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Modulation of Moloney Leukemia Virus Long Terminal Repeat Transcriptional Activity by the Murine CD4 Silencer in Retroviral Vectors

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We investigated whether CD4 gene regulatory sequences might be useful for developing transcriptionally targeted Moloney murine leukemia virus (Mo-MLV)-based retroviral vectors for gene expression specifically in $CD4^+$ cells. We could modulate Mo-MLV long terminal repeat (LTR) activity by inserting a 438-bp-long fragment containing the murine CD4 silencer in the LTR of the vector; both β-galactosidase and green fluorescent protein reporter gene activities were strongly down-regulated in both murine and human $CD8^+$ cells, but not in $CD4^+$ lymphoid cell lines and freshly isolated lymphocytes transduced with this vector, compared with the findings using a control vector carrying wild-type LTRs. Titration experiments on NIH-3T3 cells revealed that inclusion of the CD4 silencer in the LTRs did not reduce the titer of the vectors. These findings indicate that a cellular silencer can be successfully included in retroviral vectors, where it maintains its transcription-regulatory function, thus suggesting a novel approach to transcriptional targeting.

Key Words: CD4; silencer; retroviral vectors; lymphocytes; gene therapy.

INTRODUCTION

Retroviral vectors derived from the Moloney murine leukemia virus (Mo-MLV) are widely used to transfer genes of interest into a variety of animal and human cells, both *in vitro* and *in vivo* (Verma and Somia, 1997). Among the many shortcomings that hamper *in vivo* gene transfer by retroviral vectors, specificity of gene transfer and expression are of major concern (Gunzburg and Salmons, 1996). Retroviral tropism is controlled at different levels, including specificity of infection for selected cell types and transcriptional activity of the viral long terminal repeat (LTR) in the infected cell. Our group previously focused on vectors targeting T lymphocytes in terms of specificity of infection and could show the feasibility of generating both MLV/HIV and MLV/SIV pseudotypes to selectively target $CD4^+$ cells at the level of virus entry (Indraccolo *et al.,* 1998; Mammano *et al.,* 1997). As a complementary approach, we evaluated the possibility of transcriptionally targeting a retroviral vector to CD4-expressing lymphoid cells by inserting regulatory sequences from the tightly regulated CD4 gene into the viral LTR. Engineering the viral LTR with tissue-specific regulatory sequences is one of the favorite approaches to transcriptional targeting, in view of the fact that the LTR drives the expression of the marker/therapeutic genes in most retroviral vectors, and has been shown to be successful in many cases (Couture *et al.,* 1994; Fassati *et al.,* 1998; Golemis *et al.,* 1990; Mrochen *et al.,* 1997; Robinson *et al.,* 1995; Saller *et al.,* 1998; Valerio *et al.,* 1989; Vile *et al.,* 1995). We focused on the CD4 gene because detailed information on its regulated expression was available. Indeed, previous studies identified promoter sequences from the human gene that are necessary and sufficient for tissue-specific expression of a reporter gene in transient transfection assays (Salmon *et al.,* 1993); furthermore, a proximal murine CD4 enhancer, which is indispensable for correct expression of the transgene in transgenic mice, has also been characterized (Boyer *et al.,* 1997; Salmon *et al.,* 1996; Sawada and Littman, 1991). Finally, recent studies have uncovered a murine CD4 silencer, located in the first intron of the gene, which might contribute to the down-regulation of CD4 expression in $CD8^+$ cells and thus lead to the generation of single positive CD4 and CD8 cells in the thymus (Sawada *et al.,* 1994). Our findings show that it is feasible to exploit a cellular silencer from the CD4 gene to modulate the activity of the viral LTR: since previous studies with hybrid LTRs exploited exclusively either tissue-specific promoters or enhancers, this extends the repertoire of sequences that can be useful for transcrip-

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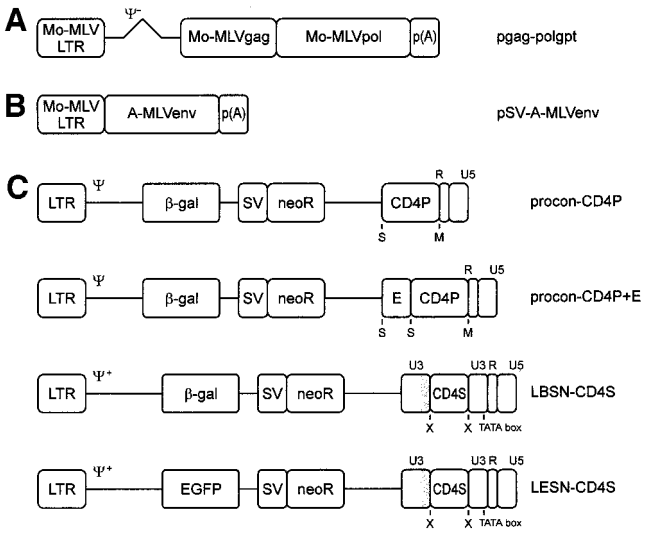

FIG. 1. Schematic representation of the constructs used to produce Mo-MLV-based vectors by transient transfection. (A) Mo-MLV Gag-Pol expression plasmid (p*gag-pol*gpt) (Markowitz *et al.,* 1988). (B) A-MLV Env expression plasmid (Page *et al.,* 1990). (C) β-gal and EGFP transfer vectors containing different CD4 gene regulatory elements. The Mo-MLV packaging signal (ψ) is deleted in p*gag-pol*gpt but is present in each vector genomic-RNA encoding construct; some constructs carry a longer encapsidation signal (ψ^+), which also comprise some gag sequences that increase packaging efficiency (Miller and Rosman, 1989). p(A), polyadenylation signal; SV, simian virus 40 promoter; neoR, neomycin phosphotransferase gene; CD4P, human CD4 promoter; E, murine CD4 proximal enhancer; CD4S, murine CD4 silencer. The gray box indicates the Mo-MLV enhancer; the approssimate position of the viral promoter in the CD4 silencer-carrying vectors is indicated by the TATA-box. Restriction sites relevant for cloning are indicated: S, *Sac*II; M, *Mlu*I; X, *Xba*I.

tional targeting of retroviral vectors. While targeting of $CD4^+$ cells *in vitro* can be easily achieved at the level of viral entry, i.e., by immunoselection of the $CD4^+$ subset or by using the above-mentioned pseudotypes, a transcriptionally targeted silencer-containing vector could be useful for gene transfer into hematopoietic stem cells, in order to direct expression of the therapeutic gene(s) in the T lymphoid compartment; furthermore a cellular silencer could be exploited in vectors for direct *in vivo* gene transfer targeting this cell subset.

