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Novel Retroviral Vectors for Efficient Expression of the Multidrug Resistance (*mdr-1*) Gene in Early Hematopoietic Cells

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We present data that retroviral gene expression in early hematopoietic cells is subjected to transcriptional controls similar to those previously described for embryonic stem cells. Transient transfection experiments revealed that both the viral enhancer region in the U3 region of the long terminal repeat as well as a repressor element coincident with the primer binding site of Moloney leukemia viruses are limiting for expression in hematopoietic cells in a differentiation-dependent manner. Within the group of Moloney leukemia virus-related viruses, only the myeloproliferative sarcoma virus showed high enhancer activity in myeloid (including erythroid) cells. In contrast, enhancer regions related to the Friend mink cell focus-forming viruses mediate much higher gene expression levels in both multipotent and lineage-committed myeloid cells. In addition, transcriptional repression related to sequences in the primer binding site of Moloney leukemia virus-derived vectors is also found in early hematopoietic cells and can be overcome by using the corresponding sequences of the murine embryonic stem cell virus. On the basis of these results, two types of novel retroviral hybrid vectors were developed; they combine the U3 regions of either the Friend mink cell focus-forming virus family or the myeloproliferative sarcoma virus with the primer binding site of the murine embryonic stem cell virus. When used to express the human multiple drug resistance gene, these vectors substantially improve protection to cytostatic drugs in transduced hematopoietic cell lines FDC-Pmix, TF-1, and K-562 in comparison with Moloney leukemia virus-derived vectors presently used for the stem cell protection approach in somatic gene therapy.

Progress in somatic gene therapy research has increased the demand for retroviral vectors that are both safe and highly efficient for gene expression in human hematopoietic cells (28, 29). One example is the stem cell protection approach, whereby chemoprotecting genes like the human multiple drug resistance cDNA (*mdr-1*), encoding the drug efflux pump P-glycoprotein (Pgp), are transduced into benign hematopoietic stem and progenitor cells to protect the bone marrow from dose-limiting side effects of cytotoxic chemotherapy (13, 27, 33, 35). Successful gene transfer into hematopoietic cells has been achieved with conventional retroviral vectors based on the Moloney murine leukemia or sarcoma virus (MoMuLV or MoMuSV, respectively); however, gene expression levels of MoMuLV-based vectors are reduced in more primitive myeloid stem cells, and long-term gene expression in hematopoietic stem cells and their derivatives is compromised by extinction (6, 7, 21). Both phenomena are most likely a consequence of insufficient interaction of the vector's *cis*-regulatory elements with the transcriptional machinery of the target cell.

Retroviral *cis* elements determining transcriptional activity are located in the U3 region of the long terminal repeat (LTR) and in the retroviral leader. The U3 region of MoMuLV is strongly lymphotropic and may not be very active in myeloid cells (23, 36). Point mutations introduced into enhancer elements of the U3 direct repeat increase the myelotropism of MoMuLV (36). Derivatives of MoMuSV such as the myeloproliferative sarcoma virus (MPSV), causing a myeloproliferative disease in adult mice, and PCMV (for PCC4-cell-passaged MPSV), selected for activity in primitive embryonic carcinoma cells, exhibit activating mutations in the regions flanking the direct repeats (2, 15, 19, 39). Exchange of the MPSV U3 with that of MoMuLV abolishes disease tropism for the stem cell compartment (40). Consequently, MPSV-derived vectors are superior to MoMuLV-derived vectors for expression in myeloid stem cell lines (6).

Retroviral gene expression in murine embryonic stem (ES) cells, which are, as shown here, possibly transcriptionally related to the most primitive multipotent hematopoietic stem cells, can be blocked by repressor elements located in the U3 region and in the primer binding site (PBS) of the retroviral leader (14, 22). Lack of MoMuLV expression in embryonic cells is a compound process resulting first in inefficient transcription and then in silencing correlated with methylation that prevents reexpression, even under permissive conditions (4, 8). Combination of the PCMV U3 with a mutated PBS from an endogenous murine retrovirus led to the development of a vector that is also active in undifferentiated ES cells, the murine ES cell virus (MESV) (14). MESV-based vectors also permit long-term maintenance of gene expression in the myeloid compartment in vivo, indicating that repressing elements located in the U3 region and/or in the PBS of MoMuLV-related vectors might be involved in silencing by yet unknown mechanisms (18).

In contrast to MoMuLV, the Friend murine leukemia virus (FMuLV) has evolved with a pronounced LTR-related erythrotropism (23). Recombinants of FMuLV with endogenous polytropic viruses lead to the generation of Friend mink cell

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focus-forming viruses (FMCF), which cooperate with FMuLV in inducing erythroblastosis (20, 31). A well-characterized, replication-defective FMCF mutant is the polycythemic strain of the spleen focus-forming virus (SFFVp), whose U3 region is important for the dynamics, but not for the specificity, of the Friend disease (1). Importantly, high activity of FMCF control elements may not be restricted to the erythroid lineage. High expression from the SFFVp LTR was also found in megakaryocytes in vivo (25). In addition, the malignant histiocytosis sarcoma virus (MHSV), containing an LTR highly related to that of SFFVp, induces a malignant histiocytic macrophage disease, indicating that FMCF-related regulatory elements have a broad host range within the myeloid compartment (12). Interestingly, exchange of the FMCF-type U3 of the transforming SFFVp or MHSV with a Moloney-type U3 drastically reduces disease progression in either of the myeloid lineages affected (1, 12).

