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Retrovirus-Mediated Gene Transfer of Human Adenosine Deaminase: Expression of Functional Enzyme in Murine Hematopoietic Stem Cells In Vivo

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Simplified Moloney murine leukemia virus-based recombinant retrovirus vectors have been constructed which transduce human adenosine deaminase (ADA) cDNA. ADA transcription is under the control of the constitutive promoter for the human X chromosome phosphoglycerate kinase (*pgk*) gene. In these simplified vectors, dominant selectable markers are not included and selection is dependent on overproduction of functional ADA enzyme. Primary murine hematopoietic cells were infected with helper-free recombinant ADA virus generated from Psi-2 packaging cells. Protein analysis revealed that human ADA enzyme was expressed in progenitor-derived hematopoietic colonies in vitro and CFU-S-derived spleen colonies in vivo. Enzyme expression was dependent on transcription from the *pgk* promoter. ADA expression in primary murine hematopoietic cells directed by the internal promoter was not adversely affected by the presence of the Moloney virus long terminal repeat enhancer sequence. Use of these vectors allows systematic evaluation of the effects of specific sequences in recombinant retrovirus vectors on expression in primary murine hematopoietic cells in vivo.

Gene transfer into hematopoietic stem cells offers a new system with which to investigate the expression of foreign gene sequences in the context of a developmentally competent cell in vivo and the effects of specific gene products on self-renewal and differentiation (4, 5, 10, 11, 17, 21). This technology also holds promise for potential somatic cell genetic therapy (22). Although gene transfer into pluripotent hematopoietic stem cells has been accomplished by using retroviruses, expression of introduced sequences has been variable (4, 10, 15, 23, 24). On one hand, expression of G418 (or neomycin) resistance in hematopoietic progenitor cell-derived colonies has been demonstrated by both in vitro selection and detection of neomycin phosphotransferase activity (4, 5, 10). However, progressive shutoff of expression has been observed in hematologically reconstituted animals or after serial transfer of bone marrow to secondary recipients as analyzed by neomycin resistance (4, 10). In addition, some retroviruses that function in tissue culture cell lines infected in vitro do not express transferred sequences satisfactorily in primary murine CFU-S or progenitor-derived colonies (15, 23, 24). For example, we have previously reported failure to express in vivo human adenosine deaminase (ADA) cDNA driven by a simian virus 40 (SV40) promoter internally in a retrovirus designated DHFR*-SVADA (23). Substitution of a variety of promoters (cytomegalovirus, metallothionein, thymidine kinase, and the endogenous ADA promoter) in this virus or very similar viruses constructed by others has yielded consistently negative results in vivo, in spite of adequate expression in tissue culture lines (15, 24; unpublished results). These vectors contain dominant selectable markers (e.g., neomycin phosphotransferase, dihydrofolate reductase [DHFR], and hypoxanthine phosphoribosyltransferase) in addition to the ADA cDNA. In contrast, in a different virus in which ADA cDNA was translated from a spliced mRNA initiated from a retroviral long terminal repeat (LTR), some human ADA enzyme was observed in primary murine progenitors in vitro (1).

The relative contributions of vector design, specific retroviral sequences, such as the Moloney murine leukemia virus (MoMuLV) enhancer, dominant selectable markers, and the nature of the hematopoietic target cells to these observations on in vivo expression are uncertain. Recently, Magli et al. showed apparent differences in expression of the transferred neomycin resistance gene, as judged by the proportion of G418-resistant progenitor cells, by using structurally different retroviral vectors (12). In our studies we have chosen to use human ADA cDNA as a reporter sequence to monitor expression because a convenient semiquantitative and sensitive in situ electrophoretic assay permits detection of enzyme activity in single progenitor- or CFU-S-derived colonies. In addition, efficient transfer and expression of human ADA in hematopoietic stem cells constitute a relevant model for somatic cell gene therapy of the inherited disease severe combined immunodeficiency, which is the result of ADA deficiency (22).

In an attempt to delineate features influencing in vivo expression of sequences transferred by recombinant retroviruses, we have explored the use of a series of simplified retroviruses containing ADA cDNA. A constitutive promoter has been employed to drive transcription internally within the viruses. We have also directly assessed the influence of the retroviral LTR enhancer element on expression, since in other primitive stem cells the enhancer sequence has been implicated as a negative regulatory element (6, 19). Here we report the expression of human ADA in primary murine hematopoietic progenitor colonies in vitro and CFU-S-derived spleen colonies in vivo following retroviral infection.