RESULTS AND DISCUSSION

Generation of retroviral vectors and transduction of NIH-3T3 cells

We constructed retroviral vectors carrying these CD4 gene regulatory elements as detailed in Fig. 1 and under Materials and Methods. Retroviral vector-containing supernatants (SNs) were first used to transduce murine fibroblast NIH-3T3 cells in order to check for vector production and determine the genomic stability of the vectors. Titer was determined by G418 selection, as all vectors used in this study carried an SV40 promoter-driven neomycin phosphotransferase gene that allows drug-mediated selection of vector-transduced cells. Both the construct containing the human CD4 promoter (procon-CD4P) and the one carrying this sequence linked to the murine CD4 proximal enhancer (procon-CD4P + E) (see Fig. 1) yielded similar virus titers. In three independent experiments, $2.2 \pm 0.9 \times 10^3$ and $2.6 \pm 1.2 \times 10^3$ cfu/ml of SN were obtained on average; these figures are about 10-fold lower than those generated by the retroviral vector LBSN carrying wild-type LTRs and the one carrying the CD4 silencer in the LTR (on average $15 \pm 2.6 \times 10^3$ and $22 \pm 1.5 \times 10^3$ cfu/ml, respectively). This difference may be explained in part by intrinsic genomic differences in the vectors, such as a longer encapsidation signal (ψ^+) in the LBSN-derived vectors, which derive from LXSN and also comprise some gag sequences that increase packaging efficiency (Miller and Rosman, 1989), compared to first-generation procon vectors, which are derived from pBAG (Mrochen *et al.,* 1997; Saller *et al.,* 1998).

Assessment of promoter conversion and vector stability in transduced NIH-3T3 cells

To rule out the possibility that cellular sequences included in the vectors might affect their genomic stability and lead to rearrangements, we analyzed genomic DNA from pools of 50–100 clones of G418-resistant NIH-3T3 cells by either polymerase chain reaction (PCR) or Southern blotting techniques. In the case of LBSN-CD4S, provirus integrity and the presence of the silencer in the 5′ end LTR were verified by Southern blot analysis; to this end, genomic DNA of transduced NIH-3T3 cells and of control transfected 293T cells was digested with either *Nhe*I or *Kpn*I, both of which cleave once in each proviral LTR, blotted, and hybridized to a neoR probe. The vectors employed and representative restriction patterns obtained are shown in Fig. 2. In the LBSN-CD4S-transduced NIH-3T3 sample, *Nhe*I digestion generated a band of 8.1 kb, which accounted for the size of a provirus containing a modified 5′ LTR derived from correct duplication of the 3′ LTR. The integrity of the 3′ LTR was determined by digestion with *Kpn*I, which generated an expected band of 8.1 kb. In the producer 293T cells, obtained by transfection of LBSN-CD4S plasmid DNA, the size of the band obtained after *Nhe*I digestion (7.7 kb) was smaller than that obtained after *Kpn*I digestion (8.1 kb), because the CD4 silencer is present only in the 3′ LTR (Fig. 2). These results indicate correct LTR duplication and integrity of the viral transcriptional unit in NIH-3T3 cells transduced by the LBSN-CD4S vector. To confirm these findings, we designed a PCR approach, based on amplification of genomic DNA with primers binding to the U3 region, 5′ to the cloning site of the CD4 silencer, and to the Ψ region of the vector (Fig. 2A), as detailed under Materials and Methods. PCR amplification yielded