To develop novel vectors based on retroviral sequences selected for myelotropism and maintenance of gene expression in vivo, we systematically analyzed the activities of *cis* elements of MoMuLV, MPSV, PCMV, MESV, and viruses with FMCF-related LTRs such as SFFVp and MHSV in several hematopoietic stages and lineages. Basic similarities in restriction of vector expression in ES cells and early hematopoietic cells were discovered. Strong transactivation in myeloid cells can be achieved by use of enhancers derived from FMCF-type viruses like SFFVp or MHSV and, to a lesser extent, also by the enhancer of MPSV. To exclude repression mediated by the MoMuLV-type PBS in multipotent stem cells, these enhancers need to be used in conjunction with the permissive leader of MESV. On the basis of these data, we cloned FMCF/MESV hybrid vectors (FMEV) and MPSV/MESV hybrid vectors (MPEV). By transferring *mdr-1* as a dominant selectable marker, FMEV and MPEV clearly are superior to conventional MoMuLV-based vectors, indicating that they will be of great value for a number of somatic gene therapy applications.

MATERIALS AND METHODS

Plasmids. Reporter gene plasmids driving the expression of the chloramphenicol acetyltransferase (CAT) gene under control of different retroviral LTR fragments were as follows. MoMuLV-CAT, PCMV(BX)/MLV-CAT, PCMV (BX)/Balp-CAT, and PCMV(BX)/Bal587-CAT (all kindly provided by M. Grez) have been described previously (14, 19). Replacing the *Nhe*I-*Kpn*I fragment of MoMuLV-CAT with the corresponding LTR fragment of MPSV (−416 to +31) generated MP-CAT. The same strategy generated SF-CAT (SFFVp clone 5.7 [cl5.7] LTR, −350 to +31) and MH-CAT (MHSV LTR, −393 to +31). Sources for LTR sequences were MPSV cl663, MHSV, and SFFVp(LS) cl5.7 (12, 24, 40). The LTR sequence of SFFVp cl5.7 corresponds to that of the previously published variant F-SFFV C4, in that its U3 region contains a truncated 42-bp second direct repeat identical to that found as a duplicate in MHSV (12, 20, 37).

The MESV-based vectors pSF-MDR and pMP-MDR are derived from p5Gneo, which contains a large 537-bp leader fragment of *dl*587rev (11, 14). Neo^r-*env* coding regions of p5Gneo have been replaced by a polylinker derived from pBluescript KS (Stratagene), and the AUG of the *gag* gene has been destroyed by point mutations. In this backbone, the MPSV/MESV basic vector p5O-M contains the MPSV U3 in the 3′ LTR (37a). The *mdr-1* cDNA was excised as a *Sac*I-*Ssp*I fragment from pMDR2000XS (kindly provided by M. M. Gottesmann, National Institutes of Health, Bethesda, Md.) representing sequences from −138 to +3878 (10). After subcloning into pBluescript II KS, the cDNA was isolated as a *Bam*HI fragment and inserted into p5O-M to yield pMP-MDR. In pSF-MDR, sequences downstream of *mdr-1* in pMP-MDR are replaced by a *Eco*RV-*Pst*I fragment derived from SFFVp(LS) cl5.7, containing residual *env* sequences downstream of *Eco*RV (position 1285) and including the complete LTR (sequence numbering according to reference 43). The MoMuLV-based proviral construct pV-MDR was kindly provided by A. B. Deisseroth, the University of Texas M. D. Anderson Cancer Center, Houston (16). Proviral vectors are termed MP-MDR, SF-MDR, and V-MDR to distinguish them from the corresponding plasmids (see Fig. 3).

Cell lines. Cell lines used in this study were NIH 3T3 (murine fetal fibroblasts), GP&env86 and GP&envAM12 (ecotropic and amphotropic retroviral packaging lines), FDC-Pmix A4 and 15S (murine multipotent myeloid stem cell lines) FDC-P1 (murine granulocyte/macrophage progenitors), F4-12 B2 (murine erythroid and Friend cell line), K-562 (human erythroleukemia cell line with monocyte and megakaryocyte potential), TF-1 (human pluripotent CD34$^+$ myeloid progenitor cell line) and CCRF-CEM (human T lymphoblastoma). Maintenance of the cell lines was as described previously (5, 38).

Transfections and reporter assays. Cells were electroporated under conditions resulting in 10 to 50% transient transfectants (5). For transient reporter assays, 10 μg of CAT vector was cotransfected with 10 μg of pCMVβ (Stratagene). Cells were harvested after 24 h to prepare cell extracts with the CAT lysis buffer supplied with the CAT enzyme-linked immunosorbent assay kit (Boehringer Mannheim), and CAT protein was determined by using the same kit according to the manufacturer's instructions. β-Galactosidase activity contained in the cell extracts was measured by the *o*-nitrophenyl-β-D-galactopyranoside assay and used for correction of transfection efficiency (34).

