High-Frequency Cell Surface Expression of a Foreign Protein in Murine Hematopoietic Stem Cells Using a New Retroviral Vector

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A retroviral vector (pSFF) derived from murine Friend spleen focus forming virus was used to transduce murine hematopoietic stem cells and express a cell surface marker protein, mutated murine prion protein, in vitro and in vivo after transplantation. To enhance retroviral vector integration in bone marrow cells, mice were treated with 5-fluorouracil (5-FU) to increase stem cell mitotic activity, which peaked on day 8 post-5-FU. The infectivity titer of the vector, pSFF-mPrP-3F4, was determined by a novel assay in which antigen-positive foci of infected cells were detected after replication and spread of the vector in cultures of mixed packaging cell lines. Infection of Sca-1+/Lineage<sup>−</sup> cells with pSFF-mPrP-3F4 resulted in marker protein expression in 40% of the progeny cells after 7 days of culture. Transplantation of marrow cells or sorted Sca-1+/Lineage<sup>−</sup> cells transduced with vector resulted in 3F4-positive mPrP expression in 11% to 37% of donor-derived peripheral blood leukocytes at 2 weeks. Though the percentage of 3F4-positive blood cells gradually declined, at 28 weeks 23% of recipient mice still maintained expression of the marker gene. Expression was observed in lymphoid, myeloid, and erythroid lineages and was detected in Sca-1+/Lineage<sup>−</sup> marrow cells. The multilineage, high-frequency expression observed suggests that pSFF may be useful in gene therapy directed at hematopoietic stem cells and their differentiated progeny.

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THE DEVELOPMENT OF replication defective viral vectors and their use to shuttle foreign genes into cultured cells has led to the introduction of genes into mammalian host cells in vivo. Retroviral vectors stably integrate into the host cell genome and are thus transferred to the progeny of transduced cells. The retroviral vectors used to shuttle genes into hematopoietic cells have been predominantly derived from Moloney murine leukemia virus. However, a variety of other murine retroviruses exist that naturally infect hematopoietic cells. The murine Friend erythroleukemia virus complex consists of the replication-competent helper virus Friend murine leukemia virus (F-MuLV) and a replication-defective spleen focus forming virus, SFFV. F-MuLV and SFFV can infect many lineages of hematopoietic cells. Tissue specificity and level of expression in infected cells is regulated in part by promoter and enhancer sequences in the viral long terminal repeat (LTR) region. While LTR sequences derived from Friend MuLV have been used in the construction of chimeric viral vectors for the transduction of hematopoietic cells, the SFFV genome has not been explored as a vector for this purpose.

We have previously shown that pSFF, an expression vector derived from SFFV, is capable of high-level expression in human and murine cell lines in vitro. SFFV is known to mediate very high preferential expression of envelope gene mRNA due to strong splicing sites. In the present report, we have evaluated pSFF for its ability to express a marker protein in hematopoietic progenitor cells. As a marker gene we have replaced the SFFV envelope gene with a mutated form of the endogenous murine prion protein (mPrP) gene, which is expressed on the cell surface and is detectable with monoclonal antibody, 3F4. In this study, we demonstrate that the vector, pSFF-mPrP-3F4, is capable of transducing murine hematopoietic stem cells with high-frequency. Transplantation of these cells resulted in long-term expression of the mPrP reporter gene in blood cells of multiple lineages.

MATERIALS AND METHODS

Animals. C57BL/6 mouse strains congenic at the Ly 5 (CD45) locus were used as donors and recipients for hematopoietic stem cell harvest and subsequent transplantation. Nucleated blood cells from these mice can be differentiated using monoclonal antibodies (MoAbs) A20.1 and AL44A2, specifically reactive with the Ly5.1 (donor) and Ly5.2 (recipient) alleles, respectively. Transplant recipient animals were irradiated with a cesium source (Mark I gamma irradiator, J.L. Shepherd and Associates, Glendale, CA) as previously described and were administered oral antibiotics for the next 2 weeks (neomycin sulfate, 2 mg/ml, ad libitum in the water source, AgriLabs, Omaha Vaccine CO, Omaha, NE). All mice were continually maintained on acidified water (pH 2.5) and autoclaved chow (Purina Mills Inc, St Louis, MO). Animal care and experiments were approved by the Institutional Animal Care and Use Committee and done in accordance with the National Institutes of Health regulations. Animal facilities and procedures for the maintenance and care of animals were accredited by the American Association for Accreditation of Laboratory Animal Care.