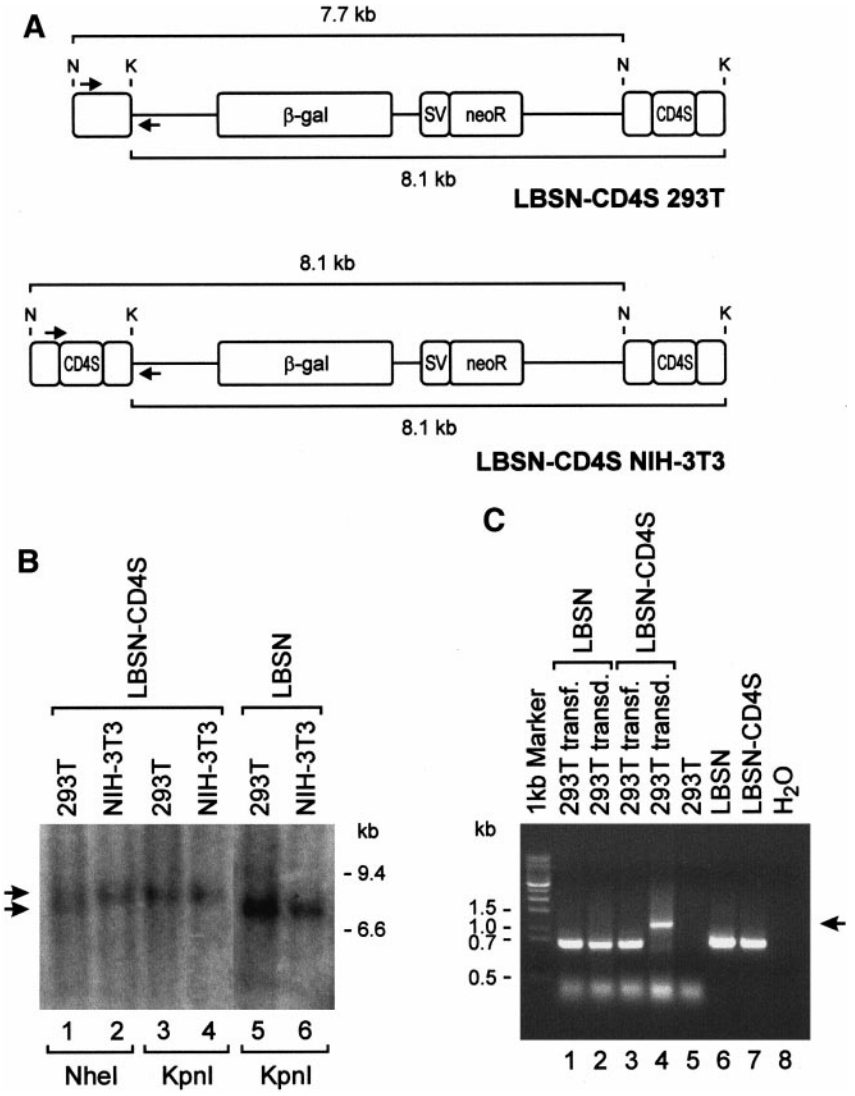

FIG. 2. Analysis of proviral DNA in transduced cells. (A) Schematic map of the LBSN and LBSN-CD4S retroviral vectors and of the expected size of restriction fragments. The approximate position of the LBSN-for (→) and LBSN-rev (←) primers used for PCR experiments is also shown. (B) Southern blot analysis of LBSN-CD4S retroviral integration in genomic DNA from vector-transduced NIH-3T3 and 293T virus producer cells. DNAs (10 μg/lane) were digested with *Nhe*I (lanes 1 and 2) and *Kpn*I (lanes 3–6) and hybridized to a neoR probe. The expected bands in *Nhe*I-digested DNA from either LBSN-CD4S-transduced cells (8.1 kb) (lane 2) or LBSN-CD4S-transfected cells (7.7 kb) (lane 1) are indicated with arrows. DNAs from cells either transfected or transduced with vector LBSN are also shown as a control for the size of the wild-type provirus (7.7 kb) (lanes 5 and 6). The molecular weight marker λ *Hin*dIII is indicated. (C) PCR analysis of vector-transduced cells. PCR was performed on genomic DNA from 293T cells either transfected or transduced with the LBSN and LBSN-CD4S vectors (lanes 1–4) or control nontransduced 293T cells (lane 5). Ten nanograms of the LBSN and LBSN-CD4S plasmids and water (lanes 6–8) were used as positive and negative controls for PCR, respectively. The band generated from 293T LBSN-CD4S-transduced cells is indicated with an arrow.

bands of 680 and 1120 bp when DNA from LBSN-CD4S-transfected and -transduced cells was used as a template, respectively (Fig. 2C). This is due to the fact that, following proviral formation, the CD4 silencer is transposed to the 5′ LTR of the vector, where it is inserted in the U3 region. On the other hand, PCR amplification of LBSN-transduced cells yielded a band of 680 bp, which was identical in size to that obtained from LBSN-transfected 293T cells, due to the lack of the CD4 silencer in this vector (Fig. 2C). In the case of the procon-CD4P and procon-CD4P + E vectors, we also verified correct promoter conversion in transduced cells by exploiting a PCR assay which enabled us to distinguish pre- and postconversion templates by the size of the PCR product (data not shown).

Determination of β-galactosidase (β-gal) activity in lymphoid and nonlymphoid cell lines

To test the activity of the different constructs carrying CD4 gene regulatory sequences, murine cell lines were transduced with the vectors and analyzed for β-gal ex-

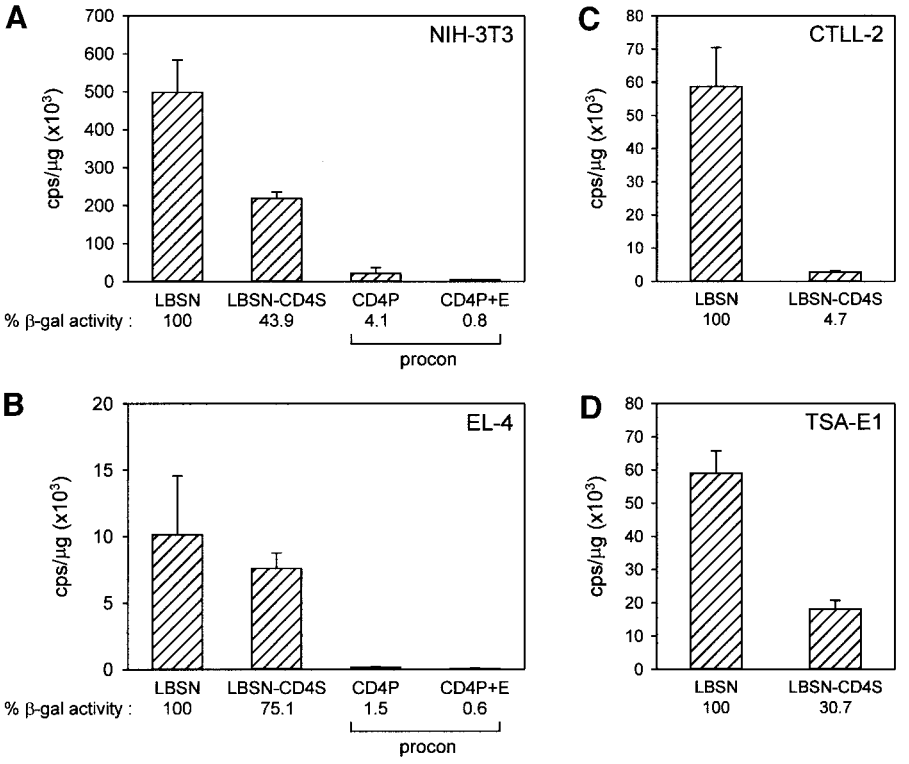

FIG. 3. Determination of β-gal activity in vector-transduced G418-selected cell lines. Murine fibroblastic NIH-3T3 (A), $CD4^+$ EL-4 (B), $CD8^+$ CTLL-2 (C), and mammary tumor TSA-E1 (D) cell lines were transduced with the indicated retroviral vectors and selected in G418-containing medium for 2–3 weeks; cell lysates were assayed for β-gal expression as described under Materials and Methods. The activity of the different constructs is expressed as counts per second (cps) per microgram protein in the cell lysates. Each bar represents the mean vector-derived β-gal activity (total activity − endogenous activity). Standard error is also indicated. Relative β-gal activities of the different vectors, compared to LBSN, are shown at the bottom of each panel. The results are representative of two experiments for CTLL-2, three for EL-4, and five for NIH-3T3 and TSA-E1 cell lines.