Retroviral transductions and selection conditions. GP&envAM12 cells were transduced with supernatants from GP&env86 mass cultures stably transfected with the proviral plasmids pMP-MDR, pSF-MDR, and pV-MDR. Clones were selected and propagated in the presence of 20 ng of colchicine (Sigma) per ml.

For infection, K-562, TF-1, and FDC-Pmix cells were cultured overnight in medium containing cell-free supernatants from amphotropic producer clones supplemented with 4 μg of Polybrene (Sigma) per ml. The multiplicity of infection was kept below 1. The following day, cells were plated in densities ranging from 2×10^3 to 5×10^4/ml in soft-agar medium containing 0, 4, 10, 20, 40, or 80 ng of colchicine per ml. Colony formation was scored after 12 to 14 days; colonies containing viable cells were counted as *mdr-1*-transduced units (MTU). Control cloning efficiencies in the absence of colchicine were 10 to 15% for K-562 and TF-1 cells and 25 to 33% for FDC-Pmix 15S cells. The assays were performed in duplicate and repeated twice, using supernatants from independently established clonal amphotropic producers. Individual colonies randomly picked from soft-agar cultures were grown in the presence of 10 ng of colchicine per ml. Viral titer was determined on fibroblasts (GP&env86 cells) in the presence of 8 μg of Polybrene per ml (6). Cells were selected in presence of 20 ng of colchicine per ml. For flow cytometric analysis of infected but unselected cells, K-562 cells were cocultivated for 2 days on top of irradiated producers releasing comparable high-titer vector supernatants.

Flow cytometry. Pgp activity was determined by using the rhodamine 123 (Rh123) efflux assay (26, 30). Cells (10^6) were incubated for 20 min at 37°C in staining medium (RPMI 1640 with 10% fetal calf serum) containing 60 ng of Rh123 (Sigma) per ml. After two washes, cells were transferred into Rh123-free medium and allowed to efflux for 10 min. In parallel experiments, efflux was performed in the presence of 10 μM verapamil, a Pgp inhibitor.

Pgp expression was analyzed by using the monoclonal antibody (MAb) 4E3 (Signet) with a phycoerythrin-conjugated goat anti-mouse immunoglobulin G2A (IgG2A) as the secondary antibody (3). As a control, an irrelevant matched-isotype MAb IgG2A (Coulter) was used. Flow cytometric measurements were carried out on a FACScan flow cytometer (Becton Dickinson) at an excitation wavelength of 488 nm, using 530/30-nm (green fluorescence) or 585/40-nm (red fluorescence) bandpass filters.

RESULTS

For gene expression in early hematopoietic and myeloid progenitor cells, U3 regions of FMCF are superior to those related to MoMuLV. To elucidate the role of retroviral U3 regions in different compartments of the hematopoietic system, a series of CAT reporter constructs was tested after transient transfection in human and murine cell lines representing different hematopoietic stages and lineages (Fig. 1). In all myeloid stem and progenitor cells studied, the FMCF-type U3 regions of SFFVp and MHSV contained in SF-CAT and MH-CAT showed the strongest activity. Differences between FMCF and MoMuLV depend on the developmental stage of the target cell. The largest difference (>10-fold) between FMCF and MoMuLV LTR constructs was found in the most primitive cells assayed, FDC-Pmix. Interestingly, even MPSV LTR-driven CAT vectors were expressed at lower levels in myeloid precursor and stem cell lines than were FMCF-driven constructs. Likewise, SF-CAT expression was found to exceed MP-CAT expression in the ES cell line CCE (4a). No lineage restriction of FMCF-type U3 regions was seen within the myeloid system; SF-CAT (SFFVp-U3) and MH-CAT (MHSV-U3) were of comparable activity in both erythroid and granulocyte/macrophage progenitors (F4-12-B2, FDC-P1, and K-562 cells).

However, the apparent myelotropism of FMCF-type U3 regions does not interfere with efficient transcription in lymphoid

