Expression of Human Adenosine Deaminase in Murine Hematopoietic Cells

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Received 13 May 1988/Accepted 30 August 1988

Multiple replication-defective retrovirus vectors were tested for their ability to transfer and express human adenosine deaminase in vitro and in vivo in a mouse bone marrow transplantation model. High-titer virus production was obtained from vectors by using both a retrovirus long terminal repeat promoter and internal transcriptional units with human *c-fos* and herpes virus thymidine kinase promoters. After infection of primary murine bone marrow with one of these vectors, human adenosine deaminase was detected in 60 to 85% of spleen colony-forming units and in the blood of 14 of 14 syngeneic marrow transplant recipients. This system offers the opportunity to assess methods for increasing efficiency of gene transfer, for regulation of expression of foreign genes in hematopoietic progenitors, and for long-term measurement of the stability of expression in these cells.

Adenosine deaminase (ADA) deficiency is a rare autosomal recessive condition which causes a form of severe combined immune deficiency (33). The pathophysiology of ADA deficiency has been intensively investigated and appears to involve selective toxicity of dATP for immature T lymphocytes. Alterations in methylation via inhibition of S-adenosylhomocysteine hydrolase have also been implicated (19, 21). Human ADA is encoded at a single 35-kilobase (kb) locus composed of 12 exons on chromosome 20q13 (24, 46, 47, 59). A full-length cDNA of 1,533 base pairs (bp) has been obtained which encodes a 362-amino-acid protein of 40.7 kilodaltons (1, 2, 40). Bone marrow transplantation can be curative (22), and enzyme replacement has been demonstrated to improve immune function (20).

Genetic diseases, such as ADA deficiency, which directly affect the blood and reticuloendothelial cells and diseases in which circulating toxic metabolites accumulate might be treated by gene transfer into bone marrow. Hematopoietic stem cells would be the most desirable target for such gene transfer because of their extensive capacity for self-renewal and proliferation. Retrovirus vectors are very efficient vehicles for gene transfer into cultured mammalian cells (54) and have been used for transfer of foreign DNA sequences into hematopoietic progenitors (5, 6, 10, 15, 23, 30, 31, 35, 37, 38, 42, 44, 61) and pluripotent stem cells (13, 14, 18, 32, 36, 60). Several replication-defective vectors have allowed expression of these introduced sequences in the differentiated progeny of infected committed progenitors and stem cells (10, 13-15, 23, 30, 31, 35-37, 44, 60), while expression with other constructions has been problematic (38, 42, 61). Specific recognition by nuclear proteins or modification via methylation has been implicated in the failure of expression of coding sequences under transcriptional control of viral long terminal repeat (LTR) promoters in both embryonic cells and transgenic mice (26, 28, 48-50, 53, 54, 57, 58). It remains unclear whether this type of mechanism affects expression after retrovirus-mediated gene transfer into he-

matopoietic stem cells. It is possible that other mechanisms, such as poor RNA stability or processing, have been involved in the failure of expression. We have previously reported transduction of human ADA to mouse hematopoietic progenitors in vitro (5). Experiments designed to test the efficacy of that particular vector in more primitive progenitors, spleen colony-forming units (CFU-S), were positive (6), but the low infection efficiency precluded consistent repetition. Lim et al. (37) have also recently shown expression of ADA in murine CFU-S, but the infection efficiency was too low to allow long-term analysis of expression stability. Therefore, we have undertaken experiments to test vectors which might allow increased virus production and increased infection efficiency of hematopoietic progenitors. We also wished to investigate vector designs that might allow consistent expression of foreign sequences in these cells. We report here the construction and testing of 16 ADA-bearing retrovirus vectors and the results of ADA expression studies with four vectors that produced high-titer virus.