Hematopoietic stem cell isolation. Mice were treated with 5-fluorouracil (5-FU) (150 mg/kg, intravenous, SoloPak Laboratories Inc, Elk Grove Village, IL) and were euthanized on day 8 post-5-FU by cervical dislocation following general anesthesia with isoﬂurane. Collection of bone marrow cells and isolation of the Sca-1+/Lineage<sup>−</sup> subset phenotype, a population highly enriched for hematopoietic stem cells, was done as previously described. Briefly, bone marrow cells that express high levels of Sca-1 antigen (Ly6A/E) and negative to low levels of the lineage markers CD2, CD3, CD5, CD8, Mac-1, B220, Gr-1, and an erythroid marker (TER-119) were analyzed and sorted by flow cytometry. All cell manipulations were performed in Hank’s Balanced Salt Solution (HBSS) with 3% fetal calf serum and 10 mmol/L Hepes buffer, pH 7.2. Flow cytometry used a FACStar instrument modified for 5-parameter operation (Becton Dickinson Immunocytometry Systems, San Jose, CA).
Dead cells were identified by staining with propidium iodide (10 μg/mL) and excluded. Cell cycle analysis of harvested bone marrow cells and sorted stem cells. To determine the time of maximal mitotic activity following in vivo 5-FU administration, bone marrow cells and Sca-1+/Lineage<sup>–</sup> cells were collected from day 0 to day 14 post-5-FU and analyzed for DNA content by staining with propidium iodide. Unsorted bone marrow cells or Sca-1+/Lineage<sup>–</sup> cells were lysed by resuspension in HBSS containing 10% fetal calf sera and lysing buffer (3 drops/10 mL, Lysing and Hemoglobin Reagent, American Scientifics Products, McGaw Park, IL), and nuclei were stained with propidium iodide (100 μg/mL). The percentage of cells with greater than diploid DNA was determined by fluorescence intensity as analyzed by flow cytometry.

Preparation of pSFF-mPrp-3F4 vector. The construction of pSFF-mPrp-3F4 (Fig 1A) from pSFF and the 1.2-kb cDNA for the 3F4-positive mPrP has been previously described by our laboratory. Briefly the vector was derived from the full length molecular clone of SFFV<sup>+</sup> as described. The pSFF vector has multiple deletions in the gag and pol genes and nearly all of the env gene has been excised to create a new cloning site. The sequence of pSFF has been reported by our laboratory (EMBL accession #Z22761). The 3F4-positive mPrP differs from endogenous mPrP by only two amino acids, and this mutation confers reactivity with MoAb 3F4.17,18,28

Cocultures of PA317<sup>+</sup> and Psi-2<sup>+</sup> packaging cell lines were transfected with pSFF-mPrP-3F4 DNA using the calcium phosphate precipitation method as previously described and used to produce supernatant stocks of the vector. It has previously been shown that use of this coculture can produce a high titer of the pSFF vector. Twenty-four hours later, cells were trypsinized and replated to allow cell growth and retroviral spread through the culture. When cells were 50% to 100% infected, as detected by reactivity with MoAb 3F4, virus stocks were collected as supernatants of the cocultures.