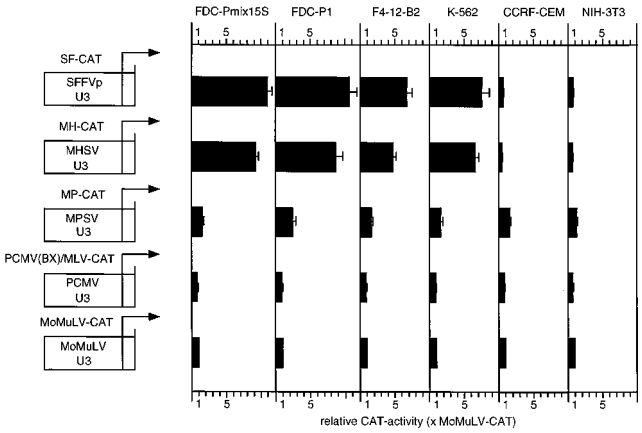

FIG. 1. The enhancer/promoter regions of SFFVp, MHSV, and, to a lesser extent, MPSV show higher transcriptional activity than those of MoMuLV in myeloid stem and precursor cells. Relative CAT expression mediated by U3 regions of SFFVp cl5.7 (SF-CAT), MHSV (MH-CAT), MPSV (MP-CAT), and PCMV [PCMV(BX)/MLV-CAT] was compared with that of the U3 region of MoMuLV (MoMuLV-CAT), whose activity was set at 1. Each value represents the mean ± standard error of three to six assays. Six (FDC-Pmix 15S and FDC-P1), five (F4-12-B2), four (CCRF-CEM), and three (K-562 and NIH 3T3) assays were done.

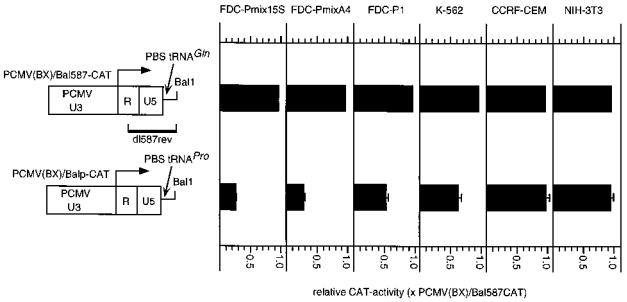

FIG. 2. Gene expression in hematopoietic stem cell lines is restricted by the PBS of MoMuSV (tRNA^{Pro}) and not by that of MESV (tRNA^{Gln}). Shown are mean values ± standard errors of four assays. The relative activity was blotted in comparison with a homologous construct containing the PBS of MESV/*dl*587rev [tRNA^{Gln}; PCMV(BX)/Balp-CAT]. MESV activity was set at 1.

cells and fibroblasts (Fig. 1). In the human T-lymphoblast cell line CCRF-CEM, the activities of SF-CAT and of MH-CAT are only slightly less than that of MoMuLV-CAT (66 and 45%, respectively). Similar ratios were seen in NIH 3T3 murine fibroblasts.

The MPSV U3 is expressed at higher levels than the MoMuLV U3 in both myeloid and lymphoid cells. Within the MoMuLV-related U3 regions, MPSV U3 consistently almost doubles the expression of CAT vectors in comparison with MoMuLV, independent of the hematopoietic stage and lineage studied (Fig. 1). The PCMV U3 with only one direct repeat element is generally weaker than the MoMuLV U3; it therefore is suboptimal for applications necessitating high gene expression in hematopoietic cells. In embryonic cells, the U3 regions of PCMV and of MPSV clearly allows higher expression than does that of MoMuLV (14, 19).

Transcriptional repression of retroviral vectors in early hematopoietic cells is mediated by the PBS and can be compensated by use of the MESV PBS. In ES cells, the MoMuLV/MPSV PBS (tRNA^{Pro}) negatively controls retroviral expression. This repression can be overcome by use of the PBS of MESV (tRNA^{Gln}) (14). We tested several constructs to determine whether a similar control mechanism can be seen in hematopoietic stem and precursor cells. The repressor activity of the PBS for tRNA^{Pro} can be monitored by comparing the activities of PCMV(BX)/Balp-CAT and PCMV(BX)Bal587-CAT (Fig. 2). Significantly, repression of the PBS for tRNA^{Pro} is strongest in the most primitive, most uncommitted myeloid cell line studied, FDC-Pmix (fourfold reduction of CAT activity). In committed myeloid precursor cells, CAT expression driven by the PCMV U3 is reduced twofold. No PBS-dependent repression could be detected upon transient transfection in T lymphoblasts and fibroblasts, concordant with earlier studies (22).

Use of tRNA^{Gln} of MESV restores the activity of LTR constructs. Vectors with tRNA^{Gln} PBS are thus fully permissive in all hematopoietic cell lines.

FMEV and MPEV retroviral vectors are released at high titer by safety-modified packaging cell lines. On the basis of these results, we cloned novel hybrid vectors, termed FMEV and MPEV, combining the LTR of FMCF-type SFFVp or of MPSV with the leader region containing the PBS for tRNA^{Gln} of MESV. The *mdr-1* cDNA was inserted as a dominant selectable marker. The construct representing FMEV is referred to as pSF-MDR; the MPEV construct is referred to as pMP-MDR (Fig. 3). For comparison, the MoMuLV-based vector pV-MDR was used (16). High-titer GP&envAM12 packaging cell lines were established upon retroviral infection with supernatants of ecotropic GP&env86 cells previously electroporated with the different constructs. The proviral DNA in the GP&envAM12 cells contains the correct and expected sequences of V-MDR, MP-MDR, and SF-MDR (Fig. 3 and data not shown). Fifteen to twenty-five independent amphotropic clones were established for each construct to monitor stability and efficiency of the new vectors in retroviral packaging cells. High-titer supernatants were obtained for all vectors (5×10^5 to 5×10^6 MTU/ml).

