*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 439–443, January 1990
Genetics

# Expression of human adenosine deaminase in mice reconstituted with retrovirus-transduced hematopoietic stem cells

**(retroviral vector/gene therapy/bone marrow transplantation)**

JAMES M. WILSON*†, OLIVIER DANOS*, MARIANN GROSSMAN*†, DAVID H. RAULET‡, AND RICHARD C. MULLIGAN*

*Whitehead Institute for Biomedical Research and ‡Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

*Communicated by Robert A. Weinberg, September 15, 1989*

ABSTRACT **Recombinant retroviruses encoding human adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) have been used to infect murine hematopoietic stem cells. In bone marrow transplant recipients reconstituted with the genetically modified cells, human ADA was detected in peripheral blood mononuclear cells of the recipients for at least 6 months after transplantation. In animals analyzed in detail 4 months after transplantation, human ADA and proviral sequences were detected in all hematopoietic lineages; in several cases, human ADA activity exceeded the endogenous activity. These studies demonstrate the feasibility of introducing a functional human ADA gene into hematopoietic stem cells and obtaining expression in multiple hematopoietic lineages long after transplantation. This approach should be helpful in designing effective gene therapies for severe combined immunodeficiency syndromes in humans.**

While somatic cell gene therapy is now being considered in the treatment of a variety of inherited and acquired diseases, genetic disorders that selectively affect the hematopoietic system have been the focus of most research, because the appropriate target cell for gene transfer, the hematopoietic stem cell, is easily accessed, manipulated *in vivo*, and transplanted. For a variety of reasons, an inherited deficiency of the enzyme adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) has been considered an ideal candidate for the initial trials of human gene therapy (1). This genetic disease causes abnormalities in the levels of specific purine metabolites that lead to the selective loss of functional lymphoid cells and result in a severe combined immunodeficiency syndrome (2). The relative success of allogeneic bone marrow transplanation and enzyme replacement therapy in selected ADA-deficient patients suggests that the introduction of a functional ADA gene into stem cells and subsequent reconstitution by the genetically modified cells may be curative in patients that are not candidates for conventional therapy. For this to occur, the patient must be reconstituted with a high proportion of stem cells that have been transduced with a functional ADA gene and the progeny of the transduced stem cells must express adequate levels of ADA activity.

Recombinant retroviruses have been used to introduce functional human ADA cDNA sequences into various types of cultured cells including human diploid fibroblasts (3), murine fibroblast cell lines (4–8), human lymphoid cell lines (5, 9, 10), and primary cultures of murine hematopoietic cells (7–11). Expression of ADA has been demonstrated in these systems from vectors that contain a variety of different transcriptional elements (3–11). However, transfer of a functional human ADA gene into the appropriate target cell, the hematopoietic stem cell, has been more difficult to demonstrate. Studies to date have primarily used a murine system in which lethally irradiated animals are reconstituted with retrovirally infected bone marrow cells. In such studies, human ADA DNA sequences and variable levels of enzyme activity have been detected 2 weeks after transplantation but, with one exception, no expression could be detected in long-term reconstituted animals (5–8). A recent study by Belmont *et al.* (8) demonstrated human ADA activity in tissues from animals 122 days after transplantation. However, the presence of replication-competent virus in the plasma of these animals made it difficult to determine whether the observed expression was dependent on stem-cell infection or simply due to the persistent reinfection of progenitor cells. In this report, we have used improved vectors and packaging cell lines to demonstrate activity of human ADA in animals engrafted for long periods of time.

## MATERIALS AND METHODS

**Recombinant Retroviruses.** Retroviral vectors designed to express human ADA (see Fig. 1) were transfected into the ecotropic packaging cell line $\psi$ cre as described (12) and selected for high level expression of ADA by the method of Kaufman *et al.* (13). Individual virus-producing clones were isolated and tested (25 clones per vector) for production of virus that transmitted the correct proviral structure by Southern analysis (12, 14). Supernatants from virus-producing cells and plasma from transplant recipients were tested for the presence of replication-competent virus and packaging of the $\psi$ genome by a modification of a previously described mobilization assay (13). The assay used in our study detected mobilization of a recombinant retroviral genome that expresses *Escherichia coli* $\beta$-galactosidase (15).

**DNA and RNA Analyses.** DNA and RNA were prepared and analyzed as described (16). Hybridization filters were probed with a 400-base-pair human ADA cDNA fragment [*Bgl* II to *Hin*dIII of mADA kindly provided by S. Orkin (17)] that was labeled to high specific activity with [$^{32}$P]dCTP by the random-primer method (18).