The titer of the pSFF vector in the supernatant stocks was determined by an infectivity assay in cocultures of the PA317 and Psi-2 packaging cell lines where the replication defective vector is capable of spread and foci of transduced cells express 3F4-positive mPrP. Vector production in transduced mixed cultures of PA317 and Psi-2 cells permits spread of the retroviral vector due to the fact that

Fig 1. Analysis of bone marrow and marrow Sca-1+/Lineage<sup>–</sup> cells after 5-FU administration. Bone marrow (A) and hematopoietic stem cell (B) (Sca-1+/Lineage<sup>–</sup>) counts were determined following 5-FU administration (150 mg/kg, intravenously, SoloPak Laboratories Inc). For each time point, data shown represents the mean and standard error of cell counts of nucleated bone marrow or Sca-1+/Lineage<sup>–</sup> cells harvested from 6 to 8 mice. Cell cycle analysis (C) of hematopoietic bone marrow stem cells (Sca-1+/Lineage<sup>–</sup>) following in vivo 5-FU administration. Sorted cells were analyzed for nuclear DNA content by staining nuclei with propidium iodide (100 μg/mL). Data shown for each time point represents the mean and standard error of data obtained from 4 to 8 mice.
the two cell lines produce different viral envelope proteins for packaging the vector, and each cell line expresses the receptor for the envelope protein produced by the other cell line. Cultures seeded with both $1 \times 10^6$ PA317 cells and $1 \times 10^5$ Psi-2 cells were plated onto TC-6 wells in RPMI-1640 with 5% fetal calf serum and 100 IU penicillin per mL. After 24 hours, the wells were incubated with 200 μL of 10-fold dilutions of viral stock with 6 μg polybrene per mL (hexadimethrine bromide, Sigma, St. Louis, MO). Four days later foci of infected cells expressing 3F4-positive mPrP were detected and quantitated using an immunoperoxidase assay (Fig 1B). Briefly, the cells monolayers were stained with MoAb 3F4, fixed with ethanol, stained with peroxidase-labeled goat antimumouse Ig antibody (Cappel, Organon Teknika Corp, West Chester, PA), and reacted with 3-amin-9-ethylcarbazole (Sigma) and H$_2$O$_2$ in acetate buffer as previously described. Three washes with phosphate-buffered saline (PBS) containing 1% fetal calf serum were done between each step. Cells expressing 3F4-positive PrP were red in color and were counted using a dissecting microscope as previously described.

As reported previously, cell-free spread of infectious pSFF-mPrP-3F4 vector produced in the packaging cells was not observed due to the early time of fixation (day 4) and the inefficiency of cell-free MuLV infection in the absence of treatment with polybrene or diethyl aminoethyl (DEAE) dextran to enhance viral adsorption. The vector titer was also assayed using NIH/3T3 cells not containing retroviral packaging sequences. In this case, no infectious progeny vector are produced and the foci of cells expressing 3F4-positive mPrP were smaller because they consisted only of the daughter cells of the original cells transduced by the vector. In both of these cell culture systems, the vector gave one-hit titration curves and similar infectivity titers (1.2 × 10$^5$ focus forming units (FFU)/mL). Contaminating replication-competent helper retroviruses were not found in the pSFF-mPrP-3F4 vector stock after transduction of NIH/3T3 cells, as no expression of reverse transcriptase or MuLV amphotropic or ecotropic envelope proteins was observed over the course of two serial passages.

**Infection/transduction and transplantation of stem cells.** Enriched hematopoietic stem cell populations and nucleated bone marrow cells isolated from mice on day 8 post-5-FU treatment were exposed to the pSFF-mPrP-3F4 vector in vitro for 2 hours before transplantation of these cells into irradiated congenic recipients. To aliquots of $8 \times 10^4$ stem cells or $2 \times 10^8$ bone marrow cells was added 1 mL of thawed vector supernatant containing $1.2 \times 10^9$ FFU in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum, 110 μL of $10^4$ concentrated WEHI-3B(D-)- cell culture supernatant as a source of cytokines, and polybrene (6 μg/mL, hexadimethrine bromide, Sigma) to enhance retrovirus-targeted cell contact. This mixture was incubated at 37°C, 5% CO2 and 100% humidity for 2 hours. Following incubation, 3 mL of DMEM with 10% fetal calf serum was added, the cells were pelleted by centrifugation at 1,200 rpm for 5 minutes and resuspended in DMEM. The suspension was then injected intravenously in the retro-orbital sinus of irradiated recipients at 0.2 mL per mouse ($1 \times 10^8$ stem cells or $5 \times 10^7$ bone marrow cells).

