Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2566–2570, April 1986
Genetics

Retrovirus-mediated transfer of human adenosine deaminase gene sequences into cells in culture and into murine hematopoietic cells *in vivo*

DAVID A. WILLIAMS†‡, STUART H. ORKIN‡, AND RICHARD C. MULLIGAN†

†Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02138; and ‡Division of Hematology-Oncology, The Children's Hospital and The Dana-Farber Cancer Institute, Department of Pediatrics, Harvard Medical School, Boston, MA 02115

Communicated by Tom Maniatis, December 12, 1985

ABSTRACT **Deficiency of the enzyme adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4; ADA) leads to severe combined immunodeficiency, a disorder that potentially could be corrected by gene transfer into hematopoietic cells. We have constructed retroviruses containing human ADA cDNA and a dominant selectable marker, a mutated dihydrofolate reductase gene (*DHFR**) encoding methotrexate resistance. Human ADA cDNA was inserted alone (DHFR*-ADA) or with a simian virus 40 (SV40) promoter (DHFR*-SVADA). Although NIH 3T3 cells infected with either construct produced human ADA activity, substantially greater levels were attained with DHFR*-SVADA. Infection of murine lymphoid cells in culture with DHFR*-SVADA led to expression of human enzyme at a level well above the mouse endogenous level. ADA activity was also increased after infection of a human ADA-deficient B-cell line. Lethally irradiated mice that were reconstituted with syngeneic marrow infected with the DHFR*-SVADA virus contained unrearranged, integrated proviral DNA in total spleen DNA or in spleen hematopoietic stem cell (CFU-S)-derived colonies. Nevertheless, no human ADA was detectable. RNA analysis showed relatively low and variable expression from the retroviral long terminal repeat, and no detectable expression from the internal SV40 promoter. These data suggest that intrinsic biologic differences exist between cultured cells and CFU-S *in vivo*.**

Severe combined immunodeficiency disease due to the deficiency of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4; ADA) (1) appears to be an ideal candidate for somatic genetic therapy. In this disorder, ADA deficiency leads to destruction of immature T lymphocytes due to accumulation of deoxyadenosine and dATP (1). Because the effects of this deficiency are primarily restricted to marrow-derived cells, the introduction of cloned and expressible human ADA cDNA (2) into the hematopoietic stem cells of patients with severe combined immunodeficiency disease and the subsequent autologous transplantation of the genetically modified cells might offer a new therapy for this severe disorder.

Because of the paucity of hematopoietic stem cells in normal bone marrow cell populations, conventional methods for gene transfer (3), which are inherently inefficient, would not appear to be appropriate for introducing a functional *ADA* gene into a substantial fraction of stem cells. However, retroviral vectors potentially offer an ideal means of gene transfer in this regard, since highly transmissible recombinant retroviruses have been shown to infect a wide variety of cells in culture efficiently and to yield single-copy proviral sequences stably integrated in host chromosomal DNA (4).

As a first step in the development of potential strategies for the correction of genetic disorders whose effects are primarily limited to bone marrow-derived cells, we have shown that retroviral vectors can be used to introduce new DNA sequences into a substantial fraction of murine hematopoietic stem cells (CFU-S) (5) and that the transduced stem cells can permanently and completely reconstitute the hematopoietic system of lethally irradiated recipients (I. R. Lemischka, D. Raulet, and R.C.M., unpublished data). Here we report our initial efforts to transfer human *ADA* sequences into both cultured cells and murine hematopoietic stem cells. We constructed a retroviral vector with which we demonstrate efficient transfer and expression of human ADA cDNA in both murine and human cell lines in culture and the transfer of *ADA* sequences into the CFU-S of intact mice. Expression *in vivo*, however, is greatly reduced relative to that seen in culture, which suggests an intrinsic difference between cell lines and hematopoietic progenitors.