**ADA Enzyme Analysis.** Lysates were prepared by freeze–thawing the cells in 10 mM Tris·HCl (pH 7.5) containing 75 mM NaCl four times followed by centrifugation (10,000 × *g* for 5 min) to remove debris and were subjected to nondenaturing isoelectric focusing (IEF) or were analyzed for total ADA activity. Nondenaturing IEF was performed with ampholytes obtained from LKB (pH 4–6) as described (19). IEF gels were analyzed for the presence of ADA enzyme activity and human ADA immunoreactivity. ADA enzyme activity

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ADA, adenosine deaminase; Mo-MLV, Moloney murine leukemia virus; LTR, long terminal repeat; IEF, isoelectric focusing; cfu, colony-forming unit(s).

†Present address: Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, MI 48109.

was detected by using a previously described overlay stain (20) while human ADA immunoreactivity was detected by electrophoretically transferring proteins from the gel to nitrocellulose paper and localizing immunoreactive proteins through the use of monospecific rabbit polyclonal antibody made to purified human ADA (kindly provided by M. S. Coleman) and alkaline-conjugated goat anti-rabbit antiserum purchased from Vector Laboratories and used according to the recommendation of the manufacturer.

Lysates were also analyzed quantitatively for total ADA activity (21) and total protein (Bio-Rad protein assay according to the recommendations of the manufacturer). Specific activity of ADA is presented as nmol of inosine produced per min per mg of total protein (21).

**Murine Bone Marrow Transplantation.** Infection and transplantation of bone marrow was performed by a slightly modified version of a previously described protocol (22). C3H/HeJ mice (7–12 weeks old, The Jackson Laboratory) were injected with 5-fluorouracil (150 mg/kg body weight) and bone marrow was harvested 5 days after injection. Bone marrow cells ($5 \pm 10^6$ cells per 10-cm plate of virus-producing cells) were cocultivated with producer cells for 48 hr in Dulbecco's modified Eagle's medium containing 10% (vol/vol) calf serum (GIBCO), penicillin/streptomycin, Polybrene (8 μg/ml), and 20% (vol/vol) WEHI-3 conditioned medium, and subsequently injected i.v. via the tail vein ($2.5 \times 10^6$ cells per recipient animal) into lethally irradiated (1100 rad; 1 rad = 0.01 Gy) C3H/HeJ male mice (7–20 weeks old).

## RESULTS

**Recombinant Retroviruses.** Two different retroviral vectors that express human ADA from cDNA sequences were constructed and tested (Fig. 1*A*). Vector pEM-ADA produces a single transcript that is responsible for viral passage and expression of ADA; transcription is initiated at the 5′ long terminal repeat (LTR) and in recipient cells is enhanced by sequences derived from the myeloproliferative sarcoma virus (24). Expression of ADA from the vector pBA-ADA is promoted by a chicken β-actin transcriptional element (25) located within the proviral transcriptional unit. Enhancer sequences were deleted from the 3′ LTR of pBA-ADA so as to reduce transcription from the 5′ LTR after reverse transcription and integration of the recombinant provirus.

Virus-producing cell lines were generated for each vector by using the improved ecotropic cell line ψ cre (12). Viral stocks from individual producer cell lines were harvested and used to infect NIH 3T3 cells to determine the efficiency of transmission of the recombinant genomes and the level of expression of ADA. The efficiency and fidelity of provirus transmission was assessed by digesting cellular DNA with a restriction endonuclease that has unique recognition sites in the LTR and by analyzing the digest according to the method of Southern (14) using ADA cDNA as a probe (Fig. 1*B*, DNA). Southern blot analysis of infected populations of NIH 3T3 cells indicated that each virus transmitted the correct proviral structure. However, the efficiency of provirus transmission differed; NIH 3T3 cells infected with the pEM-ADA virus had one provirus per cell, while those infected with the pBA-ADA virus had 5–10 proviruses per cell. Based on comparisons to viruses carrying the selectable neomycin resistance ($neo^r$) gene, these titers correspond to $1 \times 10^6$ $neo^r$ colony-forming units (cfu)/ml and $5–10 \times 10^6$ $neo^r$ cfu/ml, respectively.

Northern blot analysis of RNA isolated from cells infected with the pEM-ADA virus revealed a single band of 2.3 kilobases that represents a full-length viral transcript initiated at the 5′ LTR (Fig. 1*B*, RNA). The predominant RNA species in cultures infected with the pBA-ADA virus was the transcript initiated at the internal promoter (Fig. 1*B*, RNA). As expected, little transcription was initiated from the LTR of the pBA-ADA virus since the enhancer deletion in the 3′ LTR of the vector is transferred to the 5′ LTR of the provirus.

