up to 10-fold higher transgene expression in mouse primary T lymphocytes and T cell lines in comparison with vectors containing the Mo-MLV LTR. This effect was even more pronounced when human primary T lymphocytes and T cell lines were analyzed. The higher levels of GFP expression induced by MP71 vectors correlated with higher GFP mRNA levels and are not due to nonspecific provirus rearrangements or different copy number integration of the vector as shown by Southern blot analysis. The transduced cells expressed the transgene stably over time. In addition, we showed that high transgene expression levels can be achieved only by appropriate *cis*-regulatory elements and cannot be substituted by increasing the MOI of viruses bearing suboptimal regulatory elements. Vector-dependent differences were also maintained *in vivo* in mouse primary T lymphocytes.

The present study indicates that GFP retroviruses that efficiently transduce human and mouse primary T lymphocytes and cell lines, can be used to analyze the impact of vector *cis* elements on transgene expression. Previously, it was demonstrated that the LTR of MPSV is superior to that of Mo-MLV for gene expression in myeloid progenitor cell lines, human T lymphoblastoma cells, and human and mouse lymphohematopoietic cells (Beck-Engeser *et al.*, 1991; Baum *et al.*, 1995; Onodera *et al.*, 1998; Halene *et al.*, 1999). However, this LTR exchange alone may account for not more than a 2-fold increase in transgene expression (Baum *et al.*, 1995). Therefore, the strong increase in transgene expression observed with MP71 vectors in human and mouse T cells must also reflect features of the 5′ untranslated region and is further enhanced by the lack of an internal *neo* cassette. Interestingly, the advantage of MP71 vectors was most pronounced in primary human and mouse T lymphocytes and T cell lines, and observed to a lesser extent in non-T cells, arguing for differentiation-specific interaction with T cell transcriptional and posttranscriptional regulatory factors. GFP gene-modified TCR-transgenic T cells adoptively transferred into Rag-1-deficient mice revealed the same GFP expression level and showed the same ratio of transduced to nontransduced cells as observed *ex vivo* for the vectors LGSN and MP71GPRE after 30 days. Thus, MP71 vectors may be suitable to achieve sufficient expression levels of therapeutic genes in this important target population. This may be particularly relevant for gene therapy approaches in which the amount of expressed transgene product determines the biological effect (e.g., TCR genes, suicide genes, intracellular immunization against HIV infection). Moreover, a high level of transgene expression can be achieved with MP71 vectors, using a low MOI of vector particles, potentially reducing the risk of insertional mutagenesis. Pseudotyping of MP71 vectors with the envelope of MLV-10A1, which we have previously characterized as a suitable envelope for the efficient transduction of human T cells (Uckert *et al.*, 2000a), should further allow the generation of sufficient amounts of gene-modified T lymphocytes within a short period of time.

The insertion of the woodchuck hepatitis virus PRE into the vector MP71G to generate MP71GPRE resulted in a relatively mild increase in GFP expression in mouse T cells when compared with earlier data obtained with MLV-, HIV-, and adeno-associated virus (AAV)-derived vectors (Loeb *et al.*, 1999; Zufferey *et al.*, 1999; Schambach *et al.*, 2000). However, there was a strong increase when human T cells were analyzed. It is possible that the enhancing role of the PRE depends on the interaction with additional posttranscriptional and transcriptional regulatory elements, both being highly cell type dependent (C. Baum, unpublished data).

The introduction of the T cell-specific human *CD2* enhancer into the retroviral vector LGSN to generate LCD2GSN neither conferred lymphocyte specificity nor resulted in increased GFP expression. However, this is not surprising in view of the fact that GFP expression is driven by a viral enhancer in the former vector and by a cellular enhancer in the latter, as has also been observed by others (Indraccolo *et al.*, 2002). In a previous study, using a human *CD2* LCR-containing retroviral vector, Kaptein and coworkers have reported low vector titers (Kaptein *et al.*, 1998). We did not find decreased vector titers of LCD2GSN produced either in ecotropic GP+E86 or amphotropic PT67 packaging cells in comparison with the other vectors. These differing results may result from different vector design. Furthermore, the *CD2* enhancer included in our vector is contained in a 1-kbp truncated *CD2* LCR sequence, devoid of LCR activity, and therefore differs from the 2.1-kbp full-length *CD2* LCR used by others. Indraccolo and coworkers have also reported that only retroviral vectors carrying the full-length *CD2* LCR sequence may be instable and generate poor vector titers (Indraccolo *et al.*, 2001).

In summary, MP71 vectors efficiently transduce primary T lymphocytes and related cell lines and induce a high level of transgene expression in these cells.

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