MATERIALS AND METHODS

Construction of Retroviral Vectors and Generation of Producer Cell Lines. Construction of the retroviral vector used here, pZipDHFR* (Fig. 1), which contains a dihydrofolate reductase gene mutation encoding methotrexate resistance, will be described elsewhere. Into its unique *Xho* I restriction site, the entire coding region of ADA cDNA was introduced as a 1.1-kilobase (kb) *Nco* I–*Hin*fI fragment (2) to generate pZipDHFR*-ADA (Fig. 1). In addition, a 1.7-kb *Pvu* II–*Acc* I fragment of a pSV2-ADA construct (2), in which the simian virus 40 (SV40) early promoter is linked to the ADA cDNA, was introduced at the *Xho* I site to generate pZipDHFR*-SVADA. Recombinant plasmid DNAs were transfected into packaging cell lines $\psi 2$ (6) and ψ-AM (7) to generate helper-free viral stocks. Transfection was achieved by calcium phosphate precipitation, and transformed clones were selected in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum and 0.25 μM methotrexate. Clones producing up to 5×10^5 colony-forming units (cfu) of virus per ml ($\psi 2$) and 1×10^5 cfu/ml (ψ-AM) were obtained.

Infection of Cell Lines and Murine Bone Marrow. Infection of cultured lymphocyte lines was performed by cocultivation of $5–50 \times 10^6$ cells with a monolayer of producer cells for 24 hr in the presence of 2 μg of Polybrene per ml, followed by replating on 3 consecutive days to remove residual adherent producer cells.

Infection of murine bone marrow cells was performed as described (5) with the following modifications: donor mice (CBA/J males, 8–12 weeks) were treated with 150 mg of 5-fluoruracil per kg of body weight 2 days prior to marrow harvest; cocultivation of marrow cells was carried out in the

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; DHFR, dihydrofolate reductase; SV40, simian virus 40; kb, kilobase(s); cfu, colony-forming units; LTR, long terminal repeat.

presence of 20% WEHI-3 (8) conditioned medium. Transplantation of infected bone marrow cells and the harvesting of regenerating spleen cells and individual CFU-S-derived spleen colonies was as described (5). The recipient mice received 14 Gy of total body irradiation in two doses with a minimum of 3 hr between doses.

Molecular Procedures. Isolation of DNA and RNA samples was performed by standard methods (9). S1 nuclease analysis was by the method of Favaloro *et al.* (10).

RESULTS

Generation of Transmissible ZipDHFR*-ADA and ZipDHFR*-SVADA Retroviruses. The retroviral vector ZipDHFR* contains Moloney murine leukemia viral transcriptional sequences in the long terminal repeats (LTRs), 5′ and 3′ splice donor and acceptor sequences for the production of subgenomic (spliced) RNA, DHFR* cDNA sequences that encode a mutant enzyme with lowered binding affinity for methotrexate (11), and a unique cloning site (*Xho* I) for insertion of cDNA or genomic sequences (Fig. 1). ZipDHFR*-ADA contains ADA cDNA at the *Xho* I site, whereas ZipDHFR*-SVADA contains the SV40 early region promoter and enhancer cassette (12) of the expression plasmid pSV2 plus the ADA-encoding region (2) (Fig. 1).

Cells producing ZipDHFR*-SVADA virus contain abundant human ADA activity as compared with those producing ZipDHFR*-ADA (Fig. 2). This difference was also observed with NIH 3T3 cells infected with these recombinant viruses and selected with methotrexate (not shown). The failure of cells infected with ZipDHFR*-ADA to produce larger amounts of human ADA is most likely attributable to poor utilization of the RNA splice signals within the construct as only genomic-length RNA was seen in blot-hybridization analysis (Fig. 3). Cells producing ZipDHFR*-SVADA virus (Fig. 3) or infected with it (see below) contain two RNA species that hybridize with human ADA cDNA: one of genomic length and another initiated from the internal SV40 promoter (see below). We infer that ADA protein is translated nearly exclusively from SV40-initiated RNA. Two clones of producers, ψ2 DHFR*-SVADA6 (titer, 5×10^5 cfu/ml) and ψ-AMDHFR*-SVADA20 (titer, 10^4 cfu/ml), were shown to transmit the expected proviral structure to cells and were used in our subsequent experiments.

Retroviral Transfer and Expression of Human *ADA* in Murine and Human Lymphocyte Lines in Culture. Prior to the use of the ZipDHFR*-SVADA viruses for transferring human *ADA* sequences into murine hematopoietic stem cells, we examined the ability of the virus to express in cultured mouse and human lymphoid cell lines. This constitutes a critical control in evaluating the activity of the SV40 promoter in such cells.

