Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6733–6737, July 1995
Genetics

Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells

(transcriptional control sequences/long-term gene expression/gene therapy/hematopoietic cells)

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Communicated by Irving L. Weissman, Stanford University School of Medicine, Stanford, CA, January 20, 1995

ABSTRACT **To determine which features of retroviral vector design most critically affect gene expression in hematopoietic cells *in vivo*, we have constructed a variety of different retroviral vectors which encode the same gene product, human adenosine deaminase (EC 3.5.4.4), and possess the same vector backbone yet differ specifically in transcriptional control sequences suggested by others to be important for gene expression *in vivo*. Murine bone marrow cells were transduced by each of the recombinant viruses and subsequently used to reconstitute the hematopoietic system of lethally irradiated recipients. Five to seven months after transplantation, analysis of the peripheral blood of animals transplanted with cells transduced by vectors which employ viral long terminal repeats (LTRs) for gene expression indicated that in 83% (77/93) of these animals, the level of human enzyme was equal to or greater than the level of endogenous murine enzyme. Even in bone marrow transplant recipients reconstituted for over 1 year, significant levels of gene expression were observed for each of the vectors in their bone marrow, spleen, macrophages, and B and T lymphocytes. However, derivatives of the parental MFG-ADA vector which possess either a single base mutation (termed B2 mutation) or myeloproliferative sarcoma virus LTRs rather than the Moloney murine leukemia virus LTRs led to significantly improved gene expression in all lineages. These studies indicate that retroviral vectors which employ viral LTRs for the expression of inserted sequences make it possible to obtain high levels of a desired gene product in most hematopoietic cell lineages for close to the lifetime of bone marrow transplant recipients.**

Despite considerable progress in efforts to develop effective genetic therapies for diseases involving hematopoietic cells, significant technical hurdles remain. First, while various transduction protocols have been developed to efficiently transfer genes into murine hematopoietic stem cells (1–7), it has not been possible to achieve efficient gene transfer into reconstituting cells of large animals (8, 9). It is unclear to what extent this problem is vector related (e.g., insufficient titers, host range) or a consequence of a lack of knowledge regarding the optimal conditions for proliferation and/or efficient engraftment of appropriate target cells. A second important technical stumbling block relates to the development of retroviral vectors possessing the appropriate signals for high-level constitutive expression of inserted genes in hematopoietic cells *in vivo*. Although a number of groups have demonstrated the expression of genes in mice reconstituted with transduced bone marrow (BM) cells, others have experienced difficulties (10–12). Overall, few general principles regarding features of vector design important for gene expression *in vivo* have emerged. In particular, because of differences in vector backbones, inserted genes, viral titers, transduction protocols, and other experimental parameters, it has been impossible to directly compare the performance of different vectors and to determine the features of vector design which most critically affect gene expression in hematopoietic cells *in vivo*. In addition, few studies have examined the ability of transferred genes to be expressed for very long times (e.g., the lifetime of the transplant recipients), a clearly important goal of gene therapy for diseases involving hematopoietic cells.

We have addressed the above issues regarding gene expression *in vivo* through the construction of various retroviral vectors which encode the same gene product, human adenosine deaminase (hADA), and possess the same vector backbone yet differ specifically in transcriptional control sequences suggested by other studies to be important for vector gene expression. BM transplant (BMT) recipients engrafted by cells transduced by each vector were generated and the levels of vector expression were determined at both 5–7 months and >1 year after transplantation. An important conclusion from these studies is that simple vectors which employ viral long terminal repeats (LTRs) for the expression of inserted sequences make it possible to obtain high levels of a desired gene product in most hematopoietic lineages for close to the lifetime of a BMT recipient. Our results also suggest that the choice of viral transcriptional control sequences may be important for obtaining particularly high levels of gene expression.