MATERIALS AND METHODS

Construction of defective retrovirus vectors. A 12.75-kb human genomic DNA fragment containing exon 1 of the ADA gene was isolated from a library generated by cloning partially *Mbo*I-digested human fetal liver DNA into the vector EMBL3A. This insert contains approximately 2 kb of intron 1 and 10 kb 5' of the mRNA cap site. A human ADA promoter-ADA cDNA minigene was constructed by cloning a 1,237-bp *Eco*RI-*Dde*I fragment from pADA211 (1) into the *Hinc*II site of pUC18. A *Hind*III-*Nco*I genomic DNA fragment containing 3.9 kb 5' of exon 1 was ligated to the this cDNA subclone at the *Nco*I site (within the ATG codon). This 5.1-kb minigene was then blunt end-ligated in both orientations into the *Xho*I site of the N2 vector (3, 7, 15, 32) (vectors 7 and 8 in Fig. 2) and the *Bgl*II site of the FVXM (34) (M. Botchan; vectors 1 and 2 in Fig. 2) Δ e, Δ p, and Δ e Δ p vectors (A. Bernstein; vectors 3 to 6 in Fig. 2). The human *c-fos* minigene was constructed by ligating a 2.25-kb *Hind*III fragment from pFC1 (12) (kindly provided by R. Vincent) containing the *c-fos* cap site into the *Nae*I site of the ADA

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cDNA (56 bp 5' of the ATG). This 3.5-kb minigene was subcloned into each vector in a manner similar to the ADA minigene (vectors 9 to 13 in Fig. 2). pXT1ADA was constructed by ligating a partially *Bam*HI-cut ADA cDNA fragment from p7ADAA (a 1,237-bp *Eco*RI-*Dde*I fragment subcloned into the *Hinc*II site of pUC7) to *Bgl*III-cut and calf intestinal phosphatase-treated pXT1 (8) (kindly provided by E. Wagner; contains the 5' LTR and *gag* region from N2, a *neo* gene, and a 925-bp herpes simplex virus [HSV] thymidine kinase [TK] promoter; vector 14 in Fig. 2). pΔXT1ADA was made by subcloning the *Sall*-*Hind*III fragment from pXT1ADA (containing the TK-ADA minigene and the 3' LTR region) into *Sall*-*Hind*III-cut p5'N2 (a subclone of the 5' LTR and *gag*-containing *Eco*RI fragment from N2 in pUC18; vector 15 in Fig. 2). pΔNN2ADA was made by reconstructing the N2 vector without *neo*. A *Sall*-*Hind*III fragment from N2 (3' LTR region) was subcloned into *Sall*-*Hind*III-cut p5'N2, and then the ADA cDNA was ligated into the unique *Bam*HI site (vector 16 in Fig. 2).

Isolation of virus producer cell lines. CsCl-banded plasmid DNA (10 μg) from each of the vector constructions was transfected into the helper cell lines ψ2 (39) (ecotropic; provided by R. Mulligan) and PA317 (44) (amphotropic; provided by A. D. Miller). In the vectors lacking *neo*, the plasmid was cotransfected in a 10:1 molar ratio with pSV2neo (American Type Culture Collection [ATCC]). ψ2 and PA317 cell lines were maintained in Dulbecco modified eagle medium (Gibco, Grand Island, N.Y.) with 10% fetal calf serum (FCS; Gibco) at 37°C in 10% CO₂. Populations of transfected cells were selected in G418 (300 μg/ml) (Gibco), and then supernatant medium from those cells was used as a source of virus for infection of the packaging cell line conferring the opposite tropism. Individual infected clones were established either by selection in G418 (for *neo*-containing vectors) or by seeding at low density. Protein extracts from these clonal cell lines were checked by isoelectric focusing (IEF) for the presence of human ADA. Filtered (0.22-μm pore size) supernatant medium from the individual clones was used to infect Rat208F (A. Bernstein) target cells. After 5 days, these cells were harvested and protein extracts were analyzed by IEF. In pilot experiments there was an approximate correlation of ADA-virus titer and intensity of human ADA activity detected in the transduced target cell population. This presumably reflected an increased percentage of cells infected with the virus. Clones producing the most intense human ADA activity in the target cells were used in all subsequent experiments.