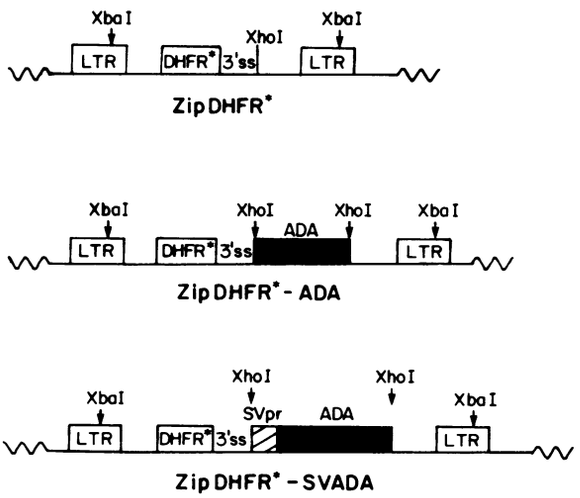

FIG. 1. Structure of the ZipDHFR* vector and ADA recombinants.

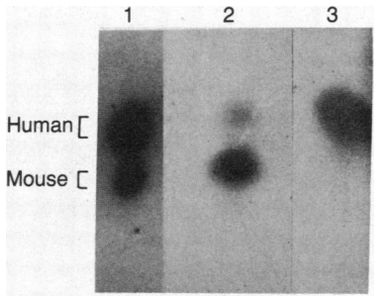

FIG. 2. Expression of human *ADA* in producer cells. Extracts of ψ2 cells producing ADA recombinant retroviruses were assayed by *in situ* starch gel analysis (2) to discriminate between human and mouse isozymes. Lanes: 1, ψ2(ZipDHFR*-SVADA; 2, ψ2(ZipDHFR*-ADA); 3, MOLT-4, a human T-cell leukemia line used as a reference for the migration of the human enzyme.

In the absence of any biochemical selection to recover transformants, infection of a murine pre-B-cell line (70Z) (13) resulted in transfer of the intact retroviral genome into 5% or less cells as judged by Southern blot analysis (data not shown). Functional human ADA was barely detectable in these unselected cells (Fig. 4). After 2 weeks of selection with methotrexate to kill uninfected cells, human ADA activity was at least 5- to 10-fold above the endogenous murine level (Fig. 4). Infection of a cultured murine thymoma (BW5127) (14) led to more efficient transfer of the retroviral genome by Southern blot analysis and to expression of human ADA enzyme at endogenous levels or above even in unselected cells (Fig. 4).

In an attempt to introduce a functional *ADA* gene into human lymphocytes derived from a patient with ADA deficiency, an ADA-deficient B-cell line (GM2471) was cocultivated with ψ-AMDHFR*-SVADA20. Even without methotrexate selection, gene transfer occurred into ≈10% of the cells, as judged by Southern blot analysis (data not shown), assuming one integration event per cell. The human ADA activity in the infected, unselected cells was considerably increased over the low level of residual enzyme activity of

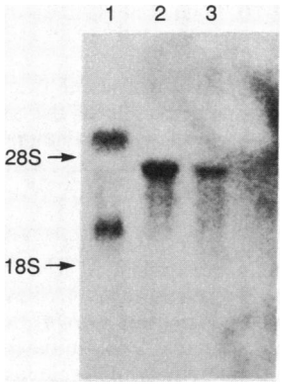

FIG. 3. Blot-hybridization analysis of ADA retroviral ψ2 producers. Total cell RNA was prepared from pooled ψ2 clones producing ZipDHFR*-SVADA (lane 1) and two clones [numbers 1 (lane 2) and 14 (lane 3)] producing ZipDHFR*-ADA. RNA (10 μg per lane) was electrophoresed in formaldehyde/agarose gels (10), transferred to nitrocellulose, and hybridized with the ADA cDNA probe. The positions of 28S and 18S ribosomal RNA species are indicated to the left.