MATERIALS AND METHODS

Construction of recombinant retrovirus vector plasmids. Three recombinant vectors were derived from the vector Zip

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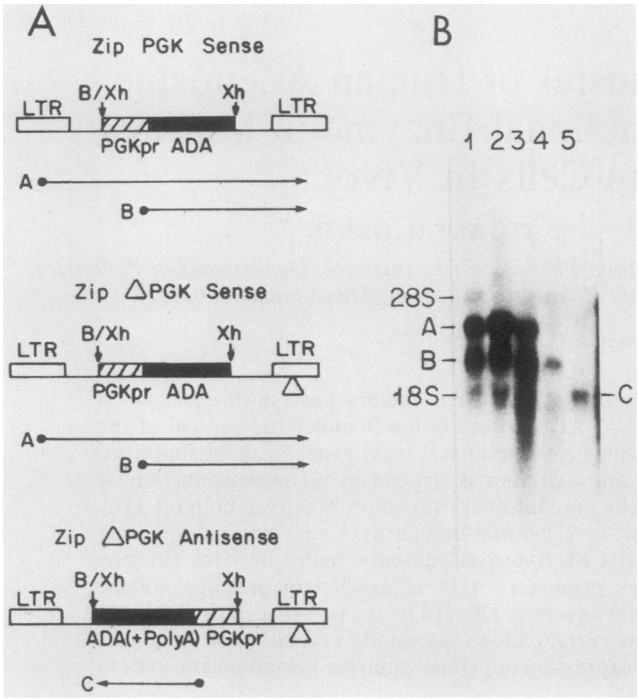

FIG. 1. (A) PGK-ADA recombinant retroviruses. The open boxes are retroviral LTRs. The hatched boxes are the promoter of the human X chromosome *pgk* gene (PGKpr). The dark boxes are human ADA cDNA. The Δ below the 3′ LTR denotes deletion of the MoMuLV enhancer sequence. Abbreviations: B, *Bam*HI; Xh, *Xho*I. The expected RNA transcripts are shown at A, B, and C below each vector. +PolyA, Polyadenylation site. (B) Northern blot of RNA probed for human ADA sequences. RNA samples were prepared from Psi-2 PGK-ADA sense (lane 1) and NIH 3T3 cells infected with the PGK-ADA sense (lane 2) recombinant viruses; from Psi-2 ΔPGK-ADA sense (lane 3) and NIH 3T3 cells infected with the ΔPGK-ADA sense (lane 4) recombinant virus; and from NIH 3T3 cells infected with the ΔPGK-ADA antisense recombinant virus (lane 5). Positions of 28S and 18S rRNAs are shown. Transcripts A, B, and C correspond to those shown in panel A. An *Nco*I-*Hin*fI fragment of the human ADA cDNA (16) was used as the probe.

DHFR* (20a). The DHFR cDNA and nonessential retroviral sequences were removed by digestion with *Bam*HI and *Xho*I, followed by insertion of *Xho*I linkers. A 1.1-kilobase (kb) *Nco*I-*Hin*fI fragment of the human ADA cDNA (16) was subcloned downstream of a 0.5-kb *Eco*RI-*Pvu*II fragment of the human phosphoglycerate kinase (*pgk*) gene in plasmid pUC19. The PGK-ADA cassette was then transferred into the *Xho*I site of the Zip retroviral plasmid (Fig. 1A, Zip PGK sense; hereafter called PGK-ADA sense). The enhancerless vector Zip ΔPGK-ADA sense was made by replacing the 3′ LTR with the 3′ LTR (enhancerless) of the SVX vector containing a *Pvu*II-*Xba*I deletion (3) (Fig. 1B, Zip ΔPGK sense; hereafter called ΔPGK-ADA sense). Finally, for the antisense construct, Zip ΔPGK-ADA antisense, the 3′ *Bgl*II-*Hin*dIII fragment of the human ADA cDNA was replaced with the corresponding genomic segment of the human ADA gene. The PGK-ADA cassette, including the natural polyadenylation signal of the ADA gene, was transferred into the Zip retroviral plasmid in reverse orientation from the 5′ LTR (Fig. 1A, Zip ΔPGK antisense; hereafter called ΔPGK-ADA antisense).