pression. The procon-CD4P and procon-CD4P + E vectors expressed very low levels of β-gal activity in NIH-3T3 cells, compared to LBSN (Fig. 3A). This result was predicted since β-gal is driven by the tissue-specific human CD4 promoter in these constructs, and this promoter is not expected to be active in NIH-3T3 cells. Unexpectedly, however, β-gal activity was also weak in the murine $CD4^+$ EL-4 cell line transduced with the procon-CD4 vectors, compared to the LBSN vector (Fig. 3B). Similar results were obtained in the human $CD4^+$, Molt-3, and Jurkat cell lines (data not shown). Thus, the human CD4 promoter, at least in this configuration, is poorly active in the context of a promoter-conversion retroviral vector, compared to the Mo-MLV promoter. On the other hand, the LBSN-CD4S vector, carrying the CD4 silencer in the LTRs, yielded measurable β-gal activity in all cell lines tested (Fig. 3 and data not shown); in particular, β-gal activity was reduced only by 25% in the $CD4^+$ murine EL-4 cell line when compared to the β-gal activity shown after transduction of the same cells with the LBSN vector (Fig. 3B). However, the CD4 silencer strongly down-modulated reporter gene expression in the $CD8^+$ murine CTLL-2 cell line; activity of the LBSN-CD4S construct in this cell line was reduced by 95%, compared to the standard LBSN vector (Fig. 3C). A marked reduction in β-gal activity (56 and 68%) was also observed in the nonlymphoid cell lines, NIH-3T3 and TSA-E1, respectively, transduced with the LBSN-CD4S, compared to figures obtained with the wild-type vector (Figs. 3A and 3D). These findings resemble previous data on CD4 silencer activity obtained after transient transfections of murine hematopoietic cell lines (Sawada *et al.,* 1994). To extend the functional studies regarding the activity of this inhibitory sequence, which has been only partially investigated to date, we transduced some human tumor cell lines, including cervical-carcinoma-derived HeLa and bladder-carcinoma-derived EJ cells. In three independent experiments, transduction of these human cell lines with the LBSN-CD4S vector yielded levels of β-gal activity that were strongly reduced (range, 70–93%) compared to LBSN-transduced controls (data not shown); thus, the murine silencer was also functional in human cells. This suggests that the still poorly characterized factors that interact in the mouse cell with the CD4 silencer might be conserved in humans as well. Interestingly, the recent identification of a human CD4 silencer, showing 77% identity with the murine sequence, indirectly supports the hypothesis that a similar regulation of CD4 gene expression might occur in humans and mice (Donda *et al.,* 1996). Based on these findings, we

conclude that our CD4 silencer-carrying vector can adequately discriminate between $CD4^+$ and $CD8^+$ cells and that the degree of suppression of gene expression is only partial in most of the other cell lines tested. Future work might entail juxtaposing the cellular silencer with a cellular promoter, as well as validation of this transcriptional targeting approach by generation of retroviral vectors carrying other cellular silencers with a marked turn-off potential for many cell types, such as the neuron-restrictive silencer element (Schoenherr *et al.,* 1996), which should lead to expression of the transgene selectively in neuronal cells.

Confirmation of silencer activity by enhanced green fluorescent protein (EGFP)-gene transfer

To analyze in greater detail silencer activity in short-term experiments and in the absence of drug selection, we cloned this element in the *Xba*I site of the 3′ LTR of a retroviral vector expressing the EGFP marker gene (see Fig. 1) and used it to transduce several human and murine cell lines; thus, the activity of a given promoter could be determined by cytofluorimetric analysis as the percentage of $EGFP^+$ cells, as well as by measuring the median fluorescence intensity (MFI). Transduction experiments were performed as detailed above with vectors carrying the β-gal gene. Seventy-two hours after infection, EGFP-expressing vector-transduced cells were analyzed on an Elite cytofluorometer (Coulter). NIH-3T3 cells transduced by the LESN-CD4S vector expressed EGFP at similar percentages (70.4% vs 72.7%) but much lower MFI (665 vs 781), compared to NIH-3T3 cells transduced by the LESN vector carrying wild-type LTRs. In these same experiments, we also evaluated the activity of a retroviral vector that carries the silencer element in the same position but in the opposite orientation, termed LESN-CD4Sα. Interestingly, this construct yielded an EGFP expression pattern in NIH-3T3 cells similar to that of the LESN vector (83.1% $EGFP^+$ cells, MFI = 782), thus indicating that CD4 silencer function might be orientation-dependent, at least in the context of retroviral vectors; this also shows that the modulation of LTR activity in $CD8^+$ cells is silencer-dependent, rather then induced by spacer effects caused by the separation of the viral promoter and enhancer by silencer insertion. Titration experiments indicated that the LESN, LESN-CD4S, and LESN-CD4Sα retroviral vectors yielded similar amounts of virus, on average $4.4 \pm 0.4 \times 10^5$, $4.2 \pm 0.5 \times 10^5$, and $4.8 \pm 0.5 \times 10^5$ TU/ml, respectively; this was also confirmed by G418 selection of transduced NIH-3T3 cells (data not shown).

