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Stable Gammaretroviral Vector Expression during Embryonic Stem Cell-Derived *In Vitro* Hematopoietic Development

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Abstract

Unlike conventional gammaretroviral vectors, the murine stem cell virus (MSCV) can efficiently express transgenes in undifferentiated embryonic stem cells (ESCs). However, a dramatic extinction of expression is observed when ESCs are subjected to *in vitro* hematopoietic differentiation. Here we report the construction of a self-inactivating vector from MSCV, MSinSB, which transmits an intron embedded within the internal transgene cassette to transduced cells. The internal transgene transcriptional unit in MSinSB is comprised of the composite cytomegalovirus immediate early enhancer-chicken β-actin promoter and associated 5′ splice site positioned upstream of the natural 3′ splice site of the gammaretroviral envelope gene, and configured such that the transgene translational initiation sequence is coincident with the envelope ATG. MSinSB could be produced at titers approaching 10^6 transducing units per ml, and directed higher levels of transgene expression in ESCs than a splicing-optimized MSCV-derived vector, MSGV1. Moreover, when transduced ESCs were differentiated into hematopoietic cells *in vitro*, MSinSB remained transcriptionally active in greater than 90% of the cells whereas MSGV1 expression was almost completely shut off. Persistent high-level expression of the MSinSB gammaretroviral vector was also demonstrated in murine bone marrow transplant recipients and following *in vitro* myelomonocytic differentiation of human $CD34^+$ cord blood stem/progenitor cells.

Keywords

self-inactivating gammaretroviral vector; embryonic stem cells; hematopoietic stem cells; differentiation; stable transgene expression

INTRODUCTION

Embryonic stem cells (ESCs) provide a powerful *in vitro* system to investigate the early stages of mammalian development [1]. Although gammaretroviral vectors derived from Moloney murine leukemia virus (MLV) are routinely used to introduce and express transgenes in differentiated cells, their utility for gene transfer into ESCs is hampered by low levels of transcription from the long terminal repeat (LTR) (for review, see [2,3]). Analysis of gammaretroviral mutants led to the development of the murine embryonic stem cell virus (MESV) that is transcriptionally active in ESCs [4]. MESV contains mutations in several repressive *cis*-acting DNA sequences: (i) a point mutation in the 5′ LTR at position -345 with respect to the transcriptional start site that destroyed a binding site for an isoform of the orphan

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nuclear receptor SF1, which acts as a transcriptional repressor of MLV; (ii) a point mutation in the enhancer at position -166 that created a functional binding site for the transcription factor Sp1, which was shown to activate the MLV LTR in ESCs; and (iii) a 5′ untranslated region from the *dl*587rev gammaretrovirus containing a primer binding site for $tRNA^{Gln}$, which removed a negative regulatory element coincident with the $tRNA^{Pro}$ primer binding site of MLV (reviewed in [2]). An alternative approach to circumvent the MLV-associated LTR transcriptional block in ESCs has involved the construction of vectors containing internal promoters from housekeeping genes exhibiting widespread transcriptional permissivity [5–8]. The murine PGK promoter (originally introduced into our HMB gammaretroviral vector) functions especially well in this context [8]. Accordingly, to efficiently express two transgenes in ESCs from a single gammaretroviral vector, we developed the dual-promoter murine stem cell virus (MSCV) vector, which incorporated the salient LTR modifications of MESV and the internal PGK promoter from HMB [9–11].

Despite their improved performance in undifferentiated ESCs, MESV/MSCV-based vectors still experience severe down-regulation of LTR- and internal PGK promoter-initiated transcription upon differentiation into embryoid bodies (EBs) and hematopoietic cells *in vitro* [12,13], although this does not always result in complete extinction of expression [9]. In this regard, it is notable that the MESV/MSCV LTR retains a so-called negative control region (NCR) that is recognized by the YY1 repressor/activator, a member of the GLI-Krüppel zinc finger transcription factor family [14]. In addition, because the MESV/MSCV LTR still contains one copy of the enhancer direct repeat (DR), which is bound by at least six *trans*-acting factors [15], differential availability of these transcription factors during differentiation could conceivably contribute to the repression observed. In support of the notion that the NCR and/or DR elements may participate in gammaretroviral vector expression failure during ESC differentiation, creation of a self-inactivating (SIN) version of MSCV by deletion of the region spanning these elements resulted in an increased probability of transgene expression directed by the internal PGK promoter in murine F9 embryonal carcinoma cells [16].

In contrast to lentiviral vectors [17], introns are generally removed from transgenes carried by gammaretroviral vectors, although not necessarily with 100% efficiency during one replication cycle [18,19]. In fact, Chang and colleagues found that the intron in an internal human EF1a promoter was efficiently propagated to target cells by an MLV-based SIN gammaretroviral vector [20]. Here we describe an MSCV-based SIN gammaretroviral vector, MSinSB, with a 303-bp deletion in the 3′ LTR encompassing the NCR and DR elements. The internal promoter incorporated into MSinSB (designated the CA promoter) is a modified version of the composite CAG promoter — which consists of the human cytomegalovirus immediate early enhancer linked to the promoter region and first intron of the chicken β-actin gene with an engineered heterologous 3′ splice site from the rabbit β-globin gene [21] — in which the rabbit β-globin gene 3′ splice site has been removed so that the natural 3′ splice site of the gammaretroviral envelope gene is used to express downstream transgene mRNAs.

RESULTS AND DISCUSSION

Construction of MSGV1-Based LTR and SIN Gammaretroviral Vectors

All of the gammaretroviral vectors used in this study were derived from the splicing-enhanced MSCV-based LTR vector MSGV1 [22,23], and contain the enhanced green fluorescent protein (GFP) gene as a fluorescence-activated cell sorter-selectable reporter [24] (Fig. 1). Position effects due to chromatin structure at the vector insertion site are believed to contribute to transcriptional silencing of transgenes shortly after integration, to expression variegation (where variable expression is observed within the clonal progeny of a target cell), and to extinction of expression (where expression is down-regulated with time or during differentiation) [2]. In an attempt to eliminate these effects, we and others have introduced

insulator elements, which function as chromatin domain boundaries, into the U3 region of retroviral LTRs. Some improvements were obtained when a 1.2-kb fragment containing a monomer of the chicken β-globin 5′HS4 insulator was used [25–28]. However, in our experience, the titers of 5′HS4 insulator-containing vectors were generally reduced [28]. Moreover, Felsenfeld and colleagues reported superior protection in cell culture transfection and *Drosophila* transgenesis experiments when the transgene was flanked by two copies of the 1.2-kb 5′HS4 fragment in tandem [29]. In further work by this group, a GC-rich core element of the 5′HS4 insulator was mapped to a 5′ 250-bp fragment, two tandem copies of which were shown to provide complete insulator function [30]. Therefore, as one approach toward alleviating ESC differentiation-associated gammaretroviral extinction, we inserted a dimer of the 250-bp 5′HS4 core element into the *Nhe*I site in the U3 region of the 3′ LTR of MSGV1 [31], creating MSGV1-C^2 (Fig. 1).