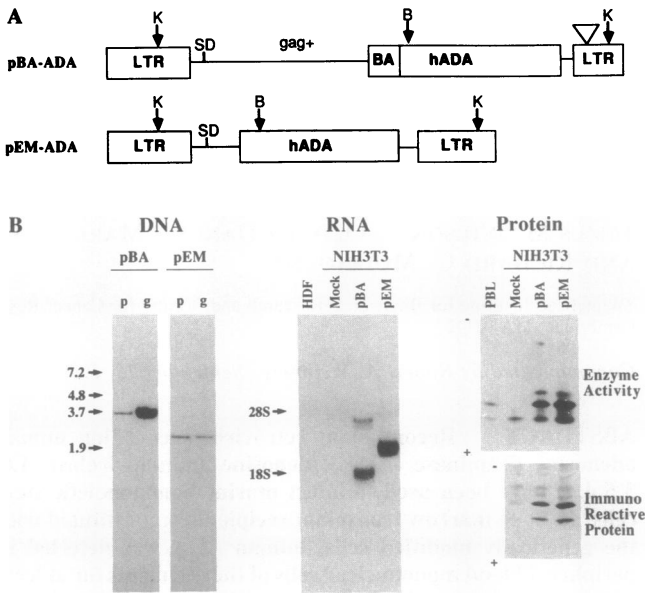


FIG. 1. Structure and characterization of retroviral vectors that express human ADA. (*A*) Structure of vectors. pEM-ADA and pBA-ADA are derived from previously described vectors LTR-LDLR and BA-LDLR, (16); the coding sequence for low density lipoprotein receptor (LDLR) in the original vectors have been replaced with sequences derived from a human ADA cDNA clone that span from the *Nae* I site at +20 to the *Acc* I site at +1120 (17). Sequences containing the Mo-MLV enhancer in the 3′ LTR (from the *Pvu* II site at nucleotide 7933 to the *Xba* I site at nucleotide 8111; see ref. 23 for numbering) have been deleted in the vector pBA-ADA and have been replaced with the homologous sequences from the LTR of the myeloproliferative sarcoma virus in the pEM-ADA vector (24). SD indicates the Mo-MLV splice donor site, and gag+ indicates the presence of additional Mo-MLV sequences that contain gag-coding sequence. BA, promoter from chicken β-actin (24); hADA, sequences from hADA cDNA; LTR, viral LTR sequences; K, *Kpn* I recognition site; B, *Bam*HI recognition site. (*B*) DNA, RNA, and ADA protein analyses of NIH 3T3 cells infected with recombinant retroviruses. DNA: high molecular weight DNA was prepared, digested with *Kpn* I, and analyzed by the method of Southern (14) using a human ADA cDNA probe. Lanes p, DNA (10 μg) from mock-infected cells supplemented with 10 pg of the appropriate retroviral plasmid (this represents approximately one copy of plasmid per cell); lanes g, genomic DNA (10 μg) isolated from infected populations of NIH 3T3 cells. RNA: total cellular RNA was isolated, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose paper as described (12); filters were hybridized with the ADA cDNA probe described above. Each lane contains 10 μg of RNA: lane HDF, human diploid fibroblasts; lane Mock, NIH 3T3 cells that were mock-infected; lanes pBA and pEM, NIH 3T3 cells that were infected with the pBA-ADA virus or the pEM-ADA virus, respectively. Protein: lysates were prepared and subjected to nondenaturing IEF in polyacrylamide gels, which were analyzed for ADA enzyme activity or immunoreactive protein. Each lane contains 75 μg of total protein: lane PBL, human peripheral blood mononuclear cells isolated on a Ficoll/Hypaque gradient; lane Mock, NIH 3T3 cells that were mock-infected, lanes pBA and pEM, NIH 3T3 cells that were infected with the pBA-ADA virus or pEM-ADA virus, respectively.

Human ADA enzyme was specifically detected in infected populations of cells by subjecting lysates to nondenaturing IEF, which separates the more basic human enzyme from the mouse enzyme (Fig. 1*B*, protein). Analysis of these gels for *in situ* enzyme activity indicated that the single mouse isozyme is clearly resolved from the major human isozyme. However, a variable fraction of the human enzyme comigrates with the mouse enzyme (Fig. 1*B*, protein, lane PBL vs. lane Mock) and, in overloaded gels, a more basic form of the human enzyme is seen (Fig. 1*B*, protein, lanes pBA and

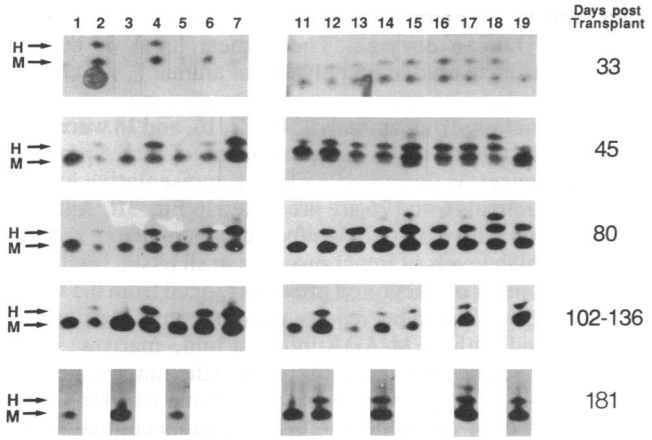


FIG. 2. Analysis of transplant recipients for human ADA activity. Recipient animals were phlebotomized retroorbitally at various times after the transplant (noted along left column) and analyzed for ADA activity as described in Fig. 1. Numbered lanes represent individual animals. H, predominant human isozyme; M, mouse isozyme.

pEM). Analysis of duplicate IEF gels for ADA immunoreactive protein, using a monospecific polyclonal antibody to human ADA, revealed a pattern consistent with the *in situ* activity assay (Fig. 1*B*, protein). The predominant form of ADA comigrates with the basic human isozyme; a less abundant form comigrates with the mouse enzyme.