IEF analysis. Rat208F target cells were harvested from 24-well tissue culture trays (Corning Glassworks, Corning, N.Y.) by trypsinization, washed once in Hanks's balanced salt solution (Gibco), and dissolved in ADA lysis buffer (50 mM KHPO₄ [pH 7.0], 1.0 mM 2-mercaptoethanol, 0.1% Triton X-100; Sigma Chemical Co., St. Louis, Mo.). The extracts were cleared by centrifugation (13,000 × *g*, 15 min, 4°C) and loaded onto a prefocused pH4-5 IEF gel (6% polyacrylamide, 0.8 mm; International Biotechnologies, New Haven, Conn.). After focusing, the gel was stained for ADA enzyme activity by standard methods (63). The gel was then fixed, dried, and photographed.

Northern analysis and RNase A protection mapping of viral transcripts. Total cellular RNA was isolated from individual cell lines by the method of Chirgwin et al. (11). RNA (10 μg) was electrophoresed through a 0.9% formaldehyde-MOPS (morpholinepropanesulfonic acid)-agarose gel. The nucleic acids were transferred, hybridized, and washed as described by Thomas (55).

RNase A protection analysis was carried out as described by Gibbs and Caskey (17). Briefly, single-stranded antisense ³²P-labeled probe was made by in vitro transcription of 1 μg of linearized (*Eco*RI) plasmid template with T7 polymerase. The template plasmid was constructed by subcloning an *Eco*RI-*Bam*HI (−136 to +150) fragment into pTZ19R (Pharmacia, Piscataway, N.J.). The probe was hybridized to 25 μg of total cellular RNA (400 mM NaCl, 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.7]) overnight at 60°C. RNA-RNA hybrids were bound to poly(U) paper and then eluted (H₂O, 65°C, 10 min) before digestion with RNase A (10 μg/ml) (67 mM NaCl, 33 mM LiCl, 1 mM EDTA, 10 mM Tris, pH 7.5). The protected strands were denatured (97% formamide, 1 mM EDTA, 10 mM Tris [pH 7.5], 0.1% sodium dodecyl sulfate, 0.001% bromophenol blue, 0.0001% xylene cyanol) and separated on a 5% acrylamide-7 M urea-TBE (Tris-borate-EDTA) sequencing gel. The gel was dried and then autoradiographed.

Bone marrow culture and transplants. Fresh bone marrow cells (6 × 10⁶) harvested from the tibias and femurs of healthy 8- to 12-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were cocultivated with 6 × 10⁶ irradiated (1,500 rad, ¹³⁷Cs source; Gammacell 1000, Atomic Energy Ltd., Zippyville, Canada) virus producer cells in Iscove modified Dulbecco medium (IMDM; Gibco) supplemented with bovine serum albumin (BSA), Fe-saturated transferrin, soybean lipids (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.), 10% FCS, 10% WEHI3B(D-) (provided by R. Phillips)-conditioned medium (interleukin-3), and 10% 5637 (ATCC)-conditioned medium (interleukin-1α, interleukin-6). After 24 h the nonadherent cells were removed into petri dishes for an additional 48 h of culture. With vectors carrying the *neo* gene, some of the cells were preselected in G418 (1.50 mg/ml). Viable cells (10⁵) were plated into 1 ml of semisolid methylcellulose cultures in supplemented IMDM. Cultured-cell colony-forming units (CFU-C) were counted and picked after 10 days. Irradiated (1,000 rad) syngeneic recipient animals were injected intravenously (i.v.) with between 5 × 10⁴ and 5 × 10⁵ viable cells for study of distinct CFU-S. Recipients were sacrificed at 14 days posttransplantation, and individual CFU-S were dissected from the surrounding stroma.

Southern analysis. High-molecular-weight DNA was isolated by standard techniques from the individual cell lines. Genomic DNA (5 μg) was cut with *Nhe*I (cuts once in each of the LTRs of the vector), electrophoresed through a 0.7% TAE (Tris-acetate-EDTA)-agarose gel, and blot transferred to nylon membrane (GeneScreen Plus; Dupont, Boston, Mass.) by the method of Southern (51). ADA cDNA was labeled with [³²P]dCTP (16) and hybridized as described before (5). Filters were washed to a final stringency of 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C. The blots were exposed to Kodak XAR-5 film overnight with an intensifying screen.