Deletion of the gammaretroviral U3 region is often associated with a 10- to 100-fold reduction in titer [32]. In the prototypical SIN vector we developed, MSinSB (Fig. 1), the NCR and DR elements were removed from the 3′ MSGV1 LTR by deletion of 303 bp between the *Nhe*I and *Sac*I sites [16]. This modification, which retains 31 bp upstream of the U3/R boundary inclusive of the TATA box, differs from our earlier SIN vectors in which all of the U3 sequences up to the U3/R boundary were deleted [8,33]. We chose this format because there continued to be some ambiguity as to whether these particular U3 sequences might be involved in 3′ vector RNA end processing [8,16,20,32–34]. MSinSB is novel in that: (i) The internal CA promoter was configured to optimally transmit an intron in the transgene transcriptional unit [35,36], maintaining the enhanced splicing and translation of transgene transcripts exhibited by the MSGV1 LTR vector [22]; and (ii) The plasmid form of MSinSB contains three poly(A) signals, two in the R region of the 3′ LTR [the natural gammaretroviral poly(A) signal and the strong SV40 late poly(A) signal] plus one [the bovine growth hormone poly(A) signal] downstream of the 3′ LTR, which is not propagated. Addition of these heterologous poly(A) signals individually to SIN vectors was previously demonstrated to result in increased levels of vector RNA either by more efficient 3′ end formation or increased transcription [37]. Our original intent was to construct variants of the R-region double-copy vector design described by Miller and colleagues [38], containing the SV40 late poly(A) signal in both LTRs. However, four vectors having this configuration exhibited dramatically reduced titers (data not shown), perhaps because of increased 5′ LTR promoter-proximal polyadenylation [39]. In any event, because there was evidence indicating that transfer of R region sequences during reverse transcription could take place prior to complete synthesis of minus-strand strong stop DNA such that mutations introduced into the R region of the 3′ LTR were copied ~10% of the time [40], we were interested in seeing whether the SV40 late poly(A) signal might be transmitted — even if at low frequency — in this position. The other vectors depicted in Fig. 1 are variations of MSGV1-C^2 and MSinSB with different combinations of the heterologous poly(A) signals and the dimererized 5′HS4 insulator core.

Transmission Properties of MSGV1-Based LTR and SIN Gammaretroviral Vectors

Replication-defective ecotropic vector particles were produced and initially used to transduce NIH3T3 cells (Fig. 1). Although inclusion of two copies of the 5′HS4 insulator core did not result in a significant drop in titer (MSGV1-C^2, $1.8 \pm 0.1 \times 10^6$ TU/ml) compared to the parental vector MSGV1 ($2.2 \pm 0.1 \times 10^6$ TU/ml), inclusion of the CA promoter was associated with a slightly greater than 50% reduction (MSGV1-CA, $0.9 \pm 0.2 \times 10^6$ TU/ml). Moreover, the titers of the MSin and MSin-C^2 SIN versions of MSGV1-CA (without or with the dimerized 5′HS4 insulator core, respectively) were a further 9-fold and 13-fold reduced compared to that of MSGV1-CA ($0.1 \pm 0.04 \times 10^6$ and $0.07 \pm 0.05 \times 10^6$ TU/ml for MSin and MSin-C^2, respectively). However, the loss in titer associated with the SIN format could be completely recovered by combinatorial inclusion of the SV40 late poly(A) signal in the R region and the

bovine growth hormone poly(A) signal downstream of the U3 region-deleted LTR (MSinSB, $0.9 \pm 0.1 \times 10^6$ TU/ml), most of which could be attributed to the R region SV40 late poly(A) signal (MSinS, $0.7 \pm 0.1 \times 10^6$ TU/ml). Based on their relatively high titers, the MSinSB and MSGV1-C^2 vectors were selected for evaluation of transcriptional properties in various stem cell models.

Expression of MSinSB and MSGV1-C^2 Gammaretroviral Vectors in Murine F9 Embryonal Carcinoma Cells and CCE ESCs

Murine F9 embryonal carcinoma cells (representing the malignant stem cell counterparts of ESCs) [8] and CCE ESCs [9] were transduced with serial dilutions of MSinSB, MSGV1-C^2 and MSGV1 replication-defective ecotropic vector stocks. The titers ranged from $0.9–1.9 \times 10^5$ TU/ml on F9 cells and from $1.2–2.0 \times 10^5$ TU/ml on CCE cells (Fig. 2). Considering that the relative titers of the parental MSGV1 vector ($1.2 \pm 0.2 \times 10^5$ TU/ml and $1.7 \pm 0.2 \times 10^5$ TU/ml) were 5–8% that on NIH3T3 cells, whereas the relative titers of MSinSB ($0.9 \pm 0.2 \times 10^5$ TU/ml and $1.2 \pm 0.1 \times 10^5$ TU/ml) and MSGV1-C^2 ($1.9 \pm 0.1 \times 10^5$ TU/ml and $2.0 \pm 0.1 \times 10^5$ TU/ml) on these undifferentiated cells were 10–13% of their respective titers on NIH3T3 cells, it appeared that the LTR modifications were conferring some degree of protection from de novo silencing [16,25,41]. The data indicated though that neither of these LTR modifications nor inclusion of the internal CA promoter was sufficient to completely ameliorate transcriptional silencing of the MSCV gammaretroviral vector backbone in these primitive cell types.

According to the Poisson distribution, transduction of a target cell population at a frequency of less than 15% should result in one copy of a randomly integrated vector in greater than 85% of the transduced cells. Therefore, to evaluate efficiency and stability of vector transcription in F9 and CCE cells, cell monolayers were transduced at low multiplicity of infection (MOI; $4.6 \pm 6.4\%$ GFP^+ and $12.0 \pm 2.6\%$ GFP^+ cells, respectively), single-cell suspensions were sorted as bulk populations, and continuously passaged cultures were analyzed for GFP expression levels by flow cytometry after one month of propagation [24,28,41]. All of the vectors continued to be expressed in these transduced polyclonal cell populations at this time point. Interestingly, the highest levels of GFP transgene expression were directed by the MSinSB vector in each case (MFI = 243 ± 27 *vs* 133 ± 19 for MSGV1-C^2 and 191 ± 31 for MSGV1 in F9 cells; Fig. 2A), with 2.5-fold higher expression levels observed in CCE ESCs than were directed by the other two vectors (MFI = 425 ± 40 *vs* 160 ± 18 for MSGV1-C^2 and 169 ± 23 for MSGV1; $P < 0.001$, Fig. 2B).