While inspection of the relative band intensities in Fig. 1*B* suggested that the level of human ADA activity was 2- to 3-fold greater than the level of endogenous mouse enzyme, quantitative enzyme assay of lysates for ADA (24) indicated the level of expression to be 8-fold [412 ± 53 ($n$ = 4) nmol·min$^{-1}$·mg$^{-1}$] and 11-fold [580 ± 78 ($n$ =5) nmol·min$^{-1}$·mg$^{-1}$] greater than the endogenous level [54 ± 8 ($n$ = 4) nmol·min$^{-1}$·mg$^{-1}$] for cells infected with pBA-ADA and pEM-ADA, respectively. Recent studies have confirmed that the *in situ* activity assay, as described here, cannot be used to accurately quantify levels of the human enzyme.

**Expression of Human ADA in Hematopoietic Stem Cells *in Vivo*.** To generate mice reconstituted with hematopoietic cells expressing the human ADA gene, bone marrow cells from C3H/HeJ female mice were infected *in vitro* with the pEM-ADA or pBA-ADA virus and injected into the tail vein of 20 lethally irradiated mice (animals 1–10 were injected with pEM-ADA infected cells while animals 11-20 were injected with pBA-ADA infected cells). Animals 8–10 and 20 died 12–16 days after the transplant, most likely because of inadequate hematopoietic reconstitution. Peripheral blood mononuclear cells were harvested from transplant recipients at various times after the transplant (i.e., 33, 45, 80, 102–136, and 181 days) and analyzed for the presence of human ADA enzyme (Fig. 2). Several important patterns emerged. Human ADA activity was detected at 33 days in 9 of 9 animals reconstituted with pBA-ADA transduced cells and in 2 of 7 animals reconstituted with pEM-ADA transduced cells. Furthermore, the relative level of expression detected at 33 days remained essentially unchanged during the course of this study (181 days). In several cases, no data regarding human ADA expression at day 33 posttransplant was available, since the lack of mouse enzyme indicated that too little extract was loaded for proper analysis (e.g., see Fig. 2, lanes 1, 3, 5, and 7). Analysis of enzyme activity at day 45 indicated that 4 of 7 animals reconstituted with pEM-ADA-infected cells expressed human ADA enzyme.

Selected animals reconstituted with cells infected with the pEM-ADA virus (animals 2, 4, 6, and 7) and the pBA-ADA virus (animals 16 and 18) were sacrificed between 108 and 123 days after the transplant, and various tissues were analyzed for proviral sequences and human ADA activity. These tissue fractions included peripheral blood erythrocytes and mononuclear cells, adherent and nonadherent spleen cells, thymocytes, and cells from lymph nodes and bone marrow. Analyses of these fractions for human ADA activity are presented in Fig. 3*A*. Human ADA enzyme activity can be detected to a variable degree in virtually all tissues of each animal studied. The ratio of human ADA to endogenous mouse ADA was consistently highest in peripheral blood erythrocytes and mononuclear cells; in the case of animal 7, the human activity exceeded the endogenous activity in both fractions. Notable exceptions to the general pattern of expression included animal 2, in which a high level of ADA was found in thymocytes, and animal 7, in which a high level of enzyme was found in bone marrow cells.

The most basic isozyme observed in overloaded activity gels of infected NIH 3T3 lysates (Fig. 1*B*) was also seen in tissues from a single animal (mouse 18); it was detected in peripheral blood mononuclear cells at various times after the transplant (Fig. 2) and in each tissue fraction that was analyzed at day 123 (Fig. 3*A*). The basis for an animal restricted heterogeneity in ADA structure remains unclear.