**Stem cell culture in vitro.** To analyze the transduction efficiency of the infection protocol in vitro, $8 \times 10^6$ Sca-1+/Lineage$^{-}$ bone marrow cells collected on day 8 post-5-FU were infected with pSFF-mPrP-3F4 as described above. Cells were washed after a 2-hour infection period and were aliquoted to TC-6 wells in 5 mL of RPMI-1640 supplemented with 20% fetal calf sera, 100 units/mL penicillin, and 0.5 mL of $10^4$ concentrated WEHI-3B(D-) supernatant. On day 7, postinfection, cells were removed from the dishes by treatment with 2.5 mmol/L sodium EDTA, incubated with mouse Ig (Pel Freezer, Rogers, AR) as a blocking agent, and stained with fluorescein-conjugated MoAb 3F4 and analyzed for expression of the 3F4-positive mPrP by flow cytometry. Dead cells were excluded during analysis on the basis of staining with propidium iodide (10 μg/mL).

**Assay for expression by flow cytometry.** Peripheral blood was collected in heparin sulfate from the retro-orbital sinus of mice anesthetized with isofluorane. One microliter of whole blood was aliquoted for assay of antigen expression on erythrocytes. Leukocytes were isolated and stained for expression of the Ly 5.1 allele and lineage markers as previously described. Briefly the protocol used to determine the percentage of 3F4-positive donor-derived leukocytes and their lineage (myeloid vs lymphoid) was as follows. Cells were stained first with MoAbs to Mac-1 (M1/70.15.15.HL) and Gr-1 (RB6-8C5) to identify myeloid cells or to B220 (RA3-6B2) and CD2 (MoAb RM-2.2) to identify lymphoid cells, then were reacted with phycoerythrin-conjugated goat antirat immunoglobulin (Biodiag Corp, Foster City, CA), incubated with rat immunoglobulin (Pel-Freez), stained simultaneously with fluorescein-conjugated MoAb 3F4 (anti-mPrP-3F4) and biotin-conjugated anti-Ly 5.1 (MoAb A20.1), and finally stained with Red613-conjugated streptavidin (ImmunoSelect, Life Technologies Inc, Grand Island, NY). The cells were washed once with HBSS with 3% fetal calf sera with an underlay of fetal calf sera between each step in the staining procedure. During analysis, dead cells were excluded on the basis of their light scatter properties and high-intensity staining with propidium iodide (10 μg/mL). In all mice tested, the fluorescence intensity of MoAb 3F4+ cells was easily distinguished from controls.

Residual recipient-derived peripheral leukocytes were identified by an anti-Ly-5 MoAb (MoAb AL44A2) and represented a mean of 10% and less than 3% of the peripheral lymphocyte population in transplant recipients at 4 to 8 and 12 to 28 weeks posttransplantation, respectively. Residual recipient cells present after 8 weeks were T lymphocytes. There was no detectable expression of the 3F4-negative mPrP in Ly 5.2 recipient-derived blood cells in mice receiving Ly 5.1 marrow cells transduced with pSFF-mPrP-3F4. MoAb 3F4 was also not reactive with blood cells in mice transplanted with hematopoietic cell populations exposed to a control pSFF vector that lacked the 3F4-positive mPrP gene.