Persistent High-Level Expression of the MSinSB Gammaretroviral Vector During *In Vitro* Hematopoietic Differentiation of CCE ESCs

The polyclonal populations of MSinSB-, MSGV1-C^2, and MSGV1-transduced CCE ESCs were differentiated into EBs and hematopoietic cells. Fig. 3 shows representative photomicrographs of vector-expressing undifferentiated CCE cells, day 9 EBs, and day 10 hematopoietic colonies. Notably, when the percentages of cells that continued to express the GFP transgene in day 9 EBs were determined by flow cytometric analysis, the majority of MSinSB-transduced cells were positive ($92 \pm 7\%$ GFP^+) while a significant proportion of the MSGV1-C^2- and MSGV-1-transduced cells were found to have down-regulated GFP expression ($61 \pm 3\%$ GFP^+ and $55 \pm 7\%$ GFP^+, respectively; $P < 0.002$, compare the histograms in Figs. 4A and B). Subsequent differentiation of day 9 EBs into hematopoietic cells was documented by expression of the early hematopoietic marker CD41 [42] and the panleukocyte antigen CD45 [9]. Whereas very few $CD41^+/CD45^+$ cells continued to express the parental MSGV1 vector (2–3% GFP^+; Fig. 4C) in day 10 hematopoietic cultures, high-level expression of the MSinSB vector was seen in a large fraction of the cells (82–92% GFP^+; $P < 0.002$, Fig. 4C). Furthermore, the dimererized 5′HS4 insulator core seemed to be partially effective at

preventing transgene silencing, with approximately 15–20% of the MSGV1-C^2-transduced cells continuing to express the GFP transgene (Fig. 4C).

Characterization of MSinSB Gammaretroviral Vector Structural Integrity and Transcriptional Activity in ESC-Derived Hematopoietic Cells

In order to rule out the possibility that the higher levels of GFP transgene expression directed by the MSinSB vector in differentiated ESCs were due to increased numbers of proviral integrants per cell, as has been observed for SIN lentiviral vectors [13], Southern blotting was performed on genomic DNA from pools of transduced CCE ESCs before and after *in vitro* hematopoietic differentiation. DNA was digested with *Bse*RI, a restriction enzyme that cleaves the vectors near the 5′ end of the GFP gene and within the LTRs, and hybridized with a GFP-specific probe. This analysis demonstrated that the MSinSB and MSGV1-C^2 vectors were present at similar copy numbers whereas MSGV1-transduced cells contained ~4-fold higher copy numbers in the experiment presented (Fig. 5A, upper left panel; shown are data for hematopoietic cell populations). It also revealed two bands in MSGV1-C^2-transduced cells, the smaller of which was present at slightly greater intensity, corresponding in size to the MSGV1 vector-specific fragment plus one (*) or two (**) copies of the 5′HS4 insulator core. This finding indicated that deletion of one copy of the 5′HS4 insulator core from the U3 region of the MSGV1-C^2 LTR occurred at high frequency during replication [43]. The effect, if any, that the deletion might have on the transcriptional properties of the MSGV1-C^2 LTR in differentiating ESCs was not investigated.

Total cellular RNA was next extracted from the same transduced CCE ESC populations before (Fig. 5A, upper middle panel) and after (Fig. 5A, upper right panel) *in vitro* hematopoietic differentiation and analyzed by Northern blotting with a GFP-specific probe to compare vector transcript levels. The transcripts detected from the vectors included full-length genomic RNAs directed by the 5′ LTR (2.7–3.2 kb, depending on the vector), a spliced form of the genomic transcript (1.8 and 2.3 kb, respectively, for MSGV1 and MSGV1-C^2) and, in the case of the MSinSB vector, a spliced (1.2 kb) transcript initiated from the internal CA promoter. A 5′-RACE PCR reaction using a reverse primer recognizing GFP sequences amplified an ~380-bp product from total cellular RNA extracted from MSinSB-transduced cells. Sequencing of the RACE DNA product confirmed correct initiation and proper splicing of RNA transcripts originating from the internal CA promoter (Fig. 5B, left). PCR analysis of genomic DNA from MSinSB-transduced cells amplified a 520-bp product, which demonstrated that the intron was maintained (Fig. 5B, right). Further analyses indicated that the MSinSB LTR U3 region deletion was preserved, but that the R region SV40 late poly(A) signal was not transferred during vector propagation (data not shown). By comparison to the MSinSB LTR- and CA promoter-initiated transcripts, the LTR-initiated transcripts originating from the MSGV1 and MSGV1-C^2 vectors were present at much lower levels in the hematopoietic cell populations (Fig. 5A, upper right panel), paralleling the flow cytometry results and suggesting that the enhancer sequences of the internal CA promoter were activating transcription from the enhancerless MSinSB 5′ LTR minimal TATA box [34].

Expression of the MSinSB Gammaretroviral Vector in Murine Bone Marrow Transplant Recipients

To determine whether the MSinSB vector was also transcriptionally active in hematopoietic stem cells and their differentiated progeny, murine bone marrow cells were transduced by coculture on ecotropic producer cell monolayers ($90 \pm 8\%$ GFP^+ input cells) and intravenously injected into irradiated recipients. Six weeks later, when donor cell engraftment is due largely to reconstitution of the hematopoietic stem cell compartment [44], peripheral blood cells of the transplant recipients were collected and analyzed for GFP expression by flow cytometric analysis. The hematopoietic systems of all MSinSB transplant recipients were efficiently

reconstituted, having 64 ± 9% GFP^+ cells in their peripheral blood, which compared favorably with the results obtained for the MSGV1-C^2 (77 ± 4% GFP^+ cells) and MSGV1 (87 ± 2% GFP^+ cells) vectors (Fig. 6A). Compared to the parental MSGV1 vector, somewhat lower GFP expression levels were achieved with the MSinSB vector (MFI = 1997 ± 828 *vs* 3064 ± 1232). However, these levels were at least 2-fold higher than those mediated by the MSGV1-C^2 vector (MFI = 910 ± 379).

Expression of the MSinSB Gammaretroviral Vector in Long-Term Myelomonocytic Cultures of Differentiated Human $CD34^+$ Cord Blood Cells

We also evaluated stability of GFP transgene expression from the MSinSB vector compared to the MSGV1-C^2 and MSGV1 vectors following *in vitro* differentiation of human hematopoietic stem/progenitor cells [28]. $CD34^+$ cells were isolated from human cord blood and transduced at low MOI with RD114-pseudotyped vectors (2.5 ± 0.9% GFP^+ cells before sorting). GFP^+CD34^+ cells were sorted and differentiated into monocytes in long-term suspension cultures containing the hematopoietic cytokines IL-3, IL-6 and GM-CSF. At the 7-week time point in the experiment presented, when 50–75% of the nonadherent cells were $CD14^+$ monocytes, all three vectors continued to be expressed at high frequency (Fig. 6B), with MSGV1 (MFI = 3,079 ± 770) directing higher levels than MSinSB (MFI = 2,138 ± 439), which was transcriptionally more active than MSGV1-C^2 (MFI = 1,495 ± 272).

Future Directions

The MSinSB design is clearly a greatly improved gammaretroviral vector backbone for transgene expression during the *in vitro* hematopoietic differentiation of murine ESCs. As such, it should prove useful for gain-of-function and RNA interference studies investigating the role of genes involved in the onset of hematopoiesis as well as those involved in lineage commitment at other early developmental stages [1]. Whether the MSinSB vector platform will also prove to be of utility for similar studies with human ESCs remains to be determined [27]. It is not unreasonable to speculate, however, that the MSinSB vector will also be less subject to silencing mechanisms operating during murine embryogenesis and thus may offer advantages over existing techniques in some transgenesis experiments [3,16,45].