MATERIALS AND METHODS

Recombinant Retroviruses and Virus-Producing Cells. Vectors were constructed by standard cloning procedures. The parental MFG-ADA vector consists of the following sequences inserted between the *Hin*dIII and *Eco*RI sites of plasmid pBR322: (*i*) 396 bp of 5′ murine chromosomal DNA (5′ flanking sequence), (*ii*) an entire Moloney murine leukemia virus (Mo-MLV) 5′ LTR and adjacent sequence up until the *Nar* I site at nt 1035 (a 6-bp *Sma* I linker was inserted at nt 624; see ref. 13 for numbering of Mo-MLV-derived sequences), (*iii*) a sequence containing the viral 3′ splice acceptor and spanning nt 5401–5780 (the A nucleotide of the *Nla* III site has been changed to a C to create an *Nco* I site at the end of this fragment), (*iv*) hADA cDNA sequence extending from the ATG initiation codon at nt 74 to an *Acc* I site at nt 1324 (14), (*v*) Mo-MLV proviral sequence extending from the *Cla* I site at nt 7674 to the end of the 3′ Mo-MLV LTR, and (*vi*) 695 bp of 3′ murine chromosomal DNA (3′ flanking sequence).

The myeloproliferative sarcoma virus (MPSV) LTR derivative of MFG-ADA was constructed by combining a 2717-bp

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Abbreviations: ADA, adenosine deaminase; hADA, human ADA; mADA, murine ADA; BM, bone marrow; BMT, BM transplant; CMV, cytomegalovirus; IEF, isoelectric focusing; LTR, long terminal repeat; Mo-MLV, Moloney murine leukemia virus; MPSV, myeloproliferative sarcoma virus.

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Ban II–*Nhe* I fragment of MFG-ADA which contains the hADA cDNA with a 6-kbp *Ban* II–*Nhe* I fragment of pC663neoR which contains the MPSV LTRs (15). This method of construction yielded proviral sequences which possessed both 5′ and 3′ MPSV LTRs. Derivatives of MFG-ADA which carry the MPSV or Friend murine leukemia virus enhancer sequence were generated by replacing the *Nhe* I–*Sac* I and *Nhe* I–*Kpn* I segments of DNA in the 3′ LTR of MFG-ADA with the corresponding sequences from the 3′ MPSV LTR plasmid (kindly provided by P. Robbins, University of Pittsburgh) and the pFr-SV(X) plasmid (16), respectively. Vectors that harbor the B2 mutation (17, 18) carry a single G-to-A mutation at position +160 of the Mo-MLV sequence.

The αSGC-ADA vector contains the following sequences and is derived from pHSG (19): (*i*) 5′ and 3′ flanking mouse chromosomal DNA sequences (see above), (*ii*) Mo-MLV 5′ LTR sequence and adjacent sequence extending to nt 1558, (*iii*) cytomegalovirus (CMV) enhancer sequence from nt 154 to nt 515 (20), (*iv*) human α-globin promoter sequence from nt −570 to nt +37 (21), and (*v*) Mo-MLV sequence extending from the *Cla* I site at nt 7674 to the end of the 3′ Mo-MLV LTR. The 3′LTR is lacking nt 7933–8111 (19).

Retrovirus-producing cell lines were generated by cotransfection of each retroviral plasmid with the plasmid pSV2-neo into the amphotropic packaging cell line ψCRIP and selection for 10 days with G418 (1 mg/ml; GIBCO) (2, 22). Cell-free supernatant was then harvested and used to infect ψCRE cells (22). An αSGC-ADA producer was isolated after direct transfection of ψCRE cells. Individual clones were screened for high-titer virus production by Southern blot analysis (2, 22, 23). To verify the presence of the B2 mutation in the selected virus-producing cell lines and its transmission to infected NIH 3T3 cells, PCR primers corresponding to Mo-MuLV nt 72–92 and 470–490 were used to amplify a fragment of 400 bp which was then sequenced (*fmol* DNA sequencing system; Promega). Producer cell clones that transmitted the mutations to recipient cells at a frequency of 25–50% were chosen for use. Supernatants from virus-producing ψCRE cells and plasma from transplant recipients were tested for the presence of replication-competent virus by the *hisD* mobilization assay (22). Less than one virus particle per milliliter can be detected by this assay. All samples tested were found to be negative.