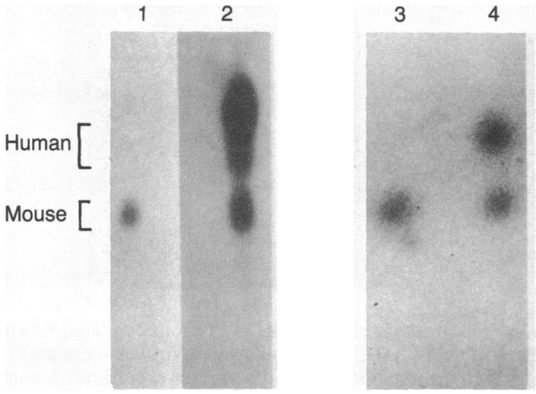

FIG. 4. Expression of human *ADA* after infection of murine lymphoid cell lines. Extracts of 70Z pre-B-cells were analyzed by starch gel analysis after infection but without selection (lane 1) or after methotrexate selection (lane 2). BW thymoma (T-) cells were studied prior to infection (lane 3) or 3 days after infection but without selection (lane 4). Cells were infected with ZipDHFR*-SVADA. Human ADA activity was weakly detectable in lane 1.

this line prior to infection [specific activity, 0.29 (prior to infection) and 7.6 (after infection) nmol/min per mg of protein].

From the infection of these cell lines, we conclude that the SV40 promoter within the ZipDHFR-SVADA construct is active in cultured lymphoid cell lines of both murine and human origin. Furthermore, in infected but not selected NIH 3T3 (not shown), BW5127, and GM2471 cell lines, the level of human ADA detected roughly approximates the percentage of cells into which we estimate proviral sequences integrate. We infer, therefore, that the increased human ADA seen in selected cells reflects enrichment for cells initially infected with retrovirus.

Transfer of Human *ADA* Sequences into Intact Mice. ψ2 cells producing ZipDHFR*-SVADA virus with a titer of 5×10^5 cfu/ml were used to transfer the retroviral genome into murine hematopoietic cells. Regenerating spleens of transplanted, lethally irradiated mice were harvested at 1 and 2 weeks after transplantation, and individual spleen colonies were isolated at 2 weeks. The efficiency of gene transfer in these experiments was similar to that seen previously (5); that is, approximately 15–40% of spleen cells contained in the ZipDHFR*-SVADA genome (Fig. 5). The entire provirus sequence was intact, as judged by Southern blot analysis of *Xba* I-digested DNA (Fig. 5 *Left*). In addition, the SV40 promoter-*ADA* region appeared structurally intact (Fig. 5 *Right*).

In spite of this relatively efficient transfer of *ADA* sequences into hematopoietic cells of the animal, no human ADA was detectable by starch gel enzyme assay in any of several retroviral DNA-positive mouse spleens (Fig. 6) or in individual CFU-S foci (B. Lim, D.A.W., and S.H.O., unpublished observations).

To investigate the lack of human ADA activity in the CFU-S-derived cells, we examined RNA from spleen cells and individual day 14 retroviral DNA-positive spleen foci. Blot-hybridization analysis (not shown) failed to detect the presence of the retroviral and SV40-initiated ADA transcripts in RNA isolated from four whole spleens, including those shown in Fig. 5. S1 nuclease assay was used to extend the sensitivity of detection and discriminate between RNA transcripts initiating from the LTR and from the SV40 promoter *in vivo*. The predicted protected DNA fragments were observed with RNA isolated from NIH 3T3 cells infected with ZipDHFR*-SVADA6 and selected with methotrexate (Fig. 7). Both the early-early and late-early SV40 transcriptional starts (13) were evident.