The splenic tissue was disrupted with several strokes in a Dounce microhomogenizer in lysis buffer. Genomic DNA was extracted from the nuclear pellet after centrifugation by standard methods, and 10 μg was cut with *Hind*III (cuts outside the vector) or with *Bam*HI (cuts once in the vector). The cut DNA was run through a 0.8% TAE-agarose gel, blotted to nylon membrane, and hybridized with random hexamer ³²P-labeled ADA cDNA probe. Estimation of copy number was performed with an Amersham video analyzer (Amersham, Arlington, Ill.). Band intensities were normalized by using the endogenous mouse ADA gene as an

FIG. 1. Protocol for screening retrovirus vector. See Materials and Methods for details. hADA, Human ADA. Insets: IEF gel analysis was used to determine whether cloned Ψ 2 or PA317 cell lines expressed human ADA and thus could produce ADA virus. IEF gel analysis was also used to assess the expression of human ADA in target Rat208F cells. A high level of expression (denoted by a heavy line) was correlated with a high-titer virus preparation from an individual virus-producing clone (individual clones denoted by numbers 1 through 3).

internal standard. To allow detection of this band, the hybridization wash temperature was reduced to 55°C.

Marker rescue assay. Supernatants from defective virus producer cell lines and serum samples were incubated with an indicator cell line (SVB-3T3). The indicator cell line was produced by transfection of NIH 3T3 (ATCC) fibroblasts with the replication-defective vector pZIPneoSV(B) (9) (provided by C. Cepko). When infected with replication-competent virus, these cells will also package a *neo*-containing defective vector. After a 2-week passage, the indicator cell line supernatant was harvested and used to infect Rat208F cells. These cells were then placed in G418 for enumeration of resistant colonies. The titer of replication-competent virus was determined by serial dilution of the initial supernatant or serum sample before infection onto SVB-3T3 cells.

RESULTS

Screening of vector constructions. The protocol for the evaluation of the ADA vectors is outlined in Fig. 1. Vectors were transfected into the packaging cell lines Ψ 2 (ecotropic) and PA317 (amphotropic). Since cell lines generated by infection have been shown to allow higher titers and more stable defective vector production (45), the transformed populations were used as a source of virus to infect the opposite packaging cell type. Clonal isolates of the infected cells were obtained and checked for expression of human

ADA. ADA-transducing virus produced from these clonal isolates was then assayed by infection of target cells. The assay was intentionally made insensitive so that vectors which either produced low titers (less than 10^4 CFU/ml) or expressed ADA poorly could be identified and set aside. Individual cell lines giving the highest level of transduction were then used for subsequent experiments on bone marrow infection. Sixteen retrovirus vectors were tested in this way (Fig. 2). These vectors were designed to evaluate the effect on titer and expression of three aspects of structure: (i) sequences 3' of the classical ψ packaging site—so-called *gag*⁺; (ii) deletions in the 3' U3 region (self-inactivating vectors [62, 64]); and (iii) internal transcriptional units in both orientations with respect to the vector LTR. Only vectors which contain the *gag*⁺ region gave positive results in the transduction assay. The *gag*⁺ region has been shown to enhance viral RNA packaging but has been associated with a high propensity for recombination to form replication-competent virus (7). Subsequent marker rescue assay showed that several of the packaging lines transfected with *gag*⁺ vectors did produce replication-competent virus. The titer of the virus in the pANN2ADA (vector 16 in Fig. 2) line was determined to be 10^4 infectious units per ml.

The human ADA promoter-containing constructs proved to be unstable, so that although very high titers of *neo*-transducing virus (1×10^6 to 5×10^6 G418^r CFU/ml) were

FIG. 2. Retrovirus vector constructs. The heavy line (vectors 7, 8, and 12 to 16) indicates the *gag*⁺ region (nucleotides 564 to 1038 in Moloney virus). Open boxes denote the Moloney virus LTR. Plasmid vector DNAs were transfected into the packaging cell lines ψ 2 and PA317. Virus produced from these cells was used to infect the packaging cell line of the opposite tropism. Those vectors which produced virus titers sufficient to be detected by IEF on unselected populations were developed further. Clonal isolates from infected populations were tested for transduction of virus to the target cell line Rat208F. pro, Promoter; NA, not applicable. Hatched boxes correspond to the human ADA promoter and 5'-flanking sequences (ca. 3.9 kb from the transcription initiation site); shaded boxes denote the *c-fos* promoter and enhancer sequences (12).