In myeloid precursor and stem cell lines, FMEV and MPEV *mdr-1* vectors provide much better protection against cytotoxic effects of colchicine than the standard MoMuLV vector. To assess the activity of SF-MDR (FMEV type), MP MDR (MPEV-type), and the standard V-MDR (MoMuLV type), the latter being used for somatic gene therapy in hematopoietic cells, human myeloid cell lines K-562 and TF-1 were infected with supernatants from high-titer amphotropic producer clones and plated in soft agar containing increasing doses of colchicine, a known substrate of Pgp. The 50% lethal doses of colchicine determined in short-term cytotoxicity assays were 4 to 6 ng/ml for K-562 cells and 6 to 8 ng/ml for TF-1 and FDC-Pmix cells (not shown). At 10 ng of colchicine per ml, a

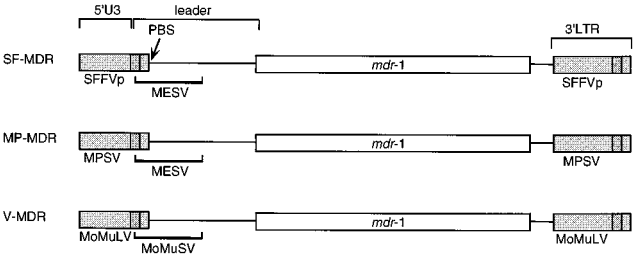

FIG. 3. Retroviral vectors with the *mdr-1* gene. Shown are proviral forms of the vectors following retroviral integration. SF-MDR contains the LTR of SFFVp cl5.7 and leader of MESV; MP-MDR contains the LTR of MPSV and leader of MESV; V-MDR contains the LTR of MoMuLV and leader of MoMuSV/MoMuLV. The position of the PBS is indicated by the arrow.

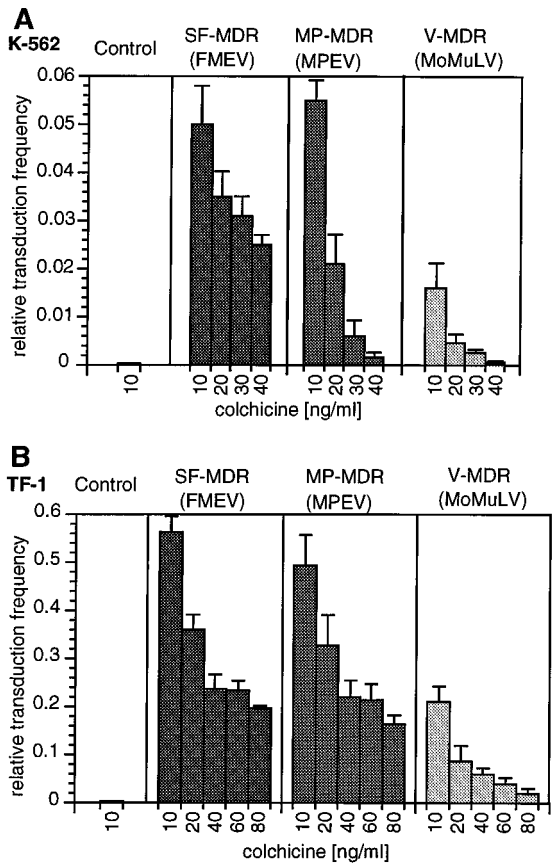

FIG. 4. FMEV and MPEV *mdr-1* vectors protect myeloid cells much better than do standard MoMuLV vectors. Mean relative transduction frequency was corrected for titer on fibroblasts and for cloning efficiency in the absence of colchicine. Titers on fibroblasts varied less than threefold between the different vectors. (A) K-562 (no endogenous Pgp-activity); (B) TF-1.

dose sufficient to suppress growth of mock-infected cells, both SF-MDR (FMEV) and MP-MDR (MPEV) showed much better expression, as measured by transfer efficiency, than did the standard vector V-MDR in K-562 cells (Fig. 4A). However, at higher concentrations (30 to 40 ng/ml), the SF-MDR vector provided up to 100-fold-greater protection to K-562 cells compared with both MP-MDR and V-MDR vectors.

Excellent transfer ratios were obtained with TF-1 cells (Fig. 4B). Viable colchicine-resistant clones could easily be selected, even at high doses. In TF-1 cells, containing a preactivated *mdr-1*, endogenously upregulated Pgp may contribute to the generally better resistance of the cells to colchicine. The differences in plating efficiency with increasing doses of colchicine between FMEV and MPEV vectors in these cells are not significant. Interestingly, in TF-1 cells, the U3 region of SFFVp is three- to fourfold more active than that of MPSV as determined in CAT assays, similar to the ratios found in myeloid progenitor cell lines K-562 and F4-12-B2 (Fig. 1 and data not shown). This finding indicates that in TF-1 cells transduced with MPEV or FMEV, *mdr-1* expression or Pgp function is saturated at a posttranscriptional level. Again, the standard MoMuLV vector is clearly inferior to both SF-MDR and MP-MDR, with 10- to 100-fold-reduced titers, as determined from high-level colchicine selection.