DNA Analysis. Genomic DNA was prepared (24) and digested with *Nhe* I or *Ecl*136II. Hybridization filters were probed with a 721-bp ^{32}P-labeled *Bam*HI–*Bgl* II fragment of the hADA cDNA (Multiprime DNA labeling system; Amersham). The copy numbers were determined on a phosphor imager (Fuji Bio-Imaging) relative to the intensity of bands corresponding to a single copy of the provirus. To account for uneven loading, the band signal was normalized to the endogenous murine ADA (mADA) band.

Murine BM Transplantation and Analysis of Cell Populations. Infection and transplantation of BM from C57BL/6J mice were performed as described (2). From 2.5 × 10^5 to 4.5 × 10^6 cells were injected per lethally irradiated (11 Gy) female host (2). Selected animals were sacrificed 12–14 months after BM transplantation and samples of peripheral blood, BM, spleen, spleen-derived B and T lymphocytes, and BM-derived macrophages were prepared. In brief, B and T cells were harvested after 72 hr of stimulation with lipopolysaccharide (10 μg/ml) and Con A (2 μg/ml), respectively. More than 85% of cells were T or B lymphocytes as judged by flow cytofluorometry using anti-Thy-1.2 (PharMingen) or B220 (Caltag, South San Francisco, CA) antibodies. BM cells were cultured in medium containing 20% (vol/vol) L929-cell supernatant to generate macrophages. More than 95% of cells harvested after 10–11 days were macrophages by morphology.

ADA Enzyme Analysis. Isozyme-specific ADA activity was detected in cell lysates by nondenaturing isoelectric focusing (IEF) (2, 25). Total protein concentration was determined for each sample with the Bio-Rad protein assay. Fixed amounts of total protein were loaded on the IEF gels (300 μg for peripheral blood cells; 150 μg for BM; 120 μg for spleen, macrophages, and B lymphocytes; 75 μg for T lymphocytes). After 12 min of staining reaction, the gels were fixed and the colorimetric intensity of each band was quantified with a computing densitometer (Molecular Dynamics). Since multiple gels were necessary to analyze all of the samples, a separate gel was run which included samples from each gel to provide a means of normalizing the data obtained from the individual gels.

RESULTS AND DISCUSSION

Generation of Recombinant Viruses Encoding hADA. The starting point for our studies of vector design was the construction of MFG-ADA (Mo-LTR construct) (Fig. 1*A*). The MFG vector, constructed in our laboratory (L. Spain, P. Robbins, and R.C.M., unpublished data; see also refs. 26–28), employs the Mo-MLV LTRs for transcription of the viral genome. Additionally, the vector retains both the ψ and ψ^+ sequences necessary for efficient encapsidation of recombinant genomes into virus particles (29–31) and the viral splice donor and acceptor sequences necessary for generation of the subgenomic viral *env* mRNA. The hADA sequence was inserted so as to position the initiation ATG codon of the ADA cDNA at the position in the subgenomic viral transcript identical to that normally occupied by the viral *env* ATG. No selectable marker exists in the vector. Because the transcriptional activity of the MPSV LTR had been demonstrated by others in vector studies involving hematopoietic cells (10, 15, 17, 18, 32), we generated derivatives of MFG-ADA which possessed either the enhancer of MPSV positioned in the 3′ Mo-MLV LTR [MFG(MPSV-Enh)ADA] or both 5′ and 3′ MPSV LTRs in place of the Mo-MLV LTRs [MFG(MPSV)ADA]. In addition, because of previous studies suggesting the potential novel properties of the Friend virus enhancer sequences (16, 33–35), MFG-ADA derivatives were generated in which the Mo-MLV enhancer sequence was replaced by analogous Friend enhancer sequence [MFG(Fr-Enh)ADA]. In