found, no transduction of ADA activity was observed. One potential explanation for this may be its relatively large size (9.4-kb mRNA). Vectors containing a shorter ADA promoter are currently being evaluated. The orientation of the *c-fos* minigene appeared to be important. Infection of ψ 2 cells with the forward-orientation construct (same direction of transcription as the LTR) did not allow ADA transduction, although the reverse orientation did. This might be explained by the presence of cryptic splice donor or acceptor signals, poly(A) addition sites, or other interactions between these sequences and the vector LTRs causing instabilities in the vector containing the forward-orientation *c-fos* promoter minigene. In contrast, the HSV TK promoter constructions produce virus well. Thus, orientation can play an unpredictable role in individual constructs.

Southern and Northern analyses of selected virus-producing lines and infected clones of Rat208F cells indicated no major rearrangements in their integrated and expressed forms (Fig. 3a and b). Interestingly, we detected only one species of human ADA mRNA in the cells infected with the pΔNN2ADA virus. The comparable parent construction, N2, which contains *neo*, produces two mRNAs, one full length and the other spliced (3). The possible utilization of an

alternative cryptic splice acceptor in pΔNN2ADA is being further evaluated by RNase protection analysis.

Infection of murine hematopoietic cells. The four high-titer viruses (designated pN2Fos2ADA, pXT1ADA, pΔXT1ADA, and pΔNN2ADA; vectors 13 to 16 in Fig. 2) were subsequently used to infect primary mouse bone marrow cells. Infection was obtained by 24 h of cocultivation with irradiated virus producer lines. Infected marrow was either plated in semisolid culture for enumeration of G418-resistant CFU-C or injected into irradiated recipients. Some recipients received graded doses of the marrow cells so that discrete CFU-S could be studied. Other animals received reconstituting doses (1×10^6 to 3×10^6 viable cells) to allow long-term studies of human ADA expression. The results of these experiments are summarized in Table 1.

Two vectors, pN2Fos2ADA (vector 13) and pXT1ADA (vector 14), containing the *c-fos* promoter and the HSV TK promoters, respectively, failed to express human ADA in CFU-C or CFU-S even though these viruses worked well in tissue culture fibroblasts and even though the infection efficiency was adequate. Both vectors expressed *neo* from the LTR promoter and conferred G418 resistance to between 1 and 20% of CFU-C. In contrast, CFU-C infected with the

FIG. 3. (a) Southern analysis on ADA vector-infected cells. Group A, vector 13, pN2Fos2ADA; group B, vector 14, pXT1ADA; group C, vector 16, pΔNN2ADA; group D, vector 15, pΔXT1ADA. The parent virus producer line (P) and five infected clones are shown. Genomic DNA from infected Rat208F clones was cut with *NheI* (cuts in the vector LTRs), blotted, and then probed with a radiolabeled ADA cDNA. (b) Northern analysis of infected cells. Group A, vector 15, pΔXT1ADA; group B, vector 16, pΔNN2ADA; group C, vector 14, pXT1ADA; group D, vector 13, pN2Fos2ADA. Lanes correspond to the parent virus producer line (P) and five infected clones. Total RNA was separated on a formaldehyde-MOPS-agarose denaturing gel, and then human ADA-containing mRNA was detected with an ADA cDNA probe.

virus by using the Moloney virus LTR promoter (pΔNN2ADA, vector 16) or the HSV TK promoter and lacking the *neo* gene (pΔXT1ADA, vector 15) expressed human ADA. This enzyme activity could be detected in pooled CFU-C in the absence of selection, indicating very high infection efficiency. Likewise, when individual CFU-S were analyzed, infection efficiencies of between 50 and 85%