Although previous work indicated that strand transfer can occasionally occur prior to complete reverse transcription of the 5′ R region of the gammaretroviral LTR [40], the SV40 late poly (A) signal inserted into the R region of the 3′ MSinSB LTR was not effectively transmitted during reverse transcription. Nonetheless, we note that incorporation of this modification results in MSinSB vector titers that are ~10-fold higher than achieved with our earlier SIN gammaretroviral vectors [8,33], facilitating transduction of murine and human hematopoietic stem/progenitor cells. However, MSinSB titers remain ~2-fold lower than MSGV1 titers. Moreover, in the current SIN form, LTR-directed transcription is observed from the minimal TATA box as was reported previously [34]. Therefore, with a view to potential hematopoietic stem cell gene therapy applications [2,23], future efforts will be directed toward improving the titer as well as the biosafety of the MSinSB design [46].

MATERIALS AND METHODS

Construction of Gammaretroviral Vectors

The gammaretroviral vectors used in this study were derived from MSGV1 (*M*SCV-based *s*plice-*g*ag *v*ector) [22,23], an LTR vector analogous to MFG in which the transgene is expressed as a spliced transcript resembling the normal envelope mRNA [35]. All vectors carry the enhanced GFP reporter gene (which is not included in the vector names to simplify nomenclature). To generate MSGV1-C^2, two 250-bp DNA fragments encompassing the core sequence of the chicken β-globin 5′ DNase I hypersensitive site 4 (5′HS4) insulator [47]

(designated C^2) were amplified by polymerase chain reaction (PCR) from the plasmid pJC13-1 (a gift from Dr. Gary Felsenfeld, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) and blunt-end ligated into the *Nhe*I site of the MSGV1 3′ LTR. The MSGV1-CA vector was constructed by blunt-end ligating an 850-bp enhancer-promoter fragment (containing 365 bp of the cytomegalovirus immediate early enhancer, 277 bp of the chicken β-actin promoter, and 208 bp of the first exon and a portion of the first intron of the chicken β-actin gene) from the SIN-CAG-GFP lentiviral vector [28, 48] into the *Bgl*II site of MSGV1. MSin was generated by introducing a 303-bp *Nhe*I to *Sac*I deletion (nucleotides -336 to -34 relative to the U3/R boundary) into the U3 region of the MSGV1-CA 3′ LTR. MSin-C^2 contains two copies of the chicken β-globin 5′HS4 insulator 250-bp core element blunt-end ligated as an *Eco*RV-*Nru*I fragment into the *Nhe*I-*Sac*I-digested MSGV1-CA 3′ LTR. The MSinS and MSinS-C^2 vectors were generated from MSin and MSin-C^2 by blunt-end insertion of a 222-bp *Afl*II-*Xba*I fragment containing the simian virus 40 (SV40) late polyadenylation [poly(A)] signal from pEYFP-N1 [24] into the R regions of the respective 3′ LTRs at the *Sma*I site. The MSinSB and MSinSB-C^2 vectors were subsequently generated from MSinS and MSinS-C^2 by blunt-end insertion of a 664-bp *Not*I-*Nco*I fragment containing the bovine growth hormone poly(A) signal from the pTV lentiviral vector [37] (a gift from Dr. Lung-Ji Chang, University of Florida, Gainesville, FL) downstream of the respective 3′ LTRs at a *Cla*I site.

Cell Lines

GP+E-86 packaging cells (No. CRL-9642; American Type Culture Collection, Manassas, VA), NIH3T3 fibroblasts (ATCC No. CRL-1658), HT1080 fibrosarcoma cells (ATCC No. CCL-121), Phoenix-RD114 packaging cells [49] (a gift from Dr. Hans-Peter Kiem, Fred Hutchinson Cancer Research Center, Seattle, WA), and F9 embryonal carcinoma cells [8] were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 4.5 g/l glucose, 4 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Cambrex BioScience Walkersville, Inc., Walkersville, MD). Undifferentiated CCE ESCs were grown on 0.1% gelatin-coated dishes in DMEM with 4.5 g/l glucose and 4 mM L-glutamine plus 150 μM β-mercaptoethanol, 15% FBS and 10% Chinese hamster ovary leukemia inhibitory factor (LIF)-conditioned medium [9]. All cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Production of Retroviral Vector Particles

Stable ecotropic or RD114-pseudotyped producer cells were generated by electroporation of linearized vector DNA (20 μg) into GP+E-86 or Phoenix-RD114 cells, respectively, followed by cell sorting on a FACSAria instrument (BD Biosciences, San Jose, CA) after 10 days of culture [8,24]. Vector titers were determined on NIH3T3 or HT1080 cells by flow cytometric analysis of GFP fluorescence using a FACScan analyzer (BD Biosciences) as described previously [48].

Transduction and *In Vitro* Hematopoietic Differentiation of CCE ESCs

Serial dilutions of vector conditioned medium from GP+E-86 producer cells were added to monolayers of CCE ESCs and F9 embryonal carcinoma cells in presence of polybrene at 8 μg/ml. GFP expression was analyzed by flow cytometry after 3 days to determine the relative end-point titers. The cells were then sorted and after 1 month in culture the mean fluorescence intensity (MFI) of GFP fluorescence levels were determined by flow cytometric analysis.

A two-step method was used for *in vitro* hematopoietic differentiation of CCE ESCs as described previously [9]. The primary differentiation of ESCs into EBs was induced by removal of LIF and plating a single cell suspension of ESCs in Iscove's modified Dulbecco's medium

(IMDM) containing 1% methylcellulose, 2 mM L-glutamine, 150 μM β-mercaptoethanol, 15% FBS and 40 ng/ml stem cell factor (SCF) (StemCell Technologies, Vancouver, Canada). Hematopoietic differentiation of EBs was performed by plating single cell suspensions of day 9 EBs in a methylcellulose-based hematopoietic differentiation medium (StemCell Technologies) containing IMDM, 1% methylcellulose, 2 mM L-glutamine, 1% bovine serum albumin, 10 μg/ml insulin, 200 μg/ml transferrin, 150 μM β-mercaptoethanol, 15% FBS, 150 ng/ml SCF, 30 ng/ml interleukin (IL)-3, 30 ng/ml IL-6 and 3 U/ml erythropoietin. Staining with anti-CD41 and anti-CD45 monoclonal antibodies conjugated to R-phycoerythrin (BD Biosciences PharMingen, San Diego, CA) and GFP expression analysis by flow cytometry was performed as described previously [9,24].

Transduction and Transplantation of Murine Bone Marrow Cells

Female C57BL/6 mice were used at 6- to 8-weeks of age as bone marrow donors and recipients. Bone marrow processing, transduction, transplantation and GFP expression analysis were carried out as described in detail previously [10,23,33]. All animal procedures were carried out in accordance with Institutional Animal Care and Use Committee guidelines.