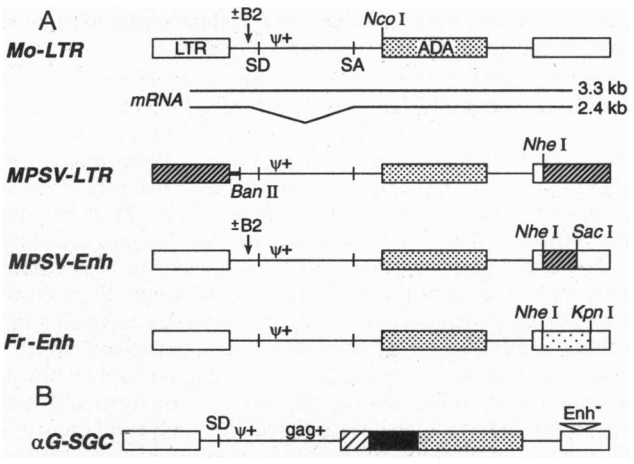

FIG. 1. Structure of retroviral vectors encoding hADA. Here and in the other figures, MFG-ADA is designated as Mo-LTR; MFG(B2)ADA as Mo-LTR/B2, MFG(MPSV-Enh)ADA as MPSV-Enh, MFG(MPSV-Enh/B2)ADA as MPSV-Enh/B2, MFG-(MPSV)ADA as MPSV-LTR, MFG(Fr-Enh)ADA as Fr-Enh, and α-SGC as αG-SGC. (*A*) MFG-derived recombinant vectors. Sequences are derived from hADA cDNA (▨), Mo-MuLV (▭), MPSV (▨), and Friend virus (▩). SD and SA, splice donor and acceptor sequences. (*B*) αG-SGC vector. In this vector, hADA expression is under the control of the human CMV enhancer (▥) and α-globin promoter (▬). For details, see *Materials and Methods*.

light of previous studies which identified a mutation in the viral tRNA primer binding site, termed B2, capable of improving vector gene expression in embryonal carcinoma cells (36, 37), we also generated derivatives of MFG-ADA and the MPSV enhancer which carried the B2 mutation [MFG(B2)ADA and MFG(MPSV-Enh/B2)ADA, respectively]. Lastly, to provide a comparison of LTR-based vectors and vectors which employ internal promoters for expression of inserted genes, we generated αSGC-ADA. This vector utilizes a hybrid transcriptional element comprising the human α-globin promoter and CMV enhancer sequences and has a deletion of enhancer sequence in the 3′ LTR. The precise structure of each of the above constructs is described in *Materials and Methods.*

Virus-producing ψCRE cell lines for each of the above constructs were selected for their high viral titer after infection of NIH 3T3 cells by Southern blot analysis. The transmission efficiency of all viruses for transduction of NIH 3T3 cells was in a range 0.7–3.6 copies of provirus per cell (data not shown).

Detection and Quantitation of hADA Activity in Murine BMT Recipients Engrafted with Genetically Modified Cells. To investigate hADA expression in cells derived from transduced hematopoietic stem cells, mice were transplanted with 2.5×10^5 to 4.5×10^6 BM cells that had been cocultured with recombinant virus-producing cells. Fifteen to 18 mice were transplanted with cells transduced by each construct. A first step in assessing vector-mediated gene expression involved the analysis of hADA enzyme activity in the peripheral blood cells of reconstituted animals 5–7 months after transplantation. Since the direct enzymatic assay of ADA activity in extracts does not provide a means of distinguishing human activity from the endogenous murine activity, we chose to employ a semiquantitative IEF enzyme assay (2, 25). In this assay, hADA activity can be readily separated from mADA activity and the relative levels of the two activities can be estimated by densitometry measurements of labeled reaction products. We have shown, using dilutions of extracts that are within the range of protein loaded in the current studies and the time of incubation employed, that the enzyme activity assay generates signals proportional to the amount of enzyme activity and reflects the tissue-specific differences in ADA expression observed previously (2) (see also Fig. 3*B*). Because a small portion (10–20%) of hADA migrates at the position of the murine enzyme, the assay does consistently underestimate the absolute amount of human activity and, therefore, the ratio of human to murine enzyme in extracts. In extracts containing large amounts of human activity, the discrepancy can be as great as 3-fold (2).