FIG. 4. (A) Southern analysis of CFU-S infected with the pΔNN2ADA vector. Genomic DNA was extracted from the nuclear pellets of homogenized individual CFU-S and cut with *HindIII* (does not cut within the provirus). Human ADA sequences were detected with a radiolabeled ADA cDNA probe. Lanes correspond to DNA isolated from CFU-S, ΔN2, Control infected cell line; ψ2, parent packaging cell line; MC, control mouse splenic DNA. (B) Expression of human ADA in CFU-S. Protein extracts from individual 14-day CFU-S taken from recipient animals injected with 5×10^4 pΔNN2ADA virus-infected marrow cells were then run on IEF gels as described in Materials and Methods. Each lane contains one CFU-S; bracketed areas indicate individual animals. The positions of murine and human ADA are shown. Lane numbers designate mouse recipient and CFU-S samples.

were observed based on Southern analysis (Fig. 4A) and expression of human ADA (Fig. 4B). There was a 100% correlation between integration events and expression with both of these vectors in this experiment. The restriction enzyme *HindIII* does not cut within the provirus, so each band on the Southern blot corresponds to a discrete integration event. The fact that some of the CFU-S had multiple integrations and some had none suggests heterogeneity in the infectability of these cells. In a series of three experi-

TABLE 1. Infection and expression of ADA-transducing retrovirus vectors in murine hematopoietic cells

Vector ^a (vector no.)	In vitro		In vivo ^b	
	G418 ^c CFU ^c (%)	IEF ^d	No. infected/total CFU-S (% infection efficiency) ^e	No. expressing hADA/total infected CFU-S (% expression efficiency)
pN2Fos2ADA (13)	8–20	Negative	2/35 (6)	0/2
pXT1ADA (14)	1.6–8.5	Negative	11/18 (61)	0/11
pΔXT1ADA (15)	NA ^f	Positive	9/15 (60)	9/9
pΔNN2ADA (16)	NA	Positive	31/45 (69)	24/31

^a See Fig. 2 for description of vectors.

^b Discrete (CFU-S) cells or whole spleens were marrow-reconstituted mice were analyzed as described in the legend to Fig. 4.

^c Infected bone marrow cells were plated in supplemented IMDM with 0.5% methylcellulose as described previously (9). CFU were counted at 10 days.

^d Individual CFUs were picked from semisolid cultures and pooled, and the protein extracts were analyzed for the presence of human ADA by use of IEF. For vectors 13 and 14, these were G418^r CFU, but for vectors 15 and 16, the CFUs were produced under nonselective conditions.

^e The number of CFU-S bearing the ADA-containing provirus was determined by Southern analysis of genomic DNA as described in the legend to Fig. 4.

^f NA, G418 selection not applicable to vectors without the *neo* gene.

FIG. 5. RNase A protection analysis on spleen RNA from animals infected with the pΔXT1ADA vector. Single-stranded RNA antisense probe was synthesized in vitro by use of the T7 promoter (T7) from a linearized (*Eco*RI-cut) plasmid (pTZ19). The total probe length is 305 nucleotides, including a part of the TK promoter, ADA cDNA to the *Bam*HI site (position +150), and the pTZ19 polylinker (poly). A transcript initiated in the LTR would be expected to protect a fragment of 268 nucleotides. A transcript initiated in the TK promoter would be predicted to protect a transcript of 189 nucleotides (although a 154-nucleotide transcript was observed). The normal hADA transcript should protect only the ADA coding sequence (130 nucleotides). Lanes: 1, molecular weight markers; 2, Molt4 cells (human T cell leukemia line, ATCC); 3, pΔXT1ADA-infected Rat208F cells; 4, spleen cells from marrow-reconstituted animal 1 14 days posttransplantation with pΔXT1ADA-infected marrow; 5, pΔXT1ADA-infected animal 2; 6, control spleen.

ments (Table 2) with the pΔNN2ADA vector, we also observed variation in expression efficiency. In the second experiment, there was adequate infection efficiency (approximately 60%), as determined by provirus integration, but very low expression efficiency in CFU-S. This indicates that beyond the variability in infectability of these cells, there is also biological variation in the regulation of expression with the same vector sequences.

To determine whether expression of human ADA in the

spleens of animals transplanted with the pΔXT1ADA vector-infected marrow originated from the LTR or the HSV TK promoter, an RNase A protection study was performed (Fig. 5). This suggested that the predominant RNA species originated in the LTR and that the HSV TK promoter contributed relatively little to production of human ADA mRNA.