Transduction and *In Vitro* Monocytic Differentiation of Human $CD34^+$ Cord Blood Cells

Human umbilical cord blood samples were obtained after informed consent in conformity with an institutionally approved protocol. $CD34^+$ cells were purified from mononuclear cell preparations by super paramagnetic microbead selection using a Miltenyi Biotec varioMACS CD34 progenitor cell isolation kit (Auburn, CA), and cultured as described previously [28]. After 48 h cytokine prestimulation, the cells were transduced for 24 h with RD114-pseudotyped vector conditioned medium (1×10^6 TU/ml; MOI, 1) in the presence of 4 ug/ml protamine sulfate (Sigma-Aldrich Corp.). Fresh medium was added and the cells were cultured for an additional 72 h. The cells were then harvested, washed and resuspended in phosphate buffered saline plus 2% FBS, and GFP^+CD34^+ cells were sorted. The cells were cultured for 7 weeks in IMDM containing 2 mM L-glutamine, 50 IU/ml penicillin, 10% heat-inactivated FBS, 20 ng/ml IL-3, 20 ng/ml IL-6 and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (PreproTech, Inc., Rocky Hill, NJ) at 37°C in a humidified atmosphere containing 5% CO_2. Staining with anti-CD34 and anti-CD14 monoclonal antibodies conjugated to allophycocyanin (BD Biosciences PharMingen) and GFP expression analysis by flow cytometry was performed as described previously [28,50].

Nucleic Acid Analysis

Southern and Northern blot analyses were carried out as described previously [48].

For 5′-RACE (rapid amplification of cDNA ends) reactions, cDNA was synthesized from total cellular RNA (1 μg) using an oligo(dT) primer according to a protocol provided by the manufacturer (BD SMART™ RACE cDNA Amplification Kit; Clontech Laboratories, Inc., Mountain View, CA). The cDNAs were amplified using a reverse primer near the 5′ end of the GFP gene [GFP-R: (5′-TCGCCCTTGCTCACCATG-3′)], and the BD SMART II™ oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCG GG-3′). The PCR products were re-amplified successively using nested primers GFP-RN (5′-TCCTCGCCCTTGCTCACCATGGC-3′) and the BD SMART NUP primer (5′-AAGCAGT GGTATCAACGCAGAGT-3′). The nucleotide sequences of the 5′-RACE PCR products were determined by automated DNA sequencing. RT-PCRs were performed using the CA-5′ (5′-CTCTGACTGACCGCGTTACTC-3′) and GFP-R (5′-TCGCCCTTGCTCACCATG-3′) primers. Total cellular RNA (2 ug) was mixed with the GFP-R primer (20 pmol) in a 9 μl volume, heated to 65°C for 10 min and chilled on ice for 2 min. The reaction volume was then increased to 20 μl and reverse transcription was performed in the presence of 2 μl of 5× buffer (50 mM Tris-HCl, 200 mM KCl, 30 mM $MgCl_2$, 50 mM dithioerythritol [pH 8.3]), 125 uM

of each dNTP and 40 U MLV reverse transcriptase (Roche Applied Science, Indianapolis, IN). Following 1 h incubation at 37°C, the reaction was stopped by 10 min heat-inactivation at 65° C. The cDNA (5 μl reaction mixture) or genomic DNA was then amplified in 50-μl PCR reactions containing 5 μl of 10x buffer [100 mM Tris-HCl, pH 8.85, 250 mM KCl, 50 mM $(NH4)_2SO_4$, 20 mM $MgSO_4$], 125 uM each dNTPs, 30 pmol forward primer (CA-5′), 30 pmol reverse primer (GFP-R), and 2.5 U of *Taq* DNA polymerase (Roche Applied Science). Following an initial denaturation step at 94°C for 2 min, the thermocycle profile, repeated 35 times, consisted of a step cycle of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min, with a final 7-min elongation step at 72°C. MSinSB plasmid DNA (100 ng) was amplified under the same conditions as a positive controld. The PCR-amplified products were separated on a 1% agarose gel.

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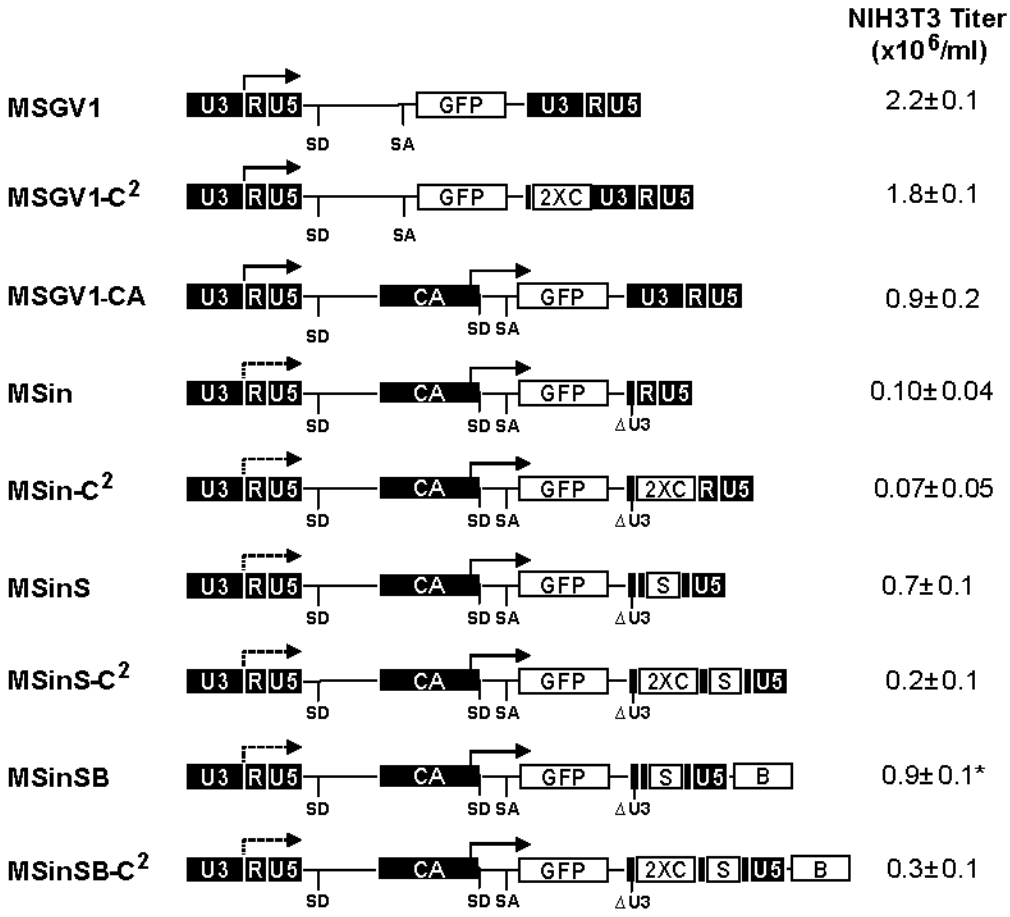

FIG. 1.