In mice repopulated with cells transduced with MFG-derived vectors, 90 out of 93 transplanted animals expressed hADA. In 93% (77/93) of those transplanted animals, the expression of hADA was equal or greater than that of endogenous mADA (data not shown). Only 3% (3/93) of the animals did not express hADA at a detectable level. In mice reconstituted with the αSGC-infected cells, only 4 mice out of 17 expressed the hADA at low levels, probably because of the low copy number of provirus that we detect in the tissues of these animals (see below).

Vector Expression in Specific Hematopoietic Lineages at Long Periods After Reconstitution. Based on the high frequency of BMT recipients which demonstrated significant gene expression in the peripheral blood 7 months after BM transplantation, we examined a subset of animals for hADA expression in various hematopoietic cell types at much longer times (>1 year) after transplantation. A first step in these studies was to reanalyze the mice previously analyzed for enzyme expression in peripheral blood. A comparison of the relative amounts of human and mouse enzymes at the two time points (shown below each IEF gel track in Fig. 2) indicated that little if any significant decrease in expression occurred over time. Approximately 80% of the expression observed at 7 months persisted after 1 year (Fig. 2). Mice engrafted with αSGC-ADA-transduced cells exhibited a greater decrease in gene expression than observed with the MFG-derived vectors.

In the next series of experiments, expression of the vectors in different hematopoietic cell types was examined through the fractionation of each cell population and subsequent quantitation of enzyme activity and proviral copy number. In light of the large number of experiments possible, we restricted this analysis to three to six animals engrafted with cells transduced by either MFG-ADA, MFG(B2)ADA, MFG(Fr-Enh)ADA, MFG(MPSV)ADA, or αSGC-ADA. The cell populations subjected to enzyme and DNA analysis included whole BM, whole spleen, fractionated macrophages, T lymphocytes, and B lymphocytes. The data may be summarized as follows.

(i) Proviral copy number achieved by different viruses. A significant proportion of the various hematopoietic cell populations carried proviral sequences even at >1 year after transplantation (Fig. 3*A*). With the exception of the αSGC-ADA vector, where fewer cells appeared to carry provirus, each of the vectors yielded comparable proviral copy number