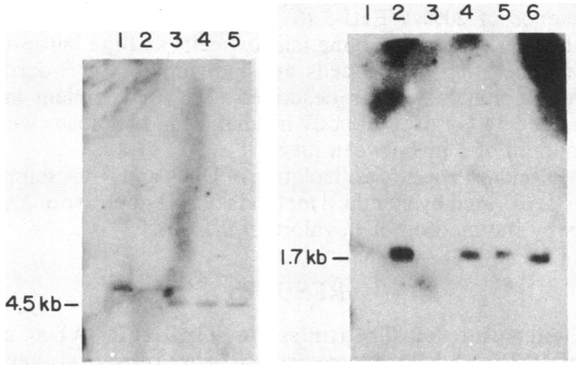

FIG. 5. Retroviral transfer of human *ADA* sequences to murine hematopoietic cells. Bone marrow was cocultured with ψ2 cells producing ZipDHFR*-SVADA and transplanted into lethally irradiated syngeneic mice. Total-spleen DNA was prepared at the specified time after transplantation, digested with either *Xba* I (*Left*) or *Xho* I (*Right*), and subjected to Southern blot analysis (9) using ADA cDNA as probe. (*Left*) Lanes: 1 and 2, negative control spleens; 3–5, CBA/J spleens harvested respectively at 2, 1, and 2 weeks after transplantation. (*Right*) Lanes: 1 and 3, negative control spleens; 2, NIH 3T3 cells infected with ZipDHFR*-SVADA and selected with methotrexate for 2 weeks (control for single-copy integration); 4–6, spleens from mice transplanted with CBA marrow cells cocultured with ZipDHFR*-SVADA6 respectively, harvested at 1, 2, and 1 weeks after transplantation.

From analysis of whole-spleen RNA and day 14 spleen colony RNA, several observations can be made. First, some RNA initiated from the LTR was detected in both types of samples (Fig. 7, lanes 5 and 6). However, the level relative to that seen in infected and selected NIH 3T3 cells is much reduced. Second, the expression from the LTR is variable, since some retroviral DNA-positive spleen RNA samples alternatively showed no expression (Fig. 7, lanes 4 and 7). Third, no RNA species initiated from the SV40 early-region promoter were clearly detected. In two instances a protected probe fragment consistent with utilization of only one of the SV40 transcriptional starts was observed. For example, in Fig. 7, lane 5, a fragment consistent with use of one of the upstream SV40 starts is apparent. In another spleen focus with single-copy integration of the ADA retrovirus (not shown), a fragment consistent with use of one of the down-

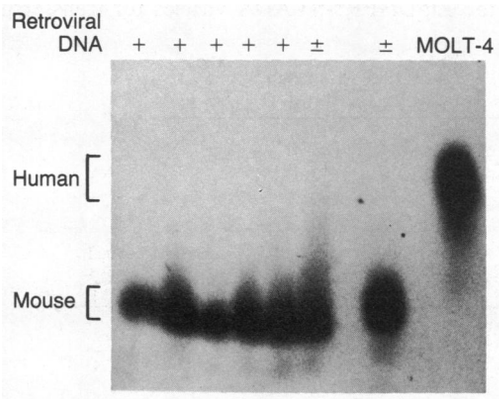

FIG. 6. ADA enzyme analysis of mouse spleens from transplanted animals. Extracts of spleen cells were assayed for enzyme in starch gel. Retroviral DNA "+" refers to the presence of the ZipDHFR*-SVADA proviral genome at 0.1 single-copy level or above. Retroviral "±" indicates the samples in which integration was <0.1 single-copy level but still detectable. Southern blot analysis of the spleens shown in lanes 3–5 from the left is presented in lanes 4–6 of Fig. 5 *Right*.