Schematic representation of MSGV1-derived gammaretroviral vectors. Plasmid forms of the vectors are illustrated. All vectors include the GFP transgene under the transcriptional control of the LTR or an internal CA promoter. The CA promoter contains a 5′ splice site positioned upstream of the natural 3′ splice site of the gammaretroviral envelope gene. Arrows depict the initiation sites of LTR- and CA promoter-directed vector RNA synthesis. The deletion in the U3 region of the 3′ LTR in the SIN configuration is indicated by U3. Other abbreviations: 2×C, dimer of the 250-bp 5′HS4 insulator core element; S, SV40 late poly(A) signal; B, bovine growth hormone poly(A) signal. Vector titers on NIH3T3 cells are shown for triplicate determinations. *Significantly higher than the MSin NIH3T3 titer ($P < 0.004$).

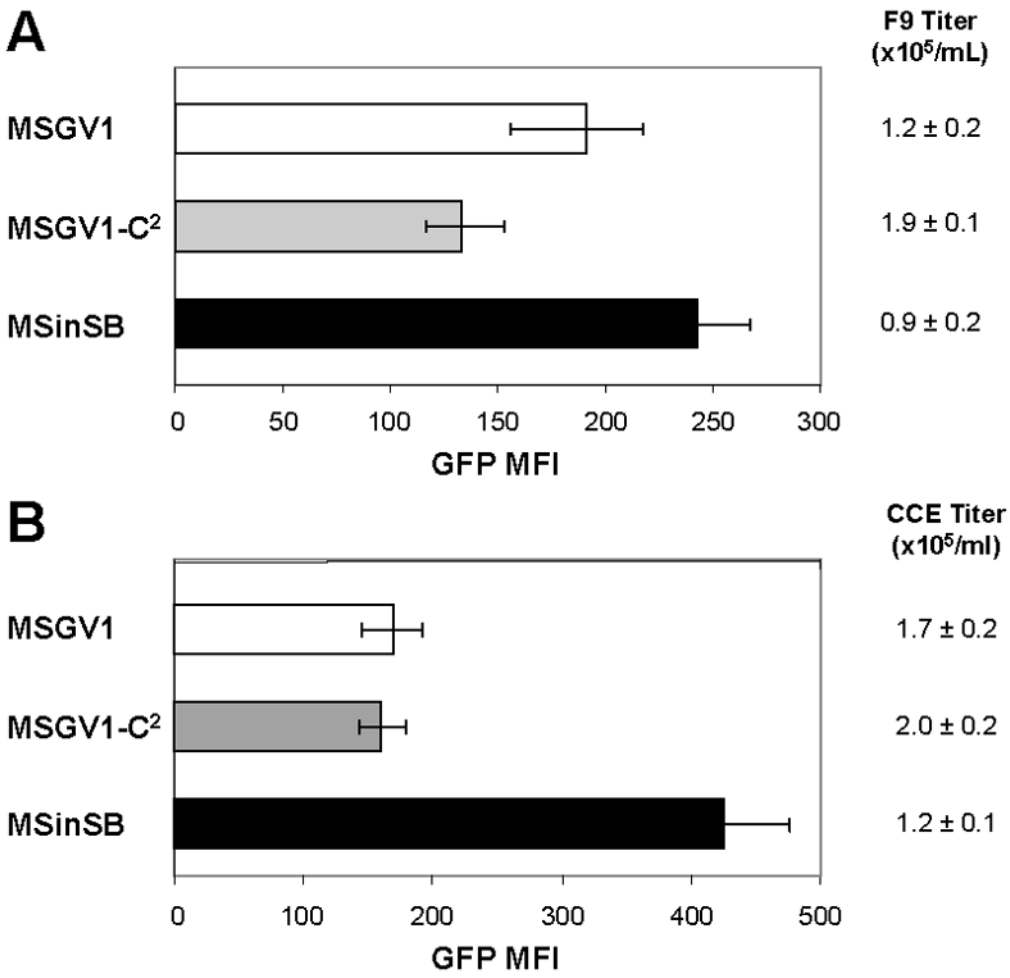

FIG. 2.

Expression of MSGV1-derived gammaretroviral vectors in F9 embryonal carcinoma cells and CCE ESCs. (A) Comparison of GFP fluorescence intensity levels (MFI values) of F9 cells transduced with MSGV1, MSGV1-C^2, and MSinSB vectors after one month in continuous culture. Vector titers determined in triplicate are shown on the right. (B) Comparison of GFP fluorescence intensity levels (MFI values) of CCE cells transduced with MSGV1, MSGV1-C^2, and MSinSB vectors after one month in continuous culture. Vector titers determined in triplicate are shown on the right.

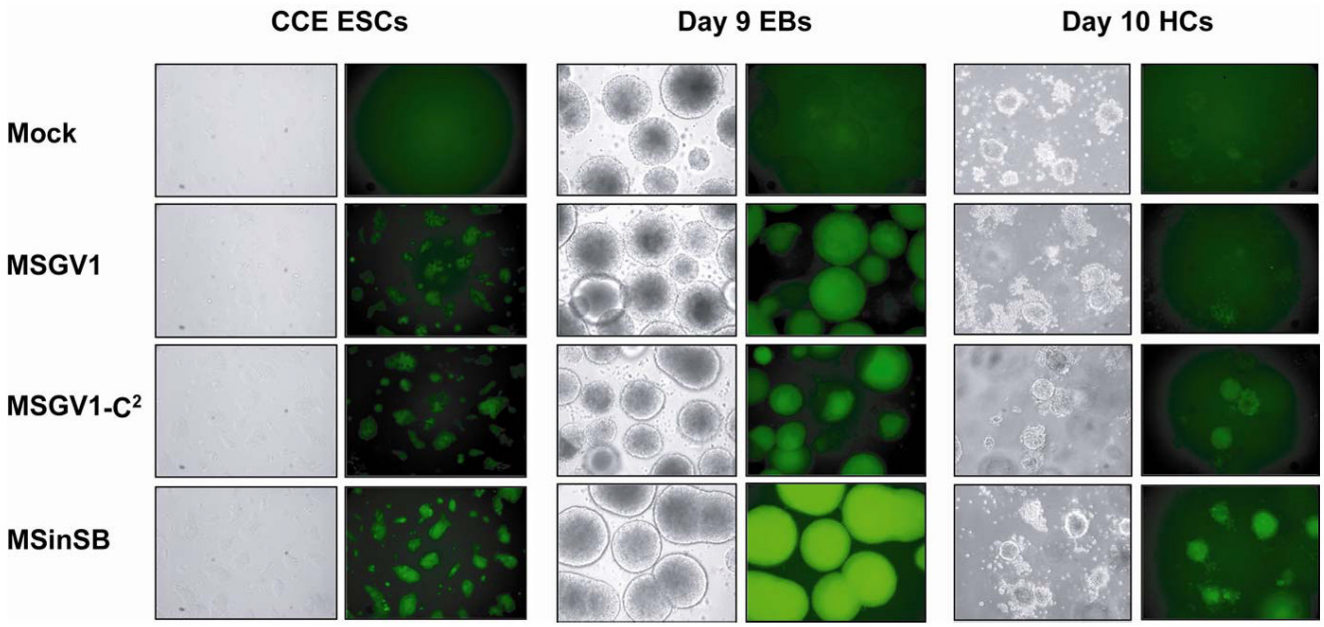

FIG. 3.