We then used these vectors to transduce lymphoid cell lines, including the $CD4^+CD8^+$ JM and the $CD4^+$ Jurkat cell lines. In preliminary experiments, we found that VSV-G pseudotyped MLV particles transduced these cell lines more efficiently than particles displaying the A-MLV Env (data not shown), and they were therefore used in the following experiments. The same cell lines transduced with the LXSN retroviral vector, not carrying EGFP, served as the negative control for EGFP-expression analysis. Gene transfer in JM cells revealed that the silencer-carrying vector expressed EGFP at much lower levels than the standard vector; 22.2% of JM cells expressed EGFP after transduction with LESN-CD4S, with MFI = 422, versus 55.7% of $EGFP^+$ JM cells (MFI = 524) with the LESN vector (Fig. 4A and 4B). Virus titration on NIH-3T3 cells confirmed that similar amounts of vector virus were present in the two preparations (4×10^5 and 3.8×10^5 TU/ml, respectively), indicating that these findings cannot be attributed to differences in titer. The reduced activity of CD4 silencer-carrying vector in JM cells is in agreement with previous findings in transgenic mice generated with different constructs carrying the CD4 silencer, indicating a lack of expression of the marker gene in $CD4^+CD8^+$ thymocytes (Duncan *et al.,* 1996). In the $CD4^+$ Jurkat lymphoid cell line, however, the LESN-CD4S construct expressed EGFP at levels similar to those generated by the standard LTR vector; 71.8% (MFI = 466) and 75.6% (MFI = 419) $EGFP^+$ Jurkat cells were detected following transduction by LESN (Fig. 4C) and LESN-CD4S (Fig. 4D), respectively. Similar figures were found following transduction of $CD4^+$ Molt-3 cells with these vectors (data not shown). These findings demonstrate that the silencer-carrying vector provides efficient EGFP expression in $CD4^+$ cell lines, but shows impaired expression in fibroblastic and $CD8^+$ lymphoid cell lines, compared to the vector carrying wild-type LTRs in the absence of drug selection, thus confirming and validating those already obtained with the β-gal gene. *In vitro* culture of these transduced cells up to 8 weeks indicated that EGFP marker expression by both the wild-type and the CD4 silencer-carrying retroviral vector was stable (data not shown).

Application to clinically relevant targets: Transduction of human primary lymphocytes with the CD4 silencer-carrying retroviral vector

Having established the activity of the LESN-CD4S vector in cell lines, it was important to verify whether the observed suppression of reporter gene activity might also be detected in primary $CD8^+$ lymphoid cells. As we had already shown that the murine silencer is also active in human cells, we transduced the relevant vector into primary human lymphocytes. We first compared infectivity for peripheral blood mononuclear cells (PBMC) of Mo-MLV particles displaying the A-MLV, VSV-G, or Gibbon ape leukemia virus (GaLV) envelopes and found the best results, in terms of the percentage of $EGFP^+$ lymphocytes, using the GaLV Env pseudotyped particles (data not shown). Therefore, we used MLV/GaLV pseudotypes to transduce human primary lymphocytes

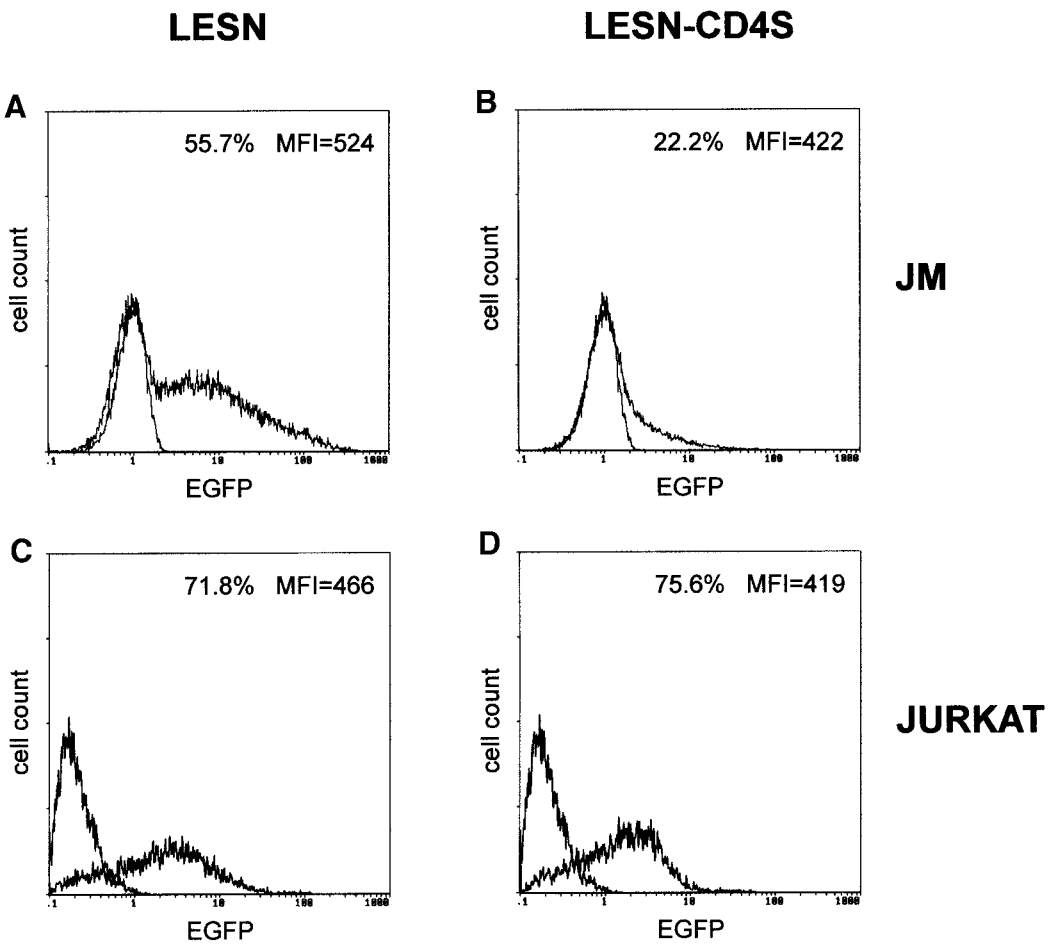

FIG. 4. Transduction of human lymphoid cell lines by different MLV/VSV-G retroviral vectors carrying EGFP as a marker gene. JM and Jurkat cells were infected with either LESN or LESN-CD4S retroviral vectors generated by transient transfection of 293T cells. Seventy-two hours after transduction the percentage of EGFP-expressing cells was quantified by FACS analysis. The mean percentage of EGFP-positive cells for each construct and the median fluorescence intensity (MFI) are indicated. The profile of the mock-transduced lymphoid cells in each panel is also shown.