In the pluripotent myeloid stem cell line FDC-Pmix, the MoMuLV-based vector V-MDR is strongly suppressed, resulting in a severe reduction of the cloning efficiency even with low doses of colchicine. At 10 ng of colchicine per ml, the relative transfer efficiency obtained with MP-MDR is 20 times higher than that obtained with V-MDR, and the level for SF-MDR exceeds that for MP-MDR by a factor of 2 to 3 (Table 1). Above 20 ng of colchicine per ml, only SF-MDR and MP-MDR give rise to viable colonies. However, increasing the dose above 40 ng of colchicine per ml completely suppresses colony formation in vitro, indicating that vector expression is generally less in more primitive cells than in committed progenitor cells. Irrespective of the vector type, relative transfer ratios obtained on the basis of *mdr-1* transduction in FDC-Pmix cells are an order of magnitude less than those obtained on the basis of selection with the neomycin resistance gene (6). This underlines the need for high gene expression levels in the *mdr-1* system.

To control for the tissue specificity of FMEV-mediated gene expression, we also infected human CCRF-CEM T lymphoblasts with V-MDR, MP-MDR, and SF-MDR and plated these cells in soft agar containing increasing doses of colchicine. In this assay, the MP-MDR vector was superior not only to V-MDR but also to SF-MDR (not shown). MESV-based vectors driving *mdr-1* from the U3 of PCMV or of the MoMuSV-related Harvey murine sarcoma virus were included in initial experiments for infection of K-562 and TF-1 cells, but further studies with these vectors were not continued since the vectors proved to be inferior to MP-MDR.

The expression level of *mdr-1* is higher in cells infected with FMEV and MPEV than in those transduced with MoMuLV vectors. Individual clones randomly picked from K-562 cells and selected at 10 ng of colchicine per ml were expanded to assess *mdr-1* expression directly. Southern blot analysis revealed, regardless of the vector used, a mean of 1.2 vector copies per clone (not shown). Fluorescence-activated cell sorting (FACS) analysis of single-copy clones, using the Rh123 efflux assay, showed significant reduction in Rh123 accumulation as well as strong efflux activity in all transduced clones (Fig. 5A and Table 2). The efflux assay was adapted to permit a rough quantification by reducing the efflux time to 10 min. For comparison, primary human bone marrow cells need efflux assays for up to 3 h to allow detection of endogenous Pgp-activity (9). Complete Rh123 depletion of SF-MDR-transduced cells, however, was seen after 15 min. FACS analysis with MAb 4E3 detected increased Pgp expression in all infected clones, corresponding to the efflux data (Fig. 5B and Table 2). Significant quantitative differences in both Pgp function (efflux assay) and Pgp expression (MAb 4E3) were seen. Not only SF-MDR- but also MP-MDR-transduced clones had higher levels of Rh123 efflux and Pgp expression than did cells

TABLE 1. Relative transfer efficiencies of *mdr-1*-transducing retroviral vectors in the early myeloid stem cell line FDC-Pmix 15S[a]

Vector	Type	Relative titer on FDC-Pmix 15S with colchicine at:	
		10 ng/ml	20 ng/ml
V-MDR	MoMuLV	1×10^{-5}	5×10^{-7}
MP-MDR	MPEV	2.3×10^{-4}	5×10^{-5}
SF-MDR	FMEV	8×10^{-4}	2×10^{-4}

[a] Mean relative transduction frequency was corrected for titer on fibroblasts (GP&env86); assigned a value of 1) selected at 20 ng of colchicine per ml obtained with the same supernatants used to infect FDC-Pmix cells and for cloning efficiency of FDC-Pmix cells after retroviral transduction in the absence of colchicine. Titers on fibroblasts varied less than threefold between the different vectors.