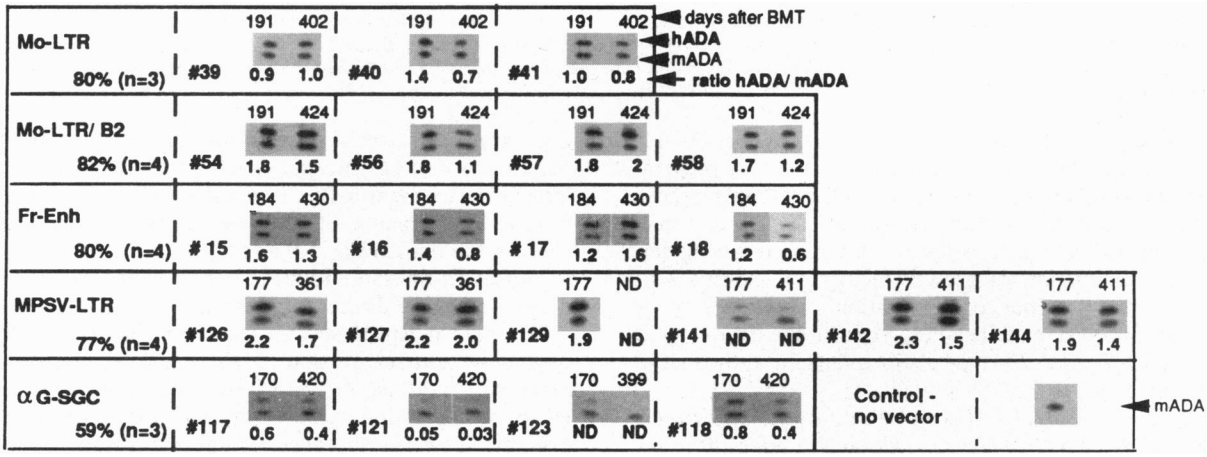

FIG. 2. Comparison of hADA expression in peripheral blood cells 5–7 and 12–14 months after BM transplantation. Blood samples were drawn at two distant time points after transplantation and analyzed for ADA activity by IEF. Individual mice are designated by their number (#). Arrows indicate mADA (lower band) and hADA (upper band) activity. The relative ADA activity indicated under each sample is determined as the ratio of the intensities of the human and mouse enzyme bands. The percentage indicated in the first column represents, for each vector, the mean hADA activity 12–14 months after transplantation compared with the original activity (100%) measured 5–7 months after transplantation; n represents the number of mice used to calculate the mean activity.