in all subsequent experiments. Freshly isolated human lymphocytes were transduced by cocultivation with a PG13-derived packaging cell line (Miller *et al.,* 1991). One representative experiment is shown in Fig. 5, while the results of six independent transductions of PBMC from healthy donors are summarized in Table 1. Overall, following transduction with the wild-type LESN vector, we found that EGFP was expressed at slightly higher levels in $CD8^+$ than $CD4^+$ T cells, in terms of both percentage of $EGFP^+$ cells and MFI (Fig. 5 and Table 1); however, following gene transfer with the LESN-CD4S vector, a significant decrease in EGFP expression in $CD8^+$ cells, compared to $CD4^+$ T cells, was observed (Fig. 5 and Table 1) ($P = 0.02$, Wilcoxon paired t test). Thus, the vector carrying the CD4 silencer did not differ significantly from the wild-type retroviral vector in its capacity to express the transgene in the $CD4^+$ lymphocyte subset, but it showed greatly impaired activity in $CD8^+$ cells. These findings indicate that the LESN-CD4S vector can also be exploited to express genes of interest preferentially in human primary $CD4^+$ T lymphocytes.

The generation of hybrid LTRs, carrying either cellular regulatory elements or heterologous viral enhancers, is an established procedure aimed at modulating the transcriptional activity of retroviral vectors (Couture *et al.,* 1994; Fassati *et al.,* 1998; Robinson *et al.,* 1995; Saller *et al.,* 1998; Vile *et al.,* 1995); the inclusion of a cellular silencer, however, is a novel approach that constitutes an alternative to the utilization of cellular promoters or enhancers for tissue-specific expression. Many workers have found that inclusion of cellular regulatory sequences in retroviral vectors is not always a successful procedure, because the functional properties of many promoters are lost or deregulated in the retroviral setting (Gunzburg and Salmons, 1996; Malik *et al.,* 1995; Miller and Whelan, 1997; Moore *et al.,* 1991); our findings with retroviral vectors carrying the human CD4 promoter and the murine proximal enhancer also indicated as much. On the other hand, this is not completely surprising in view of the fact that the physiological expression of many cellular genes is regulated by multiple sequences, often scattered throughout many kilobases of genomic DNA,

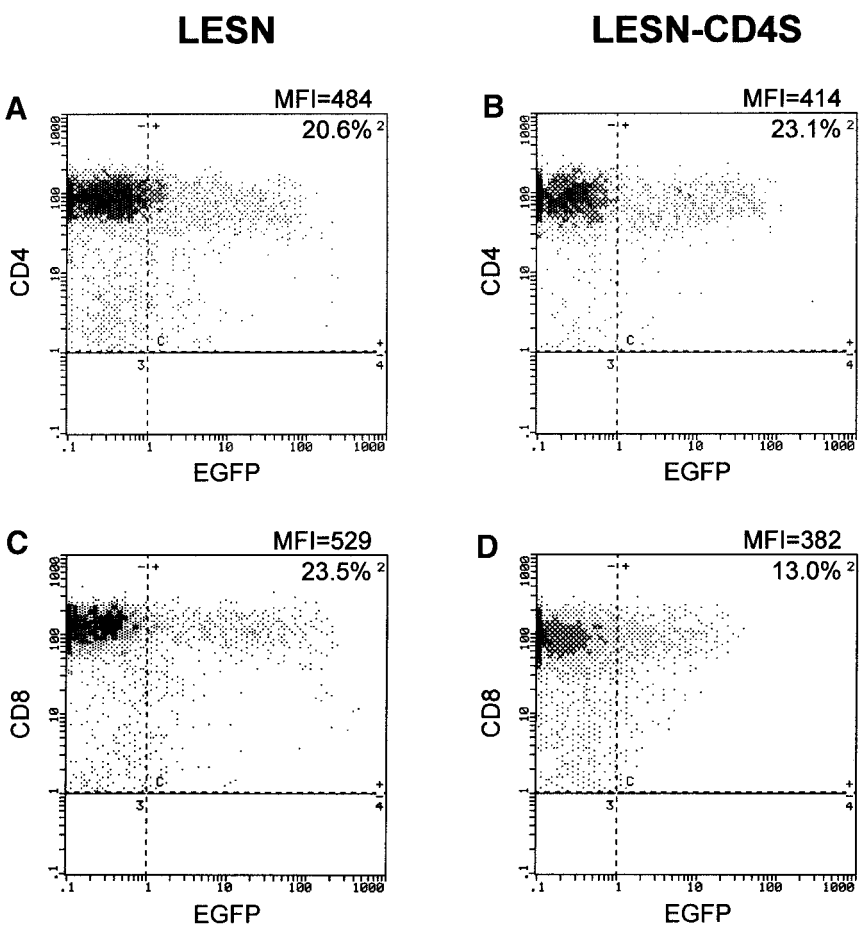

FIG. 5. Transduction of human primary T lymphocytes with different retroviral vectors carrying EGFP as a reporter gene. PHA-activated human PBMC were infected by cocultivation on PG13 packaging cells releasing either LESN or LESN-CD4S vectors. Seventy-two hours after transduction, PBMC were labeled with either anti-CD4 or anti-CD8 and the percentage of EGFP-expressing lymphoid cells was quantified by FACS analysis. FACS diagrams show either $CD4^+EGFP^+$ or $CD8^+EGFP^+$ cells (upper right of each figure). The individual constructs used for transduction, the percentage of EGFP-positive lymphoid cells, and the median fluorescence intensity (MFI) are indicated.

rather than by the promoter alone. Unfortunately, size constraints physically limit the inclusion of larger regulatory sequences in the vectors. Our findings suggest that it might be feasible to modify the function of the viral LTR, a highly complex transcriptional machinery, by adding a small inhibitory sequence that will repress transcription in defined cell types. In keeping with the results of Ferrari *et al.,* who inserted a muscle-specific enhancer into the same *Xba*I site of a similar retroviral vector (Ferrari *et al.,* 1995), the insertion of the 438-bp-long silencer into the U3 region of the viral LTR was well tolerated in that it induced neither genomic instability nor a decrease in virus titer. In conclusion, we suggest that the exploitation of cellular silencers in retroviral vectors might contribute to generate transcriptionally targeted vectors; this modification of the MLV vectors could be of general interest because it might be applied to other vector systems, including adenoviral and lentiviral vectors.