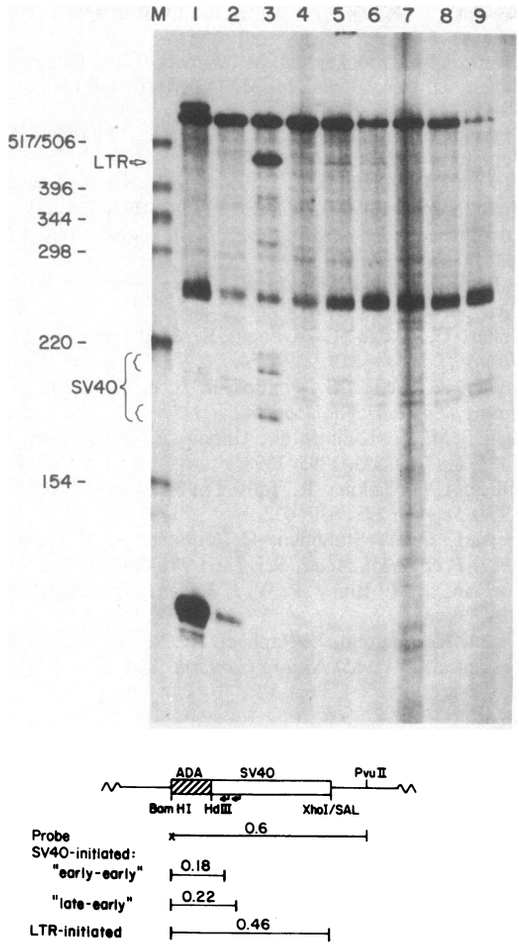

FIG. 7. S1 nuclease analysis of spleen RNAs. Total cell RNAs were prepared by guanidine hydrochloride extraction and phenol extraction (2) and were assayed with the end-labeled probe indicated in the diagram below the autoradiogram. Lanes: 1 and 2, MOLT-4 (5 μg) and HeLa (15 μg) samples (The protected fragments are shorter because of the removal of 28 nucleotides from the 5′ untranslated region of ADA cDNA in the construction of ZipDHFR*-SVADA.); 3, NIH 3T3 cells infected with ZipDHFR*-SVADA and selected with methotrexate (5 μg RNA) (The protected fragments derived from RNAs initiated from the LTR and from the SV40 early region promoter are indicated.); 4 (10 μg) and 5 (20 μg), RNAs from spleen displayed in lanes 5 and 6 of Fig. 5 *Lower*; 6 (6 μg) and 7 (10 μg), RNAs derived from spleen foci that showed single-copy integration of the proviral genome; 8 (10 μg) and 9 (10 μg), RNAs derived from retroviral DNA-negative spleens. The presence of protected fragments consistent with LTR-initiated RNA is evident in the spleen samples shown in lanes 5 and 6.

stream start sites was observed. In the absence of both sets of protected fragments expected from the SV40 promoter, we do not know the significance, if any, of these latter observations. In any event, however, expression from the retroviral transcriptional sequences is variable and apparently at a low level, and transcription from the SV40 promoter occurs at an even lower level. These findings stand in marked contrast with tissue culture experiments with the same retroviral construct in which expression from both promoters is at a high level.

DISCUSSION

The development of methods for the introduction of foreign gene sequences into hematopoietic stem cells of intact mice (5, 15) now permits systematic examination of the potential for somatic therapy of severe genetic disorders. Severe combined immunodeficiency disease due to ADA deficiency, as well as an even rarer immune deficiency due to purine nucleoside phosphorylase deficiency, has been considered a model disease for these studies principally because the condition is manifest exclusively in bone marrow-derived cells and bone marrow transplantation is curative (16). In addition, since a fraction of the normal ADA tissue level is compatible with normal immune function, precise control of the level of expression may not be critical to phenotypic correction. Our experiments demonstrate the successful transfer of *ADA* gene sequences into murine hematopoietic stem cells (CFU-S) with a new recombinant retroviral vector, pZipDHFR*-SVADA. Previously, retroviruses containing the neomycin-resistance gene and human ADA cDNA have been described that could transfer sequences and expression in culture (17, 18). The DHFR* vector has the possible advantage of containing only eukaryotic sequences, as opposed to the prokaryotic neomycin (or G418)-resistance gene. For example, it appears that prokaryotic sequences may inhibit β-globin gene expression in transgenic mice (25). The DHFR*-selectable marker may also prove useful for *in vivo* selection of hematopoietic stem cells and for *in vitro* selection of progenitor cells.

The recombinant retrovirus pZipDHFR*-SVADA efficiently transfers ADA cDNA in tissue culture to a variety of nonlymphoid and lymphoid cells of both murine and human origin and leads to the production of substantial levels of human ADA, even in the absence of selection with methotrexate *in vitro*. The retrovirus also delivers its proviral genome in an intact form to murine CFU-S. Nevertheless, *in vivo* expression of ADA mRNA initiated from an internal SV40 promoter is insufficient to generate appreciable ADA enzyme. Similarly, expression from the retroviral LTR is variable and apparently at a low level relative to that seen in NIH 3T3 cells. Preselection of CFU-S *in vitro* with methotrexate increases the proportion of DNA-positive spleen colonies, as reported by others for G418 preselection of G418-resistance (15, 19), but does not lead to production of detectable human ADA *in vivo* (B. Lim, D.A.W., and S.H.O., unpublished observations). Our findings underscore important differences in expression between that seen in tissue culture and *in vivo* with the same recombinant retrovirus. Although expression of the genes for G418 resistance (15, 19–21), hypoxanthine phosphoribosyltransferase (22), and DHFR* (D.A.W. and R.C.M., unpublished data) *in vivo* has been demonstrated, initial evidence also suggests that expression is quantitatively lower on average than that seen in NIH 3T3 cells (I. R. Lemischka, D. Raulet, R.C.M., and D.A.W., unpublished data). The level of expression of the G418-resistance and *DHFR* genes required to protect cells against drug treatment with G418 or methotrexate may be considerably less than that required to generate detectable human ADA enzyme in our experiments.