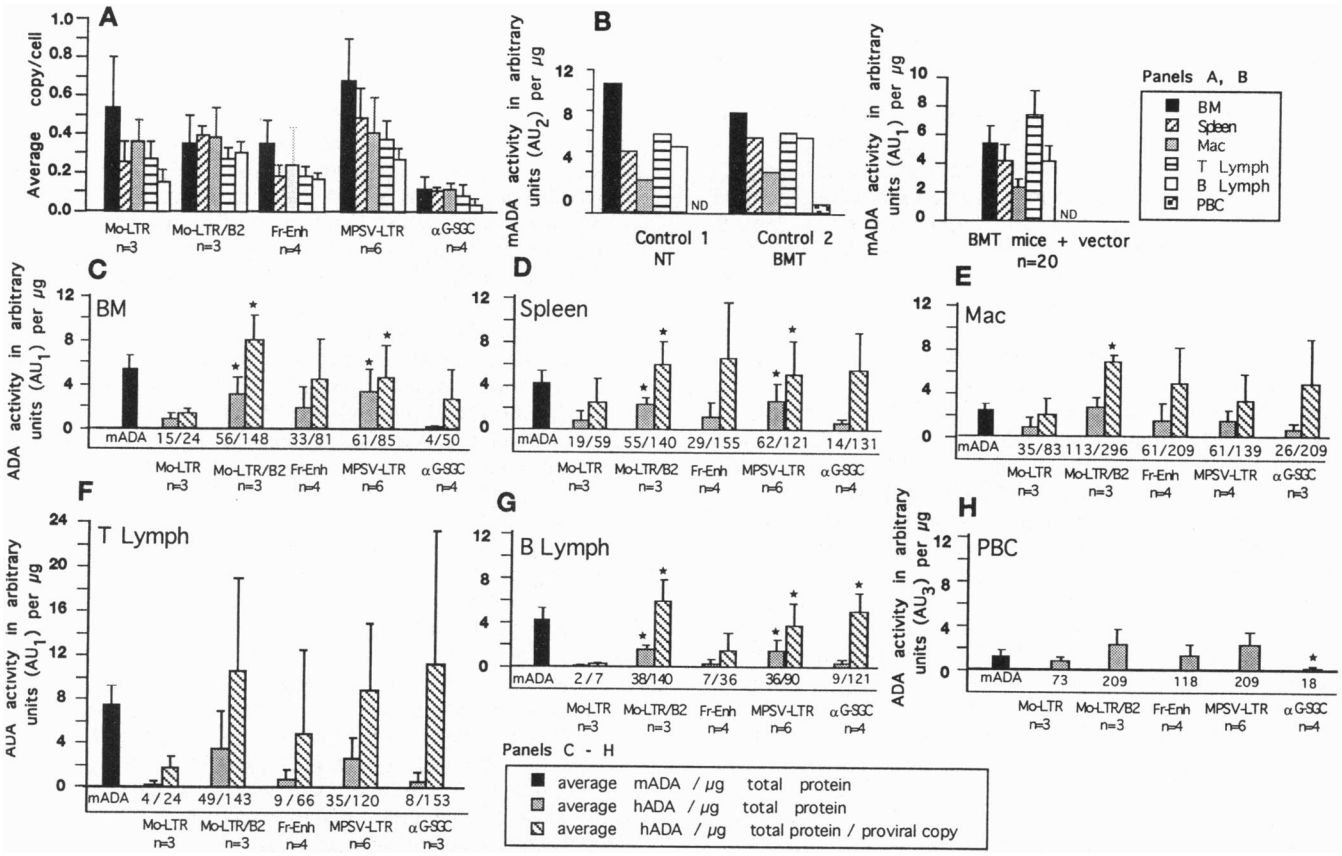

FIG. 3. Quantitation of human and murine ADA expression in hematopoietic cell populations 12–14 months after BM transplantation. (*A*) Average proviral copy number per cell. DNA was isolated from each cell fraction of all animals and analyzed by Southern blot analysis using a hADA probe. For each sample, the copy number was determined with a phosphor imager. BM, unfractionated bone marrow; Spleen, unfractionated spleen; B Lymph, splenic B lymphocytes; T Lymph, splenic T lymphocytes; Mac, macrophages derived from BM; PBC, peripheral blood cells. Each bar marked with an asterisk indicates that a distribution made of (*i*) pooled BM and spleen samples, (*ii*) B lymphocytes, (*iii*) T lymphocytes, or (*iv*) macrophages shows statistically significant differences ($P < 0.05$) when compared with the corresponding MFG-ADA distribution (Student–Fisher's *t* test). The number of mice (n) analyzed and used to calculate the mean and SD is indicated under each vector. (*B*) mADA activity in control and experimental mice. Activity is presented in arbitrary units per micrograms of total protein in all the fractions described above as well as in total PBC from a normal C57BL/6 mouse that was not a BMT recipient (control 1, NT) and from a mouse reconstituted with nontransduced BM cells (control 2, BMT). Average mADA activity was determined in every fraction of the 20 experimental animals examined and is presented in different arbitrary units (AU_1) as compared with controls 1 and 2 (AU_2). (*C–H*) Quantitation of murine and human ADA activities in various cell populations. After sacrifice of BMT recipient mice, cell fractions were harvested and ADA activity was determined by IEF. Average endogenous murine and human ADA activities are presented in the same fractions as in *A* and *B*. The number of mice (n) analyzed and used to calculate the mean ± SD is indicated below each vector. Average mADA activity is reported in arbitrary units (AU_1) per μg of total protein (black bars). Average hADA activity is reported both in arbitrary units per μg of total protein (shaded bars) and in arbitrary units per μg of total protein per proviral copy (hatched bars). The same arbitrary units (AU_1) were used in *C–G* for both murine and human enzyme. In *H*, mADA and hADA activities are reported in different arbitrary units (AU_3). Stars indicate statistical significance of values relative to MFG-ADA (Mo-LTR) vector (see above). Numbers below the shaded and hatched bars indicate the percentage of hADA activity relative to endogenous mADA activity in that cell population.

(0.2–0.7 per cell) in all lineages, in spite of differences in viral titers. There was, however, significant variation in proviral copy number from mouse to mouse. In addition to differences in hematopoietic stem-cell transduction efficiencies that may be due to intrinsic characteristics of the virus-producing cell clones (e.g., production of growth factors, adhesiveness of monolayer), the previous demonstration of the ability of specific transduced stem-cell clones to contribute unequally to different cell lineages (38) may also explain the rather large variations in copy numbers observed.