MATERIALS AND METHODS

Plasmids

The human CD4 promoter and the murine CD4 proximal enhancer and silencer were amplified from genomic DNA by PCR using the following primers:

CD4P-for: 5′-ATA CCG CGG-ACT TCC TGG GCT CAA GCA ATC CTC-3′;

CD4P-rev: 5′-GCG GCG ACG CGT-AGA CAG GAA GCC CGA TGG CAA G-3′;

CD4E-for: 5′-TCC CCG CGG-AAA TTT GAG CCC CAG CTG TTA GCC-3′;

CD4E-rev: 5′-TCC CCG CGG-CAA TCT ACC TCC ACC CTG GCT GTC-3′;

CD4S-for: 5′-ATA CCG CGG-GCT CCC ACC CAC TGC CAC CCA-3′

CD4S-rev: 5′-ATA CCG CGG-AGA TAC ATG CAG AGA ACT CAA CAC C-3′.

TABLE 1

EGFP Expression in LESN and LESN-CD4S Transduced Human Lymphocytes[a]

	% EGFP$^+$ cells (MFI)			
	LESN		LESN-CD4S	
Subject No.	CD4	CD8	CD4	CD8
1	11.5 (454)	14.5 (501)	8.4 (470)	3.4 (413)
2	16.6 (406)	16.7 (438)	16.3 (443)	15.5 (354)
3	20.6 (484)	23.5 (529)	23.1 (414)	13.0 (382)
4	3.8 (524)	5.9 (640)	2.9 (475)	1.9 (474)
5	5.9 (482)	9.1 (596)	7.5 (423)	6.1 (416)
6	7.1 (549)	9.8 (569)	6.0 (427)	3.4 (418)
Mean ± SD	10.9 ± 6.6 (483 ± 51)	13.2 ± 6.3 (545 ± 72)	10.7 ± 7.5 (442 ± 25)	7.2 ± 5.6 (409 ± 40)

[a] Human PBMC from six healthy donors were transduced with either LESN or LESN-CD4S retroviral vectors and analyzed 72 h after transduction for EGFP expression by FACS. The percentage of EGFP-positive cells for each construct and the median fluorescence intensity (MFI) in the CD4$^+$ and CD8$^+$ subsets are indicated.

PCR was performed in 50 μl of standard buffer containing 0.2 μM of each primer and 1 U of Taq polymerase (Perkin–Elmer) under the following conditions: 94°C denaturation, 1 min; 65°C annealing, 30 s; 72°C extension, 1 min; for 35 cycles, with 1 μg of genomic DNA as template. Procon-CD4P was constructed by digesting the 1083-bp PCR fragment containing the human CD4 promoter with *Mlu*I/*Sac*II restriction enzymes and cloning into the 3′ LTR of a promoter conversion vector at the corresponding unique sites (Fig. 1). Details of procon construction and characterization are reported elsewhere (Mrochen *et al.,* 1997; Saller *et al.,* 1998). Procon-CD4P + E was then generated by inserting the *Sac*II-digested 373-bp PCR fragment into procon-CD4P at the *Sac*II site (Fig. 1). Finally, LBSN-CD4S was constructed by blunt-end cloning of the 438-bp murine CD4 silencer in the *Xba*I site of the LBSN vector (Fig. 1). This *Xba*I site, positioned at bp 3186 of the parental LXSN retroviral vector genome (Miller and Rosman, 1989), from which LBSN derives, is placed between the Mo-MLV enhancer and promoter elements (Fig. 1). For EGFP experiments, the murine CD4 silencer was cloned by the same procedure used in the LESN vector (Fig. 1), which is a derivative of LXSN carrying the gene for an enhanced green fluorescent protein driven by the Mo-MLV LTR (Klein *et al.,* 1997). All constructs were verified by sequencing before functional assays were performed.

The Mo-MLV Gag-Pol expression construct *gag-pol*gpt harbors the Mo-MLV *gag* and *pol* genes under the control of the Mo-MLV long terminal repeat and a SV40 polyadenylation signal (Markowitz *et al.,* 1988). The construct lacks ψ packaging sequences as a consequence of a 134-base-pair deletion between the Mo-MLV LTR and the *gag* gene. The amphotropic Mo-MLV Env expression construct SV-A-MLV *env* has the A-MLV *env* gene inserted between Mo-MLV LTR sequences and a SV40 polyadenylation signal (Page *et al.,* 1990). A plasmid, named HCMV-G, expressing the envelope protein G of vesicular stomatitis virus (VSV-G) under the transcriptional control of the CMV promoter was used in one set of experiments to pseudotype MLV particles (Yee *et al.,* 1994) and transduce T lymphoid cell lines.