The contrast between expression in cultured cells and in murine hematopoietic cells *in vivo* most likely reflects intrinsic biologic differences in the cells themselves. Hematopoietic stem cells and their progenitors may be similar in their behavior to undifferentiated teratocarcinoma cells in which retroviral transcriptional elements and some viral enhancers function poorly (23, 24). Similarly, the SV40 early promoter and enhancer may not be optimal for expression of foreign sequences within differentiating, murine hematopoietic cells. In order to achieve high level expression of foreign sequences in these cells, it may be necessary to survey various promoters and enhancers and to examine whether specific sequences within the vectors themselves contribute to inadequate transcription.

We are grateful to Elizabeth Jensen for excellent technical assistance and Dr. David G. Nathan for his continued interest and support. We also thank Peter Daddona for his generosity in providing

quantitative ADA enzyme assays. These studies were supported by National Institutes of Health grants to S.H.O. and R.C.M.

1. Martin, D. W. & Gelfand, E. W. (1981) *Annu. Rev. Biochem.* **50,** 845–877.
2. Orkin, S. H., Goff, S. C., Kelley, W. N. & Daddona, P. E. (1985) *Mol. Cell. Biol.* **5,** 762–767.
3. Wigler, M., Pellicer, A., Silverstein, S. & Axel, R. (1978) *Cell* **14,** 725–731.
4. Cepko, C., Roberts, B. E. & Mulligan, R. C. (1984) *Cell* **37,** 1053–1062.
5. Williams, D. A., Lemischka, I. R., Nathan, D. G. & Mulligan, R. C. (1984) *Nature (London)* **310,** 476–480.
6. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33,** 153–159.
7. Cone, R. & Mulligan, R. C. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6349–6353.
8. Warner, N. L., Moore, M. A. S. & Metcalf, D. (1969) *J. Natl. Cancer Inst.* **43,** 953–962.
9. Maniatis, T., Fritsch, E. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Favaloro, J., Treisman, R. & Kamen, R. (1980) *Methods Enzymol.* **65,** 718–749.
11. Simonsen, C. C. & Levinson, A. D. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2495–2499.
12. Everett, R. D., Baty, D. & Chambon, P. (1983) *Nucleic Acids Res.* **11,** 2447–2464.
13. Paige, C. J., Kincade, P. W. & Ralph, P. (1978) *J. Immunol.* **121,** 641–647.
14. Chien, Y.-h., Becker, D. M., Linsten, T., Okamura, M., Cohen, D. I. & Davis, M. M. (1984) *Nature (London)* **312,** 31–35.
15. Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A. & Bernstein, A. (1985) *Cell* **42,** 71–79.
16. Parkman, R., Gelfand, E. W., Rosen, F. S., Sanderson, A. & Hirschhorn, R. (1975) *N. Engl. J. Med.* **292,** 714–719.
17. Friedman, R. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 703–707.
18. Valerio, D., Duyvesteyn, M. G. C. & van der Eb, A. J. (1985) *Gene* **34,** 163–168.
19. Keller, G., Paige, C., Gilboa, E. & Wagner, E. F. (1985) *Nature (London)* **318,** 149–154.
20. Joyner, A., Keller, G., Phillips, R. A. & Bernstein, A. (1983) *Nature (London)* **305,** 556–558.
21. Eglitis, M. A., Kantoff, P., Gilboa, E. & Anderson, W. F. (1985) *Science* **230,** 1395–1398.
22. Miller, A. D., Eckner, R., Jolly, D., Friedman, T. & Verma, I. (1984) *Science* **225,** 630–632.
23. Stewart, C. L., Stuhlman, H., Juhner, D. & Jaenisch, R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4098–4102.
24. Gorman, C. M., Rigby, P. W. J. & Lane, D. P. (1985) *Cell* **42,** 519–526.
25. Chada, K., Magram, J., Raphael, K., Radice, G., Lacy, E. & Costantini, F. (1985) *Nature (London)* **314,** 377–380.