Photomicrographs of gammaretroviral vector-expressing CCE ESCs during *in vitro* hematopoietic differentiation. Shown are representative phase contrast (left panels) and fluorescence (right panels) microscopic images of control (mock), MSGV1-, MSGV1-C^2- and MSinSB-transduced CCE ESCs, day 9 EBs and day 10 hematopoietic colonies (HCs). The results shown are representative of two independent experiments performed in triplicate.

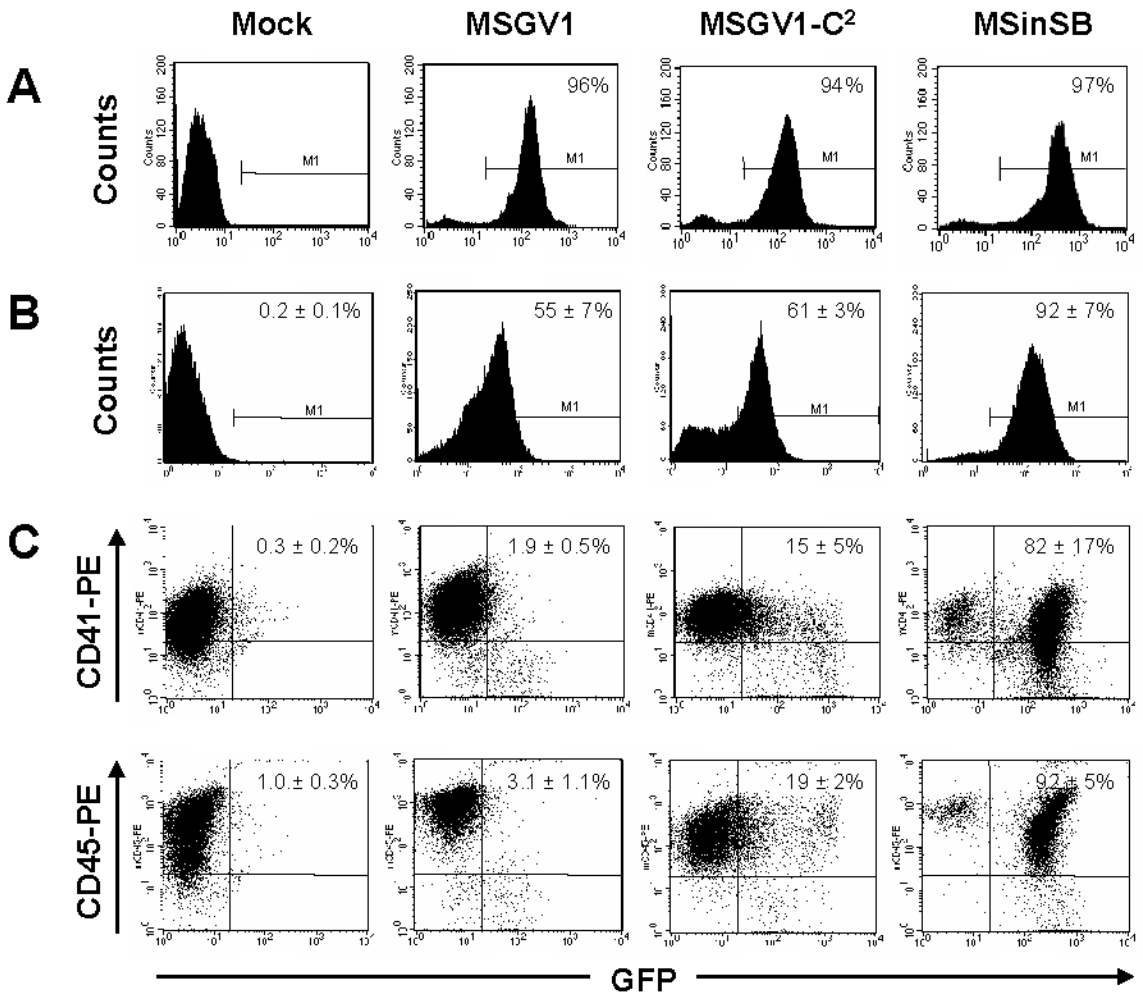

FIG. 4.

Quantitative analysis of gammaretroviral vector expression in transduced CCE ESCs during *in vitro* hematopoietic differentiation. (A) Flow cytometric analysis of GFP transgene expression in CCE cells stably transduced with the MSGV1, MSGV1-C^2 and MSinSB vectors cultured for one month after cell sorting. The percentages of GFP^+ cells are indicated. (B) Flow cytometric analysis of GFP transgene expression in day 9 EBs derived by *in vitro* differentiation of the respective transduced CCE cells. The percentages of GFP^+ cells are indicated. (C) Flow cytometric analysis of GFP transgene expression in $CD41^+$ (upper panels) and $CD45^+$ (lower panels) hematopoietic cells derived by subsequent *in vitro* differentiation of respective day 9 EBs. The percentages of GFP^+CD41^+ or GFP^+CD45^+ cells are indicated. The results shown are representative of two independent experiments performed in triplicate.

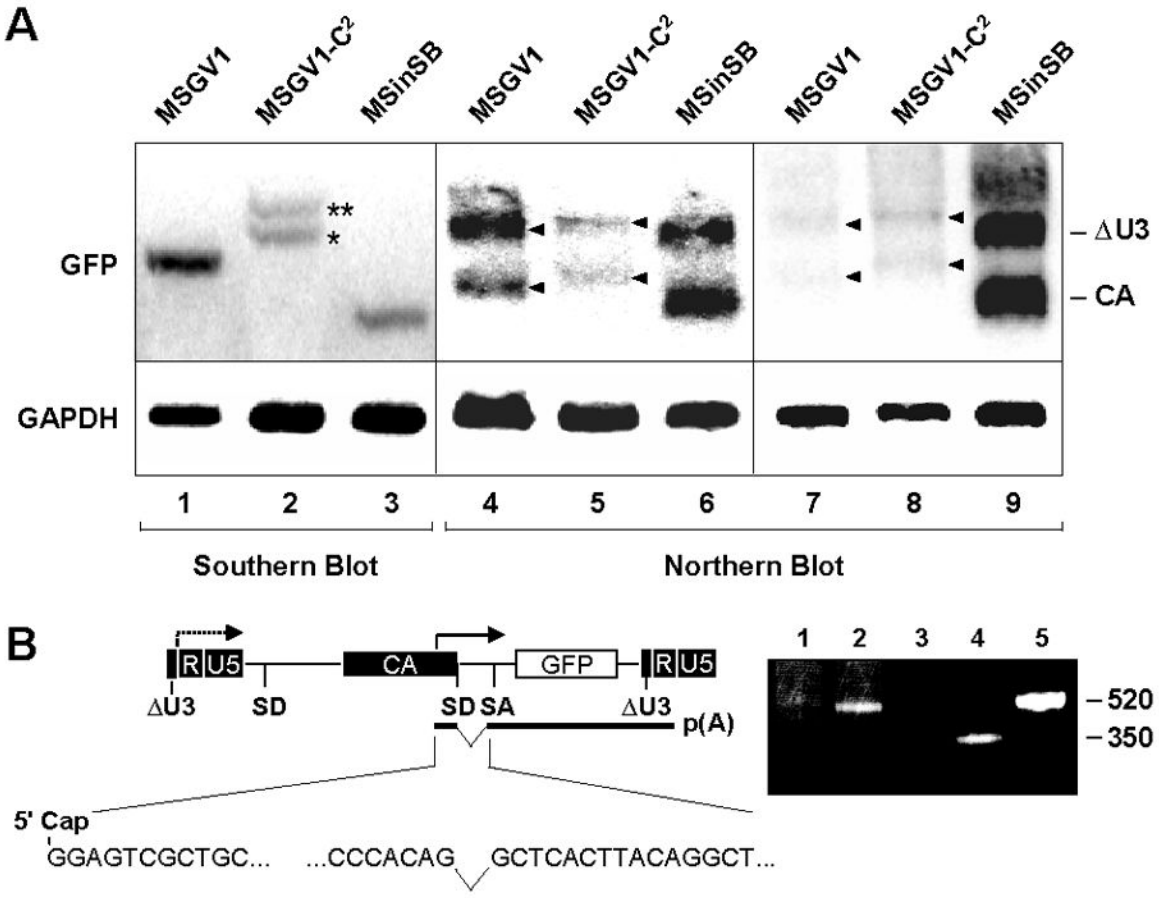

FIG 5.

Transmission and transcriptional properties of gammaretroviral vectors in ESC-derived hematopoietic cells. (A) Southern blot analysis of genomic DNA isolated from hematopoietic cell populations (lanes 1–3). *Bse*RI-digested genomic DNA (10 μg) was hybridized with a GFP-specific probe (upper left panel). MSGV1-C^2 integrants containing one (*) or two (**) copies of the 5′HS4 insulator core are indicated. The blot was rehybridized with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1.6-kb fragment) as a normalization control (lower left panel). Northern blot analysis of total cellular RNA isolated from ESC (lanes 4–6) and hematopoietic cell (lanes 7–9) populations. Total cellular RNA (20 μg) was sequentially hybridized with probes specific for GFP (upper panels) and GAPDH (1.3-kb mRNA; lower panels). U3 region-deleted LTR- (U3) and internal CA promoter (CA)-initiated MSinSB transcripts are indicated (on the left) as are LTR-directed full-length and spliced MSGV1 and MSGV1-C^2 vector transcripts (arrowheads). (B) The structure of correctly initiated and properly spliced GFP transgene mRNA originating from the internal CA promoter, determined by 5′-RACE PCR, is depicted below the schematic representation of the proviral form of the MSinSB vector (left). Shown on the right are additional PCR and RT-PCR analyses demonstrating transmission of the GFP transgene intron in the MSinSB vector and proper splicing of GFP transgene mRNA originating from the internal CA promoter. Genomic DNA from untransduced (lane 1) or MSinSB-transduced (lane 2) CCE cells was analyzed by PCR using a primer (CA-F) complementary to sequences immediately upstream of the CA promoter-associated splice site and a reverse primer (GFP-R) recognizing sequences near the 5′ end of the GFP gene (see Materials and Methods). The same primer pair was used for RT-PCR analysis of total RNA extracted from untransduced (lane 3) and MSinSB-transduced (lane

4) CCE cells. MSinSB plasmid DNA (lane 5) was included as a positive control for the PCR reactions. The sizes of the PCR (520 bp) and RT-PCR (350 bp) DNA products are indicated.

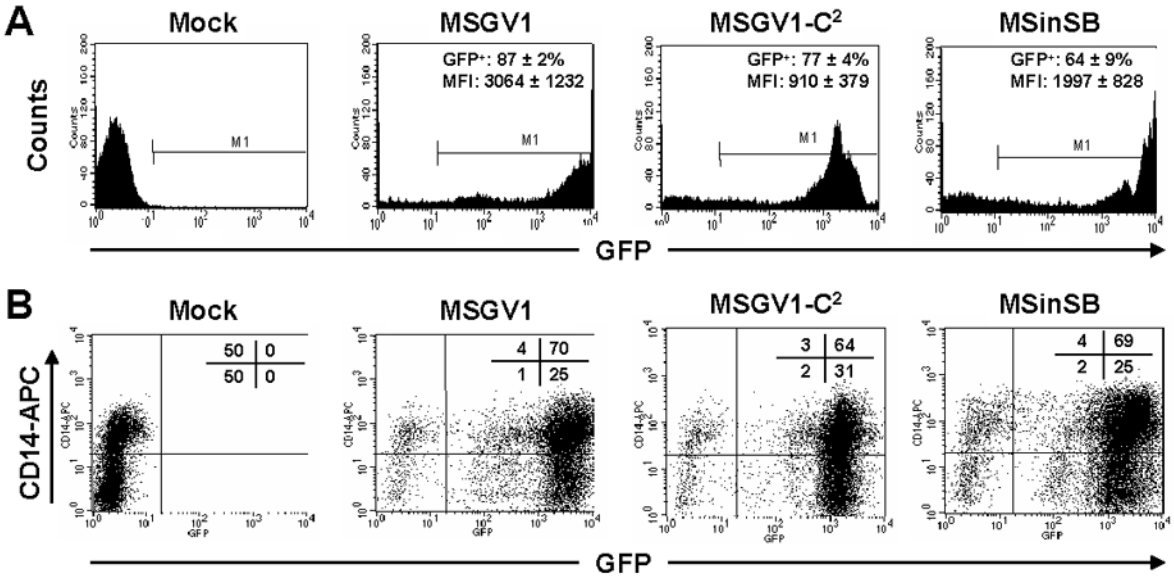

FIG. 6.

Quantitative analysis of gammaretroviral vector expression in murine bone marrow transplant recipients and in human monocytes derived from $CD34^+$ cord blood cells. (A) Flow cytometric analysis of GFP transgene expression in the peripheral blood cells of mice (6–8 per group) engrafted with MSGV1-, MSGV1-C^2- and MSinSB-transduced bone marrow cells at 6 weeks posttransplant. Representative analyses are shown. The percentages of GFP^+ cells and GFP fluorescence intensity levels (MFI values) are indicated. (B) Flow cytometric analysis of GFP transgene expression in monocytes derived from $CD34^+$ cord blood cells. $CD34^+$ cord blood cells were transduced with RD114-pseudotyped MSGV1, MSGV1-C^2 and MSinSB vectors at low MOI, sorted and cultured in the presence of IL-3, IL-6 and GM-CSF to promote monocytic differentiation. GFP fluorescence of $CD14^+$ nonadherent cells (monocytes) present in the cultures after 7 weeks was analyzed by flow cytometry. The percentages of cells in each quadrant of the dot plots are indicated. The results shown are representative of two independent experiments.