**RESULTS**

**Isolation and characterization of day 8 post-5-FU bone marrow and hematopoietic stem cells.** Stable integration of type C retroviral vectors into host chromosomal DNA optimally occurs in hematopoietic stem cells undergoing mitosis, and in previous studies mice were treated with the antineoplastic drug 5-FU to enrich and increase the mitotic activity of this typically quiescent population. In the present work, we studied bone marrow cells and Sca-1+/Lineage$^{-}$ cells of 5-FU-treated mice to determine the time of maximum cell cycle activity as an indication of susceptibility to retroviral vector infection. Following 5-FU administration, nucleated bone marrow cells were rapidly depleted and by day 8 post-5-FU, there was a 10-fold reduction in the numbers of nucleated bone marrow cells per femur (Fig 2A). Simultaneously, the marrow had a significant increase in the percentage of Sca-1+/Lineage$^{-}$ cells (Fig 2B), a population highly enriched for hematopoietic stem cells. On days 8 and 10 there was a 100-fold enrichment for Sca-1+/Lineage$^{-}$ cells (Fig 2B). The mitotic activity of Sca-1+/Lineage$^{-}$ hematopoietic cells after in vivo 5-FU administration peaked at 54% ± 6 on day 8 post-5-FU (Fig 2C). Analysis of the entire unfractonated nucleated bone marrow cell population was also done (data not
In this experiment, day 8 post-5-FU cells gave higher expression 4 weeks posttransplantation than did day 2 post-5-FU cells (Fig 3). Therefore, in all subsequent experiments, marrow cells or purified stem cells used as targets for transduction with pSFF-mPrP-3F4 were collected on day 8 post-5-FU.

Expression of 3F4-positive PrP in hematopoietic stem cell progeny in vitro. To determine if pSFF-mPrP-3F4 could express the PrP marker gene in the progeny of transduced hematopoietic stem cells after in vitro culture, Sca-1+/Linage"^c-kit"^ marrow cells were infected with the vector and cultured in vitro in the presence of cytokines. In this infection, $4 \times 10^4$ cells were incubated with $1.2 \times 10^5$ FFU of vector. On day 7 of culture, 40% of the progeny cells had surface expression of 3F4-positive mPrP (Fig 4). Recently, it has been demonstrated that because retroviral vector integration in the host cell DNA occurs after DNA replication, infection of a cell with a single vector is capable of transferring DNA to only one daughter cell. Thus a 100% infection efficiency in a one-hit infection with vector would result in transfer of the vector DNA to only 50% of the progeny cells. Therefore, the 40% expression frequency demonstrated here suggested that the infection efficiency in vitro with pSFF-mPrP-3F4 could have been as high as 80%.

Fig 2. (A) Schematic diagram of the proviral DNA encoded by pSFF-mPrP-3F4. The parent pSFF vector differs from SFFV by deletion of an EcoRI/BamHI fragment in the pol gene and a BamHI/EcoRI fragment in the env gene. The sequence of the replication defective pSFF vector has been reported (EMBL accession #2227611). The marker gene in pSFF-mPrP-3F4 is the cDNA for mPrP that was mutated to methionine at amino acids 108 and 111 to confer a unique epitope reactive with MoAb 3F4. (B) Two foci of 3F4-positive cells in a mixed culture of PA317 and Psi-2 packaging cells infected with pSFF-mPrP-3F4. In this field the monolayer of packaging cells is confluent, and only the cells expressing 3F4-positive mPrP are visible as detected by an indirect immunoperoxidase assay (magnification 75 x).

shown), and 51\% \pm 4\% of marrow cells were in the G2-S-M phase of the cell cycle on day 8 post-5-FU as compared with 33\% \pm 6\% of marrow cells in untreated mice.