(ii) Expression levels of hADA achieved by different viruses. Levels of ADA activity differ considerably among different populations of hematopoietic cells (Fig. 3*B* and ref. 2). For this reason, we have calculated both the average levels of hADA obtained with the different viral constructs in different cell populations and the corresponding level of endogenous mADA (Fig. 3 *C–H*). We have expressed the enzyme data both in terms of the actual hADA enzyme activity observed per microgram of extract (shaded bars) and the enzyme activity per microgram of extract per proviral copy (hatched bars). This latter measurement makes it possible to directly compare the relative performance of each vector. With the exception of some of the data in Fig. 3 *B* and *H*, all of the data in Fig. 3 are expressed in the same arbitrary units of enzyme activity and therefore can be directly compared.

The data illustrate several important points about vector-mediated gene expression. First, while all vectors led to expression in all tissues examined, differences in the absolute levels of expression achieved by the different viruses were evident. However, because of the significant variation in expression levels from mouse to mouse, and the relatively small numbers of animals examined, we applied statistical analysis to the data. In Fig. 3, bars marked with asterisks indicate values that are statistically significant relative to the values for the parental ADA vector. Both the MFG(B2)ADA and MFG(MPSV)ADA vectors show significant differences in ex-

pression relative to MFG-ADA. In the case of MFG(B2)ADA, significance could not be established for the T-lymphocyte lineage due to the wide variation in expression levels observed from mouse to mouse. Similarly, statistical significance could not be established for the macrophage lineage or T-lymphocyte lineage in the case of MFG(MPSV)ADA.

Direct comparison of the levels of hADA and mADA enzyme activity found in extracts prepared from different hematopoietic cell populations also indicated that physiologically significant levels of human enzyme were achieved after gene transfer (numbers under each bar indicate percent of endogenous murine enzyme activity). For example, in most cell lineages, the average level of hADA activity achieved by the MFG(B2)ADA and MFG(MPSV)ADA vectors (without correction for proviral copy number) (shaded bars) was >60% of the endogenous mADA activity. When the level of expression per proviral copy number was considered (hatched bars), several vectors led to human enzyme activity that considerably exceeded the mouse activity. The hADA activity in T cells derived from mice transplanted with cells transduced by either MFG(B2)ADA or MFG(MPSV)ADA was 35–49% of the mADA activity (Fig. 3*F*). These results are particularly noteworthy in light of the probability that efficient ADA gene expression in T cells will be critical for successful implementation of gene therapy for ADA deficiency. Since, as pointed out earlier, the *in situ* gel assay leads to an underestimate of the levels of hADA activity, the actual hADA activity that can be achieved in T cells after stem-cell transduction is likely to be even higher than we report.

A last important finding is that, perhaps with the exception of the parental MFG-ADA vector, similar levels of hADA expression were observed from tissue to tissue, in spite of the variation in endogenous mADA levels. This finding suggests that the vectors examined do not, to any great extent, exhibit tissue specificity of gene expression and therefore may well be useful for a variety of applications in which expression is required in a specific cell lineage yet permissible in nontarget cells.

Overall Conclusions. The most important overall conclusion from the above studies is that the retroviral LTR can provide long-term expression of genes in hematopoietic cells derived from transduced BM cells. Our detection of significant levels of hADA expression in all hematopoietic lineages at >1 year after transplantation is significant, since this time approximates the normal lifespan of a murine BMT recipient. Our results strongly suggest that in previous studies which have demonstrated either the inactivity or shutoff of gene expression by LTR-based vectors (10–12), specific features of vector design other than the utilization of viral LTRs may have contributed more to the observed problems in expression than was previously suspected.

The absolute magnitude of improvement of expression afforded by either the MFG(B2)ADA or MFG(MPSV)ADA vector is somewhat difficult to assess, in light of the small number of animals examined for expression in different cell lineages and the variations in expression levels observed. Nevertheless, the data suggest that those vectors offer improved expression and that the improvement appears to be general, in that it occurs in most cell lineages.

We thank Glen Paradis for performing flow cytometric analysis and Devon Young for secretarial assistance. I.R. thanks Dr. Michel Sadelain for helpful discussions and for critically reading the manuscript. This work was supported by a grant from the National Institutes of Health (HL37569) to R.C.M.

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