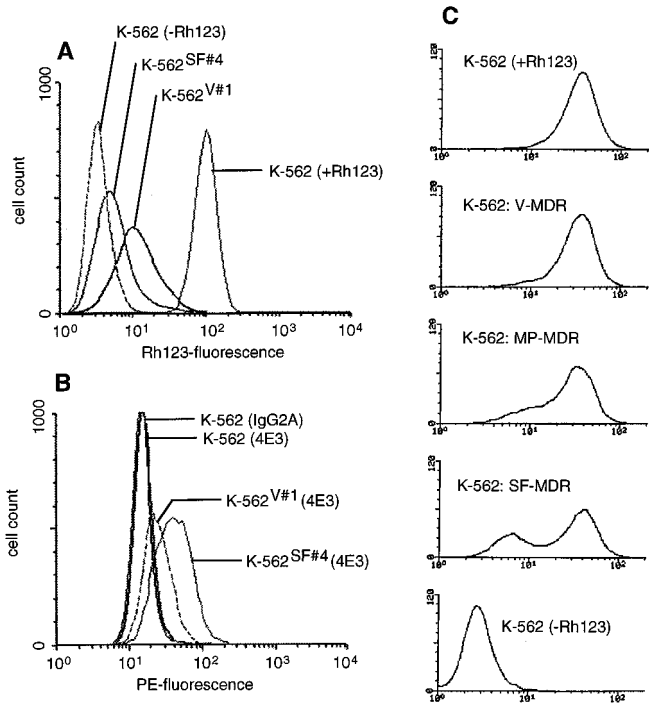

FIG. 5. Pgp expression in K-562 clones selected for expression of FMEV and MPEV *mdr-1* is significantly higher than in those transduced by standard MoMuLV vectors. Flow cytometric analysis of selected *mdr-1*-transduced clones (see also Table 1) with MAb 4E3, recognizing a surface epitope of Pgp, reflects protein expression levels. The Rh123 efflux assay, performed with limited efflux time, indicates differences in Pgp activity. (A) Retention of Rh123 after limited efflux time (10 min) in clones K-562$^{SF\#4}$ (SF-MDR transduced) and K-562$^{V\#1}$ (V-MDR transduced). Rh123 accumulation was also reduced in *mdr-1*-transduced clones (not shown). Untransduced K-562 loaded with Rh123 and autofluorescence of untransduced K-562 are shown for comparison. (B) Pgp expression as detected by MAb 4E3 in selected clones K-562$^{SF\#4}$ (SF-MDR transduced) and K-562$^{V\#1}$ (V-MDR transduced) in comparison with untransduced K-562. Staining with MAb IgG2A is shown as a negative control. PE, phycoerythrin. (C) Retention of Rh123 after limited efflux time (12 min) in unselected mass cultures analyzed after 48 h of cocultivation on GP&envAm12 clones releasing comparable high titers of V-MDR (10^6 MTU/ml), MP-MDR (5×10^5 MTU/ml), and SF-MDR (8×10^5 MTU/ml). *y* axis, cell count; *x* axis, Rh123 fluorescence. The percentages of Rh123-depleted cells (corrected for untransduced cells) were 2.6 (V-MDR), 11.9 (MP-MDR), and 26.9 (SF-MDR). Samples of the same cells plated in soft agar containing 20 ng of colchicine per ml revealed percentages of MTU per plated cell of 0.4 (V-MDR), 5.5 (MP-MDR), and 10.1 (SF-MDR).

infected with V-MDR. This difference is more pronounced in unselected infected cells (Fig. 5C), as there is a bias for selection of highly expressing V-MDR-transduced clones, reflected in the much lower recovery of selectable clones obtained with this vector even at the low concentrations of colchicine used to isolate the infected clones (Fig. 4). The percentage of unselected cells exhibiting efflux activity within the 12-min efflux time roughly corresponds to the number of clones obtained in soft agar at a selective pressure of 20 ng of colchicine per ml (Fig. 4 and 5C).

DISCUSSION

To develop retroviral vectors adapted to the requirements for efficient gene expression in myeloid stem and progenitor cells, we undertook a systematic analysis of the activities of retroviral *cis*-acting sequences in different stages and lineages of the hematopoietic system. We identified distinct activities of different retroviral U3 regions in hematopoietic cells. FMCF-type U3 regions (e.g., from SFFVp and MHSV) are expressed at much higher levels in myeloid cells, irrespective of the developmental lineage studied (erythroid, granulocyte, macrophage, or megakaryocyte) than those of MoMuLV. The differences extend up to 1 order of magnitude in primitive multipotent myeloid stem cells. Within the MoMuLV-related U3 regions, MPSV consistently exhibits highest expression levels in both myeloid and lymphoid cells.

Defining the mutations necessary for transactivation as well as maintenance of gene expression in early myeloid cells will extend our knowledge of transcriptional regulation within the hematopoietic system. Furthermore, it will be an important basis for future vector design. The complex differences between the U3 regions of MPSV and of FMCF-type viruses, however, require extensive analysis before the elements contributing to the high expression levels in hematopoietic and embryonic stem cells can be identified.

Previous studies have defined crucial *cis*-acting sequences necessary for retroviral activity in embryonic and myeloid cells. First, it has been shown that a unique Sp-1 binding site in MPSV is responsible for its high expression levels in undifferentiated embryonic cells (15). A similar Sp-1 binding site is found in the FMCF-type U3 regions studied here. Second, mutations in the enhancer core greatly contribute to the disease specificity of murine retroviruses in vivo (36). FMCF-type U3 regions contain a mutated enhancer core (20, 31, 37, 42). Preliminary data indeed indicate that these mutations are of

TABLE 2. Pgp expression in K-562 cells expressing FMEV and MPEV *mdr-1* is significantly higher than in cells transduced by standard MoMuLV vectors

Vector	Type of Pgp assay used	Pgp activity of K-562 clone:						Mean ± SEM[a]
		1	2	3	4	5	6	
SF-MDR (FMEV)	MAb 4E3[b]	32.5	31.2	20.5	ND[c]	ND	ND	
	Rh123 retention[d]	1.7	0.1	3.7	3.6	3.2	5.8	3.0 ± 0.8
MP-MDR (MPEV)	MAb 4E3	ND	93.7	14.0	ND	ND	22.9	
	Rh123 retention	5.5	1.8	8.7	8.2	10.4	7.1	7.0 ± 1.2
V-MDR (MoMuLV)	MAb 4E3	9.0	11.8	17.8	ND	ND	ND	
	Rh123 retention	8.3	10.2	7.7	15.4	6.8	6.5	10.9 ± 1.9