Transduction efficiency of day 2 versus day 8 post-5-FU enriched stem cell populations. Though the mitotic activity of Sca-1+/Linage"^c-kit"^ marrow cells increased from day 2 to day 8 after 5-FU treatment, it was not known whether this increase would result in enhanced transduction efficiency of these cells with the pSFF vector. Others have shown that efficient transduction of unsorted marrow cells requires pretreatment of donor animals with 5-FU. In one study, a comparison of the efficiency of retroviral vector mediated gene transfer to murine marrow cells isolated on day 2 versus day 5 post-5-FU demonstrated that a greater number of transplant recipient animals maintained the foreign gene when given day 5 post-5-FU marrow cells. In a preliminary experiment we isolated Sca-1+/Linage"^c-kit"^ marrow cells from mice on day 2 and day 8 post-5-FU and transduced these cells populations by incubating with vector for 2 hours as described above. The cells were then transplanted into irradiated recipients at a dose of $1 \times 10^4$ cells per mouse and the white blood cells from peripheral blood were analyzed at 4 weeks posttransplantation for expression of mPrP-3F4 by fluorescence-activated cell sorter (FACS) analysis. In this experiment, day 8 post-5-FU cells gave higher expression 4 weeks posttransplantation than did day 2 post-5-FU cells (Fig 3). Therefore, in all subsequent experiments, marrow cells or purified stem cells used as targets for transduction with pSFF-mPrP-3F4 were collected on day 8 post-5-FU.

Fig 3. Comparison of the transduction efficiency of day 2 versus day 8 post-5-FU stem cell enriched marrow cells. Sca-1+/Linage"^c-kit"^ marrow cells were isolated on day 2 and day 8 post-5-FU treatment of donor animals and were transduced in vitro with pSFF-mPrP-3F4. The cells were transplanted into irradiated mice and at 4 weeks posttransplantation donor-derived leukocytes were analyzed for cell surface expression of mPrP-3F4 by FACS. Each dot represents data obtained from a single transplant recipient.
purified Sca-1+/Lineage<sup>−/low</sup> cells were infected in vitro by incubation with 1.2 × 10<sup>5</sup> FFU of the pSFF-mPrP-3F4 vector and 2 hours later cells were injected into groups of lethally-irradiated recipient mice. A 2-hour infection time was used to minimize the potential deleterious effects of culture on stem cell function and viability before transplantation. The 3F4-positive mPrP was readily detected on transduced peripheral blood cells by flow cytometry. The percentage of donor-derived peripheral white blood cells that expressed 3F4-positive mPrP was greatest at 2 weeks after transplantation, the earliest time point examined (Fig 5A and B). The high-frequency of expression at 2 to 4 weeks demonstrated that the pSFF vector was highly efficacious in transducing post-5-FU hematopoietic cells. Furthermore, there was no detectable expression of 3F4-positive mPrP in Ly<sup>5.2</sup> recipient-derived blood cells in mice that received Ly 5.1 donor marrow cells that had been exposed to pSFF-mPrP-3F4. At subsequent times, the percentage of 3F4-positive donor cells gradually decreased (Fig 5A and B). However, even at the latest time point analyzed, 28 weeks, 4 of 14 (29%) mice that received marrow cells (Fig 5A) and 3 of 18 (17%) mice that received Sca-1+/Lineage<sup>−/low</sup> cells (Fig 5B) maintained detectable 3F4-positive mPrP expression. There was no detectable expression of 3F4-positive mPrP in Ly<sup>5.2</sup> recipient-derived blood cells at any time. There appeared to be no significant difference in the percentage of donor-derived cells with 3F4-positive mPrP expression regardless of whether mice were transplanted with unsorted marrow cells or sorted Sca-1+/Lineage<sup>−/low</sup> cells.

We also examined the lineage specificity of expression of 3F4-positive mPrP in donor-derived peripheral blood cells in transplant recipients. The percentages of donor-derived peripheral blood myeloid, lymphoid, and erythroid cells expressing 3F4-positive mPrP at various times after transplantation are shown in Fig 6. At 2 weeks posttransplantation, expression was greatest in myeloid cells. 3F4-positive peripheral blood lymphoid and erythroid cells were rarer at this time period due to the longer time required for their differentiation. However, by 4 weeks, there were equivalent frequencies of expression in myeloid, erythroid, and lymphoid lineages.