Selected extracts were quantitatively analyzed for total ADA enzyme activity in an attempt to quantify the level of human ADA (Table 1). Marked tissue-specific variation in the level of endogenous ADA was noted ranging from 4.6 nmol·min$^{-1}$·mg$^{-1}$ in erythrocytes to 531 nmol·min$^{-1}$·mg$^{-1}$ in thymocytes. This is in agreement with previous studies on the tissue distribution of ADA (26). However, while the absolute amount of human ADA measured in this analysis was qualitatively similar to the amount estimated by the *in situ* gel

Table 1. ADA enzyme activity in tissue fraction of transplant recipients

| Animal | Specific activity of ADA, nmol·min$^{-1}$·mg$^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| | RBC | WBC | Spleen | Thymus | Lymph nodes | Bone marrow |
| Control | | | | | | |
| 1 | 5.8 ± 0.4 (3) | 14 (2) | — | 656 ± 80 (3) | 153 (1) | 85 ± 6 (2) |
| 2 | 4.8 ± 0.4 (3) | — | 40 (1) | 406 ± 11 (2) | 140 ± 15 (4) | 106 ± 21 (2) |
| 3 | 3.1 ± 1.1 (3) | — | 42 (1) | — | 135 ± 11 (2) | 62 ± 16 (2) |
| Average | 4.6 ± 1.0 (3) | | 41 (2) | 531 ± 125 (2) | 143 ± 10 (3) | 84 ± 18 (3) |
| Experimental | | | | | | |
| 2 | 10.5 ± 2.1 (3) | — | — | — | 185 ± 1 (2) | 64 ± 20 (2) |
| 4 | 8.4 ± 1.2 (3) | — | 44 ± 5 (3) | — | 128 ± 11 (4) | 88 ± 14 (2) |
| 6 | 7.3 ± 1.1 (3) | — | 71 ± 3 (2) | — | — | — |
| 7 | 45 ± 9 (2) | 29 (1) | 67 ± 4 (3) | — | 169 ± 10 (4) | 155 (1) |
| 16 | 5.1 ± 0.7 (3) | — | 33 ± 2 (2) | — | 182 ± 32 (4) | 81 ± 18 (2) |
| 18 | — | — | 44 ± 6 (3) | 370 ± 40 (3) | 183 ± 19 (2) | 111 ± 25 (2) |

RBC, erythrocytes from peripheral blood, WBC, mononuclear cells from peripheral blood. Other tissue fractions are described in the legend to Fig. 3. Each value represents the mean ±1 SD for the number of determinations in parentheses.

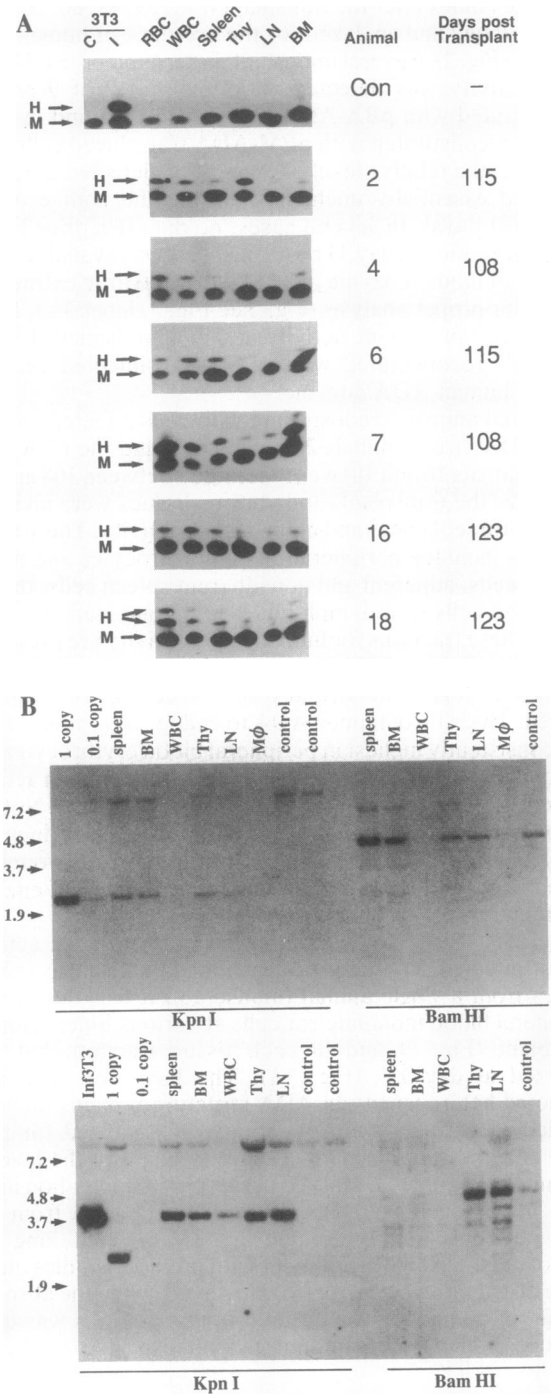


FIG. 3. ADA activity and proviral sequences in hematopoietic tissues of long-term reconstituted mice. At selected times after the transplant, individuals animals were sacrificed and tissues were harvested. Peripheral blood was fractionated on a Ficoll/Hypaque gradient into the erythrocyte fraction and mononuclear fraction. Cell suspensions were made from spleen, thymus, lymph nodes, and bone marrow. Spleen cells were further separated into nonadherent and adherent fractions as described (25). Each fraction was analyzed for total viable cells and aliquoted for preparation of protein lysates as well as high molecular weight ADA as described in the legend to Fig. 1. (*A*) Analysis of ADA activity. Individual animals (designated as control or by the appropriate number under the column labeled Animal) were harvested at the indicated time after transplant and the following fractions were analyzed for ADA activity as described in the legend to Fig. 1: RBC, hemolysate (300 $\mu$g of protein); WBC, peripheral blood mononuclear cells ($2.5 \times 10^6$ cells, 40 $\mu$g of protein); Spleen, nonadherent spleen cells ($5 \times 10^6$ cells, 80 $\mu$g of protein); Thy, thymocytes ($2.5 \times 10^6$ cells, 10 $\mu$g of protein); LN, lymph node suspension ($2.5 \times 10^6$ cells, 15 $\mu$g of protein); BM, bone marrow cells ($2.5 \times 10^6$ cells, 50 $\mu$g of protein). NIH 3T3 cells mock infected (lane 3T3/C) or infected with the pBA-ADA virus (lane 3T3/I) were also analyzed. H, human isozyme; M, mouse isozyme. (*B*) Analysis of proviral genomes. High molecular weight DNA was isolated from tissue fractions of each animal in *A*, digested with *Kpn* I or *Bam*HI (indicated below each autoradiograph), and analyzed by the method of Southern (19) for sequences complementary to human ADA cDNA (see legend to Fig. 1 for details). Analyses of animals 6 (*Upper*) and 16 (*Lower*) are presented. DNA from the following fractions was analyzed (10 $\mu$g per lane): spleen, nonadherent spleen cells; BM, bone marrow cells, WBC, peripheral blood mononuclear cells; Thy, thymocytes; LN, lymph node suspension; M$\phi$, adherent spleen cells or macrophages. Additional samples include DNA from NIH 3T3 cells that contains 1 provirus per cell (lane 1 copy) or 0.1 provirus per cell (lane 0.1 copy) of the pEM-ADA provirus or that had been infected with the pBA-ADA virus (lane Inf3T3). Lanes labeled control contain DNA from nonadherent spleen cells of mock-transfected animals. Selected molecular size markers (expressed in kilobases) are noted on the left.

stain, the latter analysis consistently underestimated the amount of human enzyme. The highest level of human enzyme was measured in hemolysate of animal 7, which had 10 times the level of endogenous mouse enzyme.

Tissue fractions from animals 2, 4, 6, 7, 16, and 18 were also analyzed for the presence and abundance of intact proviral DNA sequences as described earlier. Representative experiments for animals 6 and 16 are presented in Fig. 3*B*. Animals reconstituted with pBA-ADA-infected bone marrow consistently had high levels of intact provirus in all tissue fractions; the copy number of integrated provirus ranged from 0.5 to 1.0 provirus per cell. Similar results were obtained for animals reconstituted with pEM-ADA-infected bone marrow; however, the efficiency was generally lower (the range was from undetectable to 0.5 provirus per cell). Tissue fractions were also analyzed for cell populations that contain common sites of proviral integration. This was accomplished by digesting the DNA with a restriction endonuclear that has a single recognition site in the provirus (Fig. 1*A*) and analyzing the digest by the method of Southern (14) using human ADA cDNA as the probe. Populations of cells that have identical sized bands in the Southern blot are presumably derived from a common stem cell. As shown in Fig. 3*B*, most tissue fractions are populated with cells that contain a limited number of common proviral integrants, which are represented in most fractions. This provides evidence for the reconstitution of these animals with a small number of transduced stem cells. We have also performed secondary transplantation studies with marrow from primary recipients engrafted with genetically modified cells. Human ADA activity was detected in reconstituted recipients at levels comparable to those in the primary recipients (data not shown).

Because of the importance in documenting the absence of helper virus both in the virus stocks used for the bone marrow cell infection and in the plasma from transplanted animals, we first compared two methods for the detection of wild-type virus in terms of relative sensitivity. For these assays, a Moloney murine leukemia virus (Mo-MLV) virus stock previously determined to contain $>10^6$ infectious particles per ml by the XC assay was used (27). Dilutions of the virus were applied to (*i*) wild-type NIH 3T3 cells, which were subsequently overlaid with XC cells and analyzed for foci of syncitium (27), and (*ii*) NIH 3T3 cells harboring a single copy of a recombinant retroviral genome encoding *E. coli* $\beta$-galactosidase, which were subsequently analyzed for mobilization of the $\beta$-galactosidase genome. The results of the assays indicated that in the XC assay, a $10^6$ dilution of virus yielded detectable XC plaques, while a $10^7$ dilution yielded no plaques. In contrast, the $\beta$-galactosidase assay was more sensitive, yielding 50 blue colonies per plate when cells were infected with a $10^7$ dilution of virus. Accordingly, all virus and

sera samples were tested by the β-galactosidase assay. In every case, no infectious virus or transmission of packaging functions could be detected in either the virus used for the infections or the plasma from transplanted animals.

## DISCUSSION

Previous attempts by us and others to express cDNAs in animals reconstituted with retrovirus-infected bone marrow have failed to convincingly demonstrate consistent and high level expression when analyzed at various times after bone marrow transplantation (5–7, 28–34). While these problems in expression have been ascribed both to the inactivity of vector transcriptional signals and difficulties in obtaining stem-cell transduction, the relative importance of these two factors has not been carefully assessed. In the current studies, we attempted to address the issue by comparing the performance of two different types of retrovirus vectors (pBA-ADA and pEM-ADA), which differ both in the transcriptional signals used to promote ADA expression and in the viral titers obtainable.

Both vectors yielded stable expression of ADA sequences, albeit at different levels. The pEM-ADA vector yielded comparatively large amounts of human enzyme, particularly in animals engrafted by efficiently transduced cells. An important finding was that both vectors appeared to function as well in the progeny of infected stem cells as they did in murine fibroblasts. For example, the level of expression of the pBA-ADA provirus, as measured by the ratio of human ADA enzyme activity per proviral copy number was 500 $nmol \cdot min^{-1} \cdot mg^{-1}$ per provirus in NIH 3T3 cells and 400 and 77 $nmol \cdot min^{-1} \cdot mg^{-1}$ per provirus in erythrocytes and bone marrow cells of animal 7. (Calculated as the ratio of total ADA activity divided by the proviral copy number as determined by Southern analysis. A proviral copy number equal to 0.1 copy per cell was used for all tissues of animal 7.)

Previous studies that purported to demonstrate the inactivity of the Mo-MuLV transcriptional unit and a variety of internal promoters have rarely distinguished the transfer of proviral sequences into reconstituting stem cells from the transfer of sequences into shorter lived progenitor cells (28, 29, 31–33). In light of the studies reported here and those of Bowtell *et al.* (35), in which a vector similar to pEM-ADA was used, it is likely that the inability to efficiently transduce reconstituting stem cells accounts for a majority of expression problems seen by other workers. It is important to point out that the pBA-ADA viruses used in these studies possessed titers equivalent to $5 \times 10^6$–$10^7$ neo cfu/ml. Such high titers are not commonly obtained with recombinant genomes and, consequently, many recombinant virus stocks may not be suitable for efficient hematopoietic stem-cell infection. Alternatively, in the case of LTR-based vectors, the choice of viral enhancer could be important.

These studies have important implications for future gene expression studies in hematopoietic cells. In addition, our results represent an important step in the development of gene replacement therapy for ADA deficiency. We specifically demonstrated reproducible and efficient infection of murine hematopoietic stem cells and expression of human ADA enzyme in recipient animals long after transplantation. No helper virus or transfer of packaging functions was detectable in either the virus stocks used for the infection or the serum from transplant recipients. An important question is whether the level and distribution of ADA expression obtained with the vectors studied here will effectively restore immune function in ADA-deficient patients. Particularly in the case of enzyme-deficient patients with an absolute deficiency of ADA, whose activity shows a poor response to enzyme replacement (3), it may be necessary to express the ADA gene at developmentally appropriate levels in stem cells and/or during T-cell ontogeny when levels are highest (2).

We thank B. Guild and P. Robbins for plasmids, R. Kellems for help with the ADA selection system, E. Leung and G. Parsons for technical assistance, S. H. Orkin for review of the manuscript, and B. Mitchell for help with the ADA assay. This work was supported by the National Institutes of Health (D.H.R. and R.C.M.); Foundation pour la Recherche Medicale, National Institutes of Health–Centre National de la Recherche Scientifique Program for Scientific Collaboration, and the European Molecular Biology Organization (O.D.); and the Howard Hughes Medical Institute (J.M.W.).

1. Belmont, J. W., Henkel-Tigges, J., Wager-Smith, K., Chang, S. M. W. & Caskey, C. T. (1986) *Ann. Clin. Res.* **18,** 322–326.
2. Kredich, N. H. & Herschfield, M. S. (1983) in *The Metabolic Basis of Inherited Diseases*, eds. Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S. (McGraw–Hill, New York), 5th Ed., pp. 1157–1183.
3. Palmer, T. D., Hock, R. A., Osborne, W. R. A. & Miller, A. D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1055–1059.
4. Valerio, D., Duyvesteyn, M. G. C. & van der Eb, A. J. (1984) *Gene* **34,** 163–168.
5. Williams, D. A., Orkin, S. H. & Mulligan, R. C. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2566–2570.
6. McIvor, R. S., Johnson, M. J., Miller, A. D., Pitts, S., Williams, S. R., Valerio, D., Martin, D. W., Jr., & Verma, I. M. (1987) *Mol. Cell. Biol.* **7,** 838–846.
7. Lim, B., Williams, D. A. & Orkin, S. H. (1987) *Mol. Cell. Biol.* **7,** 3459–3465.
8. Belmont, J. W., MacGregor, G. R., Wager-Smith, K., Fletcher, F. A., Moore, K. A., Hawkins, D., Villalon, D., Chang, S. M.-W. & Caskey, C. T. (1988) *Mol. Cell. Biol.* **8,** 5116–5125.
9. Friedman, R. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 703–707.
10. Kantoff, P. W., Kohn, D. B., Mitsuya, H., Armentano, D., Sieberg, M., Zwiebel, J. A., Eglitis, M. A., McLachlin, J. R., Wiginton, D. A., Hutton, J. J., Horowitz, S. D., Gilboa, E., Blaese, R. M. & Anderson, W. F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6563–6567.
11. Belmont, J. W., Henkel-Tigges, J., Chang, S. M. W., Wager-Smith, K., Kellems, R. E., Dick, J. E., Magli, M. C., Phillips, R. A., Bernstein, A. & Caskey, C. T. (1986) *Nature (London)* **322,** 385–387.
12. Danos, O. & Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6460–6464.
13. Kaufman, R. J., Murtha, P., Ingolia, D. E., Yeung, C.-Y. & Kellems, R. E. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3136–3140.
14. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
15. Price, J., Turner, D. & Cepko, C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 156–160.
16. Wilson, J. M., Johnston, D. E., Jefferson, D. M. & Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4421–4425.
17. Orkin, S. H., Goff, S. C., Kelley, W. N. & Daddona, P. E. (1985) *Mol. Cell. Biol.* **5,** 762–767.
18. Feinberg, A. P. & Vogelstein, B. (1984) *Anal. Biochem.* **132,** 6–13.
19. Wilson, J. M., Baugher, B. W., Mattes, P. M., Daddona, P. E. & Kelley, W. N. (1982) *J. Clin. Invest.* **69,** 706–715.
20. Yeung, C.-Y., Ingolia, D.E., Bobonis, C., Dunbar, B. S., Riser, M. E., Sicilianol, M. J. & Kellems, R. E. (1983) *J. Biol. Chem.* **258,** 8338–8345.
21. Van der Weyden, M. B., Buckley, R. H. & Kelley, W. N. (1974) *Biochem. Biophys. Res. Commun.* **57,** 590–595.
22. Lemischka, I. R., Raulet, D. H. & Mulligan, R. C. (1986) *Cell* **45,** 917–927.
23. Van Beveren, C., Coffin, J. & Hughes, S. (1985) in *RNA Tumor Viruses*, eds. Weiss, R., Teich, N., Varmus, H. & Coffin, J. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed. pp. 766–783.
24. Stacey, A., Arbuthnott, C., Kollek, R., Coggins, L. & Ostertag, W. (1984) *J. Virol.* **50,** 725–732.
25. Kost, T. A., Theodorakis, N. & Hughes, S. N. (1983) *Nucleic Acids Res.* **11,** 8287–8301.
26. Van der Weyden, M. B. & Kelley, W. N. (1976) *J. Biol. Chem.* **251,** 5448–5456.
27. Rowe, W. P., Pugh, W. B., & Hartley, J. W. (1970) *Virology* **42,** 1136–1139.
28. Miller, A. D., Eckner, R. J., Jolly, D. J., Friedman, T. & Verma, I. M. (1984) *Science* **225,** 630–632.
29. Keller, G., Paige, C., Gilboa, E. & Wagner, E. F. (1985) *Nature (London)* **318,** 149–154.
30. Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A. & Bernstein, A. (1985) *Cell* **42,** 71–79.
31. Eglitis, M. A., Kantoff, P., Gilboa, E. & Anderson, W. F. (1985) *Science* **230,** 1395–1398.
32. Kantoff, P. W., Gillio, A. P., McLachlin, J. R., Bordignon, C., Eglitis, M. A., Kernan, N. A., Moen, R. C., Kohn, D. B., Yu, S.-F., Karson, E., Karlsson, S., Zwiebel, J. A., Gilboa, E., Blaese, R. M., Nienhuis, A., O'Reilly, R. J. & Anderson, W. F. (1987) *J. Exp. Med.* **166,** 219–234.
33. Magli, M. C., Dick, J. E., Huszar, D., Bernstein, A. & Phillips, R. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 789–793.
34. Stead, R. B., Kwok, W. W., Storb, R. & Miller, A. D. (1988) *Blood* **71,** 742–747.
35. Bowtell, D. D. L., Johnson, G. R., Kelson, A. & Corey, S. (1987) *Mol. Biol. Med.* **4,** 229–250.