Cell culture and transfections

293T human kidney cells were obtained by ATCC and used as a packaging cell line (Pear *et al.,* 1993). The day before transfection, 1.5×10^6 293T cells were seeded in 25-cm^2 tissue culture flasks. The cultures were transfected with plasmid DNA using a calcium phosphate precipitation technique (Sambrook *et al.,* 1989). 293T, PG13, murine fibroblastic NIH-3T3, human cervix carcinoma HeLa, human bladder carcinoma EJ, murine mammary adenocarcinoma TSA-1, and murine T lymphoma CD4$^+$ EL-4 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS, Gibco-BRL) and 1% L-glutamine. Murine CD8$^+$ CTLL-2, human CD4$^+$CD8$^+$ JM, CD4$^+$ Molt-3, and Jurkat cells were grown in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine. Finally, PBMC were isolated by Ficoll–Hypaque (Pharmacia-LKB) gradient centrifugation as described (Indraccolo *et al.,* 1995a) and cultured for 48 h in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine in the presence of phytohemagglutinin (PHA-P, Difco), prior to retroviral vector transduction.

Transduction of cells with retroviral vectors

Infectious particles were generated by transfection of 293T cells with 3 μg of the Mo-MLV Gag-Pol expression construct p*gag-pol*gpt, along with 6 μg of procon-CD4P, proconCD4P + E, LBSN, LBSN-CD4S, LESN, or LESN-CD4S as transducing vectors and 3 μg of the A-MLV env expression construct. In one set of experiments, retroviral vector pseudotypes with VSV-G envelope protein were generated as reported (Burns *et al.,* 1993) and used to transduce the EGFP gene into JM, Molt-3, and Jurkat lymphoid cell lines. Fresh medium was added to the cultures 12–18 h before the SN was collected and passaged through 0.45-μm-pore-size filters. To assess the ability of the virions to transduce NIH-3T3 cells, serial dilutions of the filtered SNs were layered over NIH-3T3 target cells that had been seeded into six-well culture plates the day before infection at 1.5×10^5 cells per well. Protamine sulfate (8 μg/ml) (Sigma) was added to the wells, and the cells were kept in a total volume of 2 ml. After 6–9 h at 37°C, 3 ml of medium was added to dilute the protamine sulfate; 36 h later, the cells were split 1:10 in 10-cm-diameter petri dishes, and new medium containing G418 (Gibco-BRL, 500 μg/ml active compound) was added. Following 2 weeks of culture in G418 selec-

tive medium, the viral titer was determined as described (Klein *et al.,* 1997) and expressed as colony forming units per milliliter of SN. In one set of experiments, titer was calculated by counting EGFP-expressing cells following transduction of NIH-3T3 cells by serial dilutions of vector-containing SN and expressed as transducing units per milliliter.

Transduction of lymphoid cell lines was performed by using cell-free SN. To this end, 3 ml of retroviral vector-containing SN was incubated with 2×10^5 target cells for 6–9 h at 37°C in the presence of protamine sulfate (8 μg/ml) with occasional stirring. Lymphoid cells were then pelleted, resuspended in fresh medium, and grown for an additional 72 h before FACS analysis or selected in G418-containing medium prior to assessment of β-gal expression.

Freshly isolated human lymphocytes were transduced by cocultivation with a PG13-derived packaging cell line (Miller *et al.,* 1991). Briefly, 4×10^6 PBMC were layered on irradiated (1000 rads) confluent PG13 cells, releasing either the LESN or the LESN-CD4S retroviral vectors, in a 60-mm petri dish and cocultivated for 48 h in complete RPMI supplemented with PHA and 100 U/ml of recombinant IL2 (EuroCetus); subsequently, PBMC were transferred to 24-well plates (Becton–Dickinson) and cultured for an additional 72 h prior to EGFP detection.

Proviral DNA analysis

Genomic DNA was obtained from vector-transduced 293T and NIH-3T3 cells using the QIAamp tissue kit (Qiagen), according to the manufacturer's protocol. For PCR analysis, in the case of LBSN and LBSN-CD4S vectors, 0.5 μg of genomic DNA was PCR-amplified with primer LBSN-for (5′-CAG ATC AAG GTC AGG AAC AG-3′) and LBSN-rev (5′-CCG CCA GAT ACA GAG CTA GT-3′), using standard PCR buffer and the following conditions: 94°C denaturation, 1 min; 60°C annealing, 30 s; 72°C extension, 1 min; for 35 cycles. PCR products were then run on a 1.2% agarose gel and visualized by ethidium bromide staining.

For analysis of the LESN and LESN-CD4S vectors, 10 μg of genomic DNA was digested to completion with either *Kpn*I or *Nhe*I restriction enzymes (New England Biolabs), electrophoresed on 0.8% agarose gels, transferred to nylon membranes (Hybond-N, Amersham) by Southern blotting as described (Indraccolo *et al.,* 1995b), and hybridized to 2×10^7 cpm of a ^{32}P-labeled 1.2-kb *Hin*dIII/*Sma*I DNA probe from plasmid pSV2-neo. Filters were washed under high-stringency conditions and exposed to autoradiography for 5–7 days at −70°C.

Determination of β-gal activity

Two to three weeks following G418 selection, pools of about 100 clones of NIH-3T3, TSA-1, HeLa, and EJ cells, as well as bulk cultures of lymphoid cell lines, were lysed in 100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol lysis buffer. The protein concentration in the cell lysates was determined spectrophotometrically using the Bio-Rad Dc protein assay kit (Bio-Rad) according to the manufacturer's instructions, and the samples were then analyzed in duplicate for β-gal activity using the Galacto-Light Plus kit (Tropix) and a plate luminometer (Packard). β-Gal activity was expressed as counts per second per microgram of protein in the sample.

Cytofluorimetric analysis

EGFP-expressing vector-transduced lymphoid cells were analyzed on an EPICS-Elite cytofluorimeter (Coulter). At different times after infection, cells were pelleted, washed, and fixed. PBMC were labeled with either anti-CD4PE or anti-CD8PE (Dako) mAbs. Two-color immunofluorescence was carried out as reported (Indraccolo *et al.,* 1993) and analyzed using the PRISM parameter of the Elite cytofluorometer; the negative control setting for each mAb was determined by using labeled Ig of the corresponding isotype. Mock-transduced cell lines served as the negative control for EGFP-expression analysis. The median fluorescence intensity was calculated by the following formula: $\mathrm{MFI} = \log_{10}(\mathrm{mean} * 10) * (1024/4)$.

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