Multilineage expression of the marker gene in donor-derived blood cells in vivo demonstrated that lineage differentiation and blood cell maturation did not restrict expression of the vector in the progeny of transduced hematopoietic cells. Furthermore, due to the short half-life of committed myeloid progenitor cells and of blood myeloid cells, 3F4-positive mPrP expression in peripheral blood myeloid cells beyond 12 weeks posttransplantation strongly suggested that multipotent hematopoietic stem cells were transduced by the vector.

Analysis of transplant recipients for expression in marrow hematopoietic stem cells. To test directly whether hematopoietic stem cells expressed the 3F4-positive mPrP gene, we isolated the stem cell population (Sca-1+/Lineage<sup>−/low</sup> cells) from bone marrow cells of mice (n = 4) at 8 and 16 weeks posttransplantation. By flow cytometry, 3F4-positive mPrP was expressed in 4% to 9% of bone marrow cells, and of these, 1.7% to 6.5% had the Sca-1+/Lineage<sup>−/low</sup> pheno-
Fig 5. Analysis of 3F4-positive mPrP expression in vivo. Cell surface expression of 3F4-mPrP was analyzed in donor-derived peripheral blood leukocytes after transplantation of nucleated bone marrow cells (A) or marrow hematopoietic stem cell enriched populations (Sca-1+/Lineage<sup>neg</sup>-low cells) (B), which were infected in vitro with pSFF-mPrP-3F4 on day 8 post-5-FU. Each dot represents data obtained from a single transplant recipient.

Fig 6. Lineage specificity of donor-derived peripheral blood cells expressing 3F4-positive mPrP after transplantation. Peripheral blood leukocytes and red blood cells were isolated posttransplantation of pSFF-mPrP-3F4 infected bone marrow or Sca-1+/Lineage<sup>neg</sup>-low marrow cells and stained for Ly 5.1 allelic expression, 3F4-positive mPrP expression, and for lineage specificity including myeloid (Mac-1 and Gr-1 positive cells), lymphoid (CD2 and B220 positive cells), and erythroid (gated by size). At each time point the data was obtained from 8 to 12 transplant recipients. The data represents the mean and standard errors of the percentage of donor-derived leukocytes in each lineage or of total red blood cells that expressed 3F4-positive mPrP at the denoted time periods after transplantation. In analyses of erythroid expression, donor-derived and recipient-derived erythroid cells could not be distinguished due to the absence of Ly 5 allelic expression in mature cells of this lineage. Thus, the frequency of 3F4-positive donor erythroid cells reported here underestimates the true transduction frequency in the erythroid lineage.

DISCUSSION

The Friend murine leukemia virus complex is known to infect erythroid, myeloid, lymphoid, and megakaryocytic lineages. Therefore, it seemed likely that the pluripotent hematopoietic stem cell precursor of all these lineages might also be susceptible to infection by this virus complex. Using the endogenous LTR promoter/enhancer of SFFV resulted in multilineage, high-frequency expression of a marker gene in vitro and in vivo following infection of marrow cells with the retroviral vector, pSFF-mPrP-3F4. It is noteworthy that such a high-frequency of expression occurred in the absence of any form of positive selection and after only a 2-hour in vitro infection period. The high level of expression noted here is consistent with the reported high level of expression of the SFFV envelope protein, gp55, by a similar vector in mixed packaging cell cultures. Differences between pSFF and the commonly used Moloney murine leukemia viral vec-
tors in the splicing efficacy or in cell-type specific functions of the LTR or 5' gag gene sequences might account for the increased expression in cells of hematopoietic origin.

Although all mice had high-frequency of expression of the marker gene shortly after transplantation, a gradual decrease was noted with time. However, at 28 weeks posttransplantation, an average 23% of mice still had detectable expression of 3F4-positive mPrP. This decrease in expression frequency has also been reported with the use of other retroviral vectors. The decrease in the percentage of cells expressing the marker in these experiments could be due in part to loss of short-term progenitor cells that were activated by 5-FU treatment and subsequently transduced by the vector, or to selection against transduced long-term repopulating stem cells due to the presence of a new expressed gene product, or to inactivation of the marker gene, possibly as a result of DNA methylation. Nevertheless, our evidence suggests that hematopoietic stem cells were transduced by the vector. For example, expression was present in myeloid cells far beyond 12 weeks posttransplantation. By this time, committed myeloid precursor cells present in the initial cell population transplantation would have been replaced, and thus any 3F4-positive myeloid cells present in the peripheral blood beyond 12 weeks posttransplantation could only have arisen from transduced hematopoietic stem cells.