[a] For the six clones analyzed for each vector.
[b] Pgp expression is given as the difference between mean fluorescences detected by MAb 4E3 (Pgp specific) and by control MAb IgG2A.
[c] ND, not determined.
[d] Percent Rh123 retention in transduced clones after 10 min of efflux time was calculated as $[(F^{10}\text{-}F^{minus})/(F^{plus}\text{-}F^{minus})] \times 100$. F^{10} is the mean fluorescence after 10 min of efflux time (e.g., K-562$^{SF\#4}$ and K-562$^{V\#1}$ in Fig. 5A); F^{minus} is the mean fluorescence of untransduced, unstained cells [K-562 (−Rh123) in Fig. 5A]; F^{plus} is the mean fluorescence of untransduced, stained cells [K-562 (+Rh123) in Fig. 5A].

outstanding importance for their strong activity in myeloid cells (4a).

The retroviral sequences located in the region of the PBS of MoMuLV and of MPSV vectors also act as a target for transcriptional repression in primitive hematopoietic cells, similarly as previously shown for ES cells (14, 22, 44). We do not know whether the same proteins that appear to act as repressors in embryonic cells are also active in early hematopoietic cells. However, the restriction of the LTR and of the region coincident with the PBS in both hematopoietic and ES cells indicates that similar control mechanisms are operative in the two cell types. In addition, these results suggest that the silencing of retroviral vectors that has been observed in hematopoietic cells may be caused by mechanisms similar to those found in embryonic cells (4, 7, 8, 14). MESV-based vectors that escape silencing in embryonic cells could thus potentially escape extinction in hematopoietic stem cells as implied by our experiments and those of other groups (8, 14, 18). Thus, to obtain high expression of transduced sequences in myeloid stem and precursor cells, vectors need to contain both FMCF- or MPSV-type U3 regions and MESV-derived PBS sequences.

On the basis of these observations, we have cloned novel types of retroviral vectors, termed FMEV (FMCF/MESV hybrid vector) and MPEV (MPSV/MESV hybrid vector), designed to overcome at least one of the major restrictions associated with retroviral gene transfer into myeloid progenitors and stem cells, i.e., transcriptional inefficiency and silencing. The experimental system used in this study clearly manifests the advantageous features of these vectors.

Expression of *mdr-1*-encoded Pgp confers resistance to a variety of cytotoxic agents used in cancer chemotherapy, a phenomenon known as multidrug resistance (10, 13). Transduction of hematopoietic cells with vectors mediating high expression levels of Pgp is used to widen the therapeutic index of cancer chemotherapy (13, 27, 33, 35). The myelosuppressive side effects of cytotoxic agents affect hematopoietic progenitors and precursors in the first instance, resulting in severe short-term defects of the myeloid lineages (neutropenia, thrombocytopenia, and anemia). With increasing or repetitive doses, stem cells will also suffer significantly, and fatal long-term side effects like myelodysplasia or induction of secondary hematopoietic malignancies are among the consequences. Vector-mediated chemoprotection must therefore be highly efficient in all developmental stages of the hematopoietic system.

Conventional MoMuLV-related vectors bearing *mdr-1* have been cloned and shown to mediate elevated levels of Pgp and a selectable phenotype in transduced cells. In vitro and in vivo (mouse) studies performed with these vectors have indicated that chemoprotection of bone marrow may be feasible (16, 17, 27, 32, 33, 35). However, the expression of *mdr-1* obtained with MoMuLV-related vectors is only slightly above the endogenous background of human myeloid stem and precursor cells. Hence, under doses just about sufficient to completely suppress growth of untransduced cells, only some 10% of transduced cells survive, indicating leakiness of chemoprotection (41). Doubling the drug dose completely suppresses the clonogenic capacity of these transduced cells (17).

Here we show that *mdr-1* expression in myeloid progenitor cells mediated by FMEV and MPEV vectors is associated with excellent chemoprotection, as determined by selection under increasing doses of colchicine. With the best construct developed so far (SF-MDR), drug concentrations up to 10 times the 50% lethal dose result in only 50% reduction of clonogenic capacity of transduced hematopoietic progenitor cells, compared with 90 to 99% reduction of cells which could be selected after infection with a conventional MoMuLV *mdr-1* vector. In the early myeloid stem cell line FDC-Pmix, the MoMuLV-based vector is heavily suppressed, and only the MESV leader-based vectors FMEV and MPEV confer significant chemoprotection.

We predict that FMEV and MPEV, relying exclusively on retroviral sequences selected in vitro and in vivo for high levels and long-term maintenance of gene expression, should prove extremely beneficial for transduction of myeloid stem and progenitor cells in stem cell protection protocols or for correction of metabolic disorders involving myeloid lineages. Preliminary in vitro data obtained so far for human primary bone marrow cells support these conclusions.

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