Expression of human ADA in blood of marrow-reconstituted animals. Following transplantation with infected marrow, the presence of human ADA in marrow-reconstituted

TABLE 2. Bone marrow infection determined by use of the pANN2ADA vector^a

Expt	CFU-S		No. expressing hADA in blood/ total no. of transplant recipients:	
	No. infected/total no. of CFU-S	No. expressing hADA/ total no. of CFU-S	4	12
1	16/22 (72)	16/22	5/5	2/5
2	8/15 (61)	1/15	5/5	0/5
3	7/8 (85)	7/8	4/4	4/4

^a See Materials and Methods and legends to Fig. 4 and 6 for experimental details.

recipient mice was monitored by serial bleeding at 2-week intervals. The results of one of these experiments are shown in Fig. 6. Human ADA was detected in the blood in 100% (14 of 14) of the mice transplanted with the pANN2ADA virus. The ADA enzyme from whole blood was derived almost entirely from the erythrocytes (RBC) and thus did not indicate the level of expression or the longevity of expression in the lymphocyte, granulocyte/monocyte, or platelet lineages. Over time the level of ADA decreased, suggesting that the cells originally expressing the human ADA originated from more mature progenitors which were infected with higher efficiency or in which no negative regulatory effects on expression had intervened. However, several of the animals continued to express human ADA, although at diminished levels, up to 6 months posttransplant (these animals were then sacrificed). The average life span of murine RBC is 45 days (4, 62), so the human ADA detected probably originated from cells whose primitive progenitors were infected with the virus. To evaluate more fully the distribution of human ADA in these recipient animals, two were sacrificed at 12 weeks posttransplantation. Hematopoietic and lymphoid tissues were analyzed by IEF for the presence of the human enzyme (Fig. 7A). Substantial amounts of human ADA were detected in the spleen, with lesser amounts in other tissues. Preliminary analyses for the presence of proviral sequences in these tissues indicated a very low copy number. In an attempt to quantify the average number of copies per cell, the analysis was repeated with various quantities of DNA compared with a known single-copy provirus insertion cell line (Fig. 7B). This study allows estimation of a copy number of less than 1/20 per cell. When the DNA was cut with *Hind*III, which does not cut in the vector, only a single band was observed. A band of similar molecular weight was detected in the bone marrow of this animal (not shown). This indicates that at least one multipo-

FIG. 6. Expression of human ADA in the blood of marrow-reconstituted recipients. Infected viable bone marrow cells (10^6) were injected i.v. into lethally irradiated recipients. These animals were bled at intervals posttransplantation, and protein extracts from blood cells were analyzed by IEF. Time after transplantation (in days) is indicated above the panels. This set of animals corresponds to those used in experiment 3 of Table 2. Lane numbers designate individual bone marrow transplant recipient animals from experiment 3 in Table 2.

FIG. 7. (A) Expression of human ADA in tissues from animals reconstituted with marrow infected with the pANN2ADA vector. Thy, Thymus; LN, lymph node; BM, bone marrow; SPL, spleen; BL, blood. Animals 6 and 9 are the same as those designated in Fig. 6 and were part of experiment 3 in Table 2. (B) Southern analysis on the spleen of animal 9. Genomic DNAs from the vector producer cell line pANN2ADA and from the spleen of the long term recipient animal 9 were digested with either *Bam*HI or *Hind*III. The blotted DNAs were then probed with an ADA cDNA. The wash stringency was adjusted to allow detection of the endogenous mouse gene to provide an internal control for quantitation. Band intensities were measured with an Amersham video imaging system. Lane numbers indicate the micrograms of DNA loaded.

tent progenitor was infected with the provirus, and its progeny may be the source of the observed ADA activity. Because the vector-producing cell lines also produced replication-competent virus, it is also formally possible that replication-competent virus introduced at the time of infection with the ADA-transducing defective virus allowed spread of the defective virus *in vivo*. Serial assay of serum from the p Δ NN2ADA and p Δ XT1ADA recipients by marker rescue assay indicated viremia with the replication-competent virus in all these animals, including those animals which expressed human ADA only transiently (data not shown). The titer of this virus in the serum of one animal (no. 9 from the third experiment, Table 2, Fig. 6) was determined to be 10^2 infectious units per ml. ADA-transducing virus was not detected by IEF analysis on the indicator cell lines or by immunohistochemical staining for human ADA (a technique which can identify single infection events; manuscript in preparation).