Furthermore, at 8 and 16 weeks posttransplantation, stem cells (Sca-1+/Lineage<sup>neg</sup>-low cells) isolated from bone marrow of transplant recipients were found by direct analysis to express the marker gene.

The results obtained when either transduced unfractionated marrow cells or sorted Sca-1+/Lineage<sup>neg</sup>-low marrow cells were transplanted into lethally-irradiated recipients were markedly similar. This occurred despite the difference in the ratio of vector to cells used in the infection protocol of isolated Sca-1+/Lineage<sup>neg</sup>-high cells versus unfractionated marrow cells. Although the actual number of cells used in these two groups differed, both cell pools, when transplanted, had similar numbers of Sca-1+/Lineage<sup>neg</sup>-low cells due to the fact that the post-5-FU marrow was highly enriched for Sca-1+/Lineage<sup>neg</sup>-high cells and that a 10-fold higher number of unfractionated marrow cells were transplanted. Although we expected to see some degree of short-term expression of the vector after transplantation of unfractionated marrow cells due to transduction of committed progenitor cells present in this population, the similarity in the degree of short-term expression after transplantation of purified Sca-1+/Lineage<sup>neg</sup>-low marrow cells was surprising. Previous work by others has demonstrated that 5-FU enriches for long-term repopulating hematopoietic stem cells and does not appear to affect their function. Although it has been shown that this phenotype is enriched for stem cell activity on day 4 post-5-FU, it is not known for marrow cells collected 8 days post-5-FU what proportion of Sca-1+/Lineage<sup>neg</sup>-low cells are long-term, multilineage repopulating stem cells. Our results suggest that the 8-day post-5-FU Sca-1+/Lineage<sup>neg</sup>-low cell population contained some committed progenitor cells, as well as long-term repopulating cells. If so, it is not clear that there was any advantage in our preselection of stem cells before transplantation with the retroviral vector.

Omitting this step should simplify future experiments using these protocols.

Similar to recent reports describing the use of other cell surface membrane proteins to mark transduced cells, the 3F4-positive mPrP gene has use as a marker gene to identify transduced hematopoietic stem cells. In the future, the 3F4-positive mPrP marker system might be useful for increasing the efficiency of hematopoietic cell gene therapy by immunoselection and subsequent transplantation of a purified population of transduced stem cells selected for gene expression by flow cytometry. The power of this technique has recently been demonstrated in studies of transplantation of transduced peripheral blood lymphocytes. In these reports, after transduction of human lymphocytes to express both a non-functional surface marker protein and the thymidine-kinase gene, positive immunoselection in vitro for the marker protein resulted in transplantation of virtually 100% gene-modified lymphocytes into the human recipients.

High-frequency in vivo expression of a foreign gene in blood cells was also recently reported with a retroviral vector using the 3' LTR of the murine myeloproliferative sarcoma virus (MPSV).

Use of this vector resulted in an unusually high-frequency of expression in blood cells in one mouse 16 weeks after transplantation of transduced bone marrow cells. In this mouse, the MPSV vector was expressed at high levels in myeloid, erythroid, and B lymphocyte lineages, but had a low level of expression in T lymphocytes. In contrast, the pSFF vector was expressed equally well in myeloid, erythroid, and lymphoid cells. It is likely that differences in the regulatory sequences of these vectors will result in unique advantages for each vector due to differential expression in certain blood lineages.

**ACKNOWLEDGMENT**

The authors thank Drs Kim Hasenkrug, Sue Priola, and Wendy Maury for their comments.

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