Establishment of PGK-ADA producer clones and quantitation of virus production. The Psi-2 packaging cell line (14) and all cell clones were maintained in alpha-modified essential medium (α-MEM) supplemented with 5% fetal calf serum and 5% calf serum (GIBCO Laboratories), streptomycin (100 μg/ml), and penicillin (GIBCO) (100 U/ml). PGK-ADA recombinant virus producer clones were generated from all three vectors by transfection with standard calcium phosphate precipitation methods. Briefly, Psi-2 cells were split 1:20 from a confluent plate 24 h prior to transfection. These cells were exposed to calcium phosphate precipitate containing 10 μg of vector DNA for 24 h at 37°C and 5% CO_2, after which the cells were fed with fresh medium. The cells were split into selection medium 48 h after transfection. The selection medium contained 4 μM xylofuranosyl arabinoside (Xyl-A) and 0.005 to 0.006 μM deoxycorformycin (dCF) (9). The concentration of dCF was chosen because it was the minimal level which killed all uninfected NIH 3T3 cells and Psi-2 cells. Surviving clones were then individually removed by trypsinization and expanded.

Initial screening for virus production by Psi-2 clones was accomplished by a semiquantitative assay. NIH 3T3 cells (5×10^4 per 100-mm plate) were infected with 1 ml of harvest (plus polybrene [8 μg/ml]) from each producer clone. After 3 h at 37°C and 5% CO_2, the cells were rinsed and fresh medium was added. Cells were allowed to grow to confluence, pelleted after trypsinization, and lysed in 20 μl of lysing buffer (see below). One microliter of lysate was assessed for human ADA enzyme activity. The relative titer of recombinant virus from each clone was estimated from the intensity of human ADA activity in these infected NIH 3T3 cells. Apparent high-titer producers were selected for quantitative assessment of titer based on Xyl-A and dCF selection. NIH 3T3 cells (10^4) were infected with dilutions of viral harvest (plus polybrene) at 37°C and 5% CO_2. After 2.5 h, fresh medium was added and the cells were further incubated for 2 days. Xyl-A (4 μM) and dCF (0.005 to 0.006 μM) were then added, and the cells were incubated for an additional 14 days. Surviving colonies were then counted.

Infection of primary murine bone marrow cells: generation of spleen colonies in vivo and progenitor colonies in vitro. Bone marrow cells were harvested as previously described (21) from the hind limbs of C3H/HeJ mice 48 h after intravenous injection of 150 mg of 5′-fluorouracil (5-FU) per kg. A total of 5×10^6 5-FU-resistant bone marrow cells were cocultured for 24 h at 5% CO_2 and 37°C in 75-cm^2 flasks (Corning Glassworks) containing monolayers of irradiated (15 Gy) Psi-2 producer cells. After 24 h, nonadherent marrow cells were collected, and 1×10^5 to 3×10^5 cells were injected into lethally irradiated (13 Gy, split dose, with a minimum of 3 h between doses) mice to generate CFU-S-derived spleen colonies (20). For marrow-derived progenitor colonies, 2×10^5 cells per ml were plated in α-MEM containing 1% methylcellulose (Fluka), 24% fetal calf serum, 1% bovine serum albumin (Boehringer Mannheim), 1% penicillin, 1% streptomycin, 10^{-5} M mercaptoethanol (Sigma Chemical Co.), and 5% pokeweed mitogen spleen-conditioned medium with erythropoietin (Caunnaught) (2 U/ml) at 37°C and 5% CO_2 (2, 8). Some bone marrow cells were preselected with Xyl-A (4 μM) and dCF (0.005 to 0.006 μM) in the presence of 10% WEHI-3b-conditioned medium for 48 h in liquid culture. These preselected cells were then harvested and used as described above to obtain spleen colonies in vivo or progenitor colonies in vitro. Xyl-A (4 μM) and dCF (0.005 to 0.006 μM) were added for continuous selection to some progenitor plates. Injected mice were sacrificed on day 14 posttransplantation, and individual spleen colonies

were isolated under a dissection microscope. Spleen colonies were cut out with fine scissors and then teased apart and triturated to obtain a single-cell suspension. From each spleen colony, one-half of the cells were used for enzyme analysis and the remaining cells were used to prepare DNA.

Molecular procedures. High-molecular-weight DNA was prepared as previously described (13) from infected NIH 3T3 fibroblasts and CFU-S-derived spleen colony cells. Cytoplasmic RNA was prepared from infected NIH 3T3 and Psi-2 producer clones by cell lysis, removal of intact nuclei from the lysed cells, and proteinase K digestion, followed by phenol extraction and ethanol precipitation.

For Southern blot analysis, DNA was digested with *Xho*I and electrophoresed in a 1% agarose gel. For Northern blot (RNA blot) analysis, RNA was electrophoresed in a 1% agarose–formaldehyde gel. The DNA or RNA was transferred to nylon filters (Zetabind, AMF voit) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and hybridized with a ^{32}P