DISCUSSION

These experiments demonstrate long-term expression of a human disease-related gene product in mouse hematopoietic cells. The results are consistent with previous published reports of expression of neomycin phosphotransferase in these cells (15, 32) with the parent vector N2. Recently, Dzierzak et al. (14) reported low-level expression of human β -globin in mouse blood cells and were able to demonstrate expression in the progeny of infected pluripotent stem cells. These experiments also demonstrated expression of *neo* in lymphoid cells under the control of the viral LTR promoter. Lim et al. (37) have also recently shown that vectors which utilize the phosphoglyceratekinase promoter can be used to obtain expression of human ADA in CFU-S. Together, these results provide direction and encouragement for the refinement of retrovirus expression vectors.

The results presented here suggest that the Moloney LTR promoter has no adverse effect on expression in murine hematopoietic progenitors. However, if *in vivo* spread of the defective virus occurs, the observed expression may have originated from infection of more mature cells, in which the LTR is known to function well. We do not believe that *in vivo* spread contributes much to human ADA expression in the blood for the following reasons: (i) the orderly and consistent reduction in expression seen in all animals at ca. 10 to 12 weeks postreconstitution; (ii) the stability of expression observed beyond 12 weeks in individual animals without apparent fluctuation; (iii) the presence of replication-competent virus in animals failing to express human ADA in the blood; and (iv) the lack of multiple, less-than-single-copy insertions in individual CFU-S. Definitive proof awaits repetition of these experiments with virus preparations that are free of replication-competent virus. The availability of a new packaging cell line which splits the *gag-pol* and *env* genes (41) should aid in this. The expression from LTR promoter vectors in hematopoietic cells contrasts with the poor expression from this promoter observed in embryonal carcinoma cells, early mouse embryo cells, and retrovirus transgenic animals (26, 28, 48–50, 52, 53, 57, 58). The regulation of expression in the differentiated progeny of true pluripotent stem cells may, however, be governed by the same mechanisms which restrict expression in the transgenic animals. This problem can be approached by further analysis of the types of vectors which are expressed in embryonal carcinoma cells and comparison with those that are expressed in the differentiated progeny of infected hematopoietic stem cells.

Further studies will be directed toward the achievement of consistent levels of expression and tissue-specific regulated expression. Inclusion of natural splice signals and tissue-specific enhancers may be of importance. Obtaining consistent and efficient infection of stem cells is another important goal. Further experiments will focus on optimization of stem cell infection by enrichment with monoclonal antibodies and cell sorting and by hormonal stimulation. The recent description of growth factors which appear to directly affect very primitive lymphoid and myeloid progenitors (25, 27) suggests that these cells might be propagated *in vitro* for infection and drug selection protocols. The nature of the cell surface receptor for viral adsorption remains unknown (29), but it may be possible to increase infection efficiency by modulating receptor density via hormonal stimulation of these cells. Molecular characterization of this receptor as well as a more detailed understanding of the most important factors governing "infectability" would be very desirable. We are optimistic that improvements can be made that will allow routine exploitation of this set of methods for analysis of hematopoiesis and eventually for the therapy of human ADA deficiency.

ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Institute (HHMI) and by Public Health Service grant HD21452 from the National Institutes of Health and a grant from the Cystic Fibrosis Foundation. G.R.M. is the recipient of an Arthritis Foundation postdoctoral fellowship; J.W.B. is an Assistant Investigator and CTC is an Investigator at HHMI.

We thank Jenny Henkel-Tigges and Michelle Rives for technical assistance and Elsa Perez for help in preparation of the manuscript. We also thank H. Zogbi and J. Noebels for help with video quantitation of Southern analyses and J. Dick and L. Donehower for useful discussions during the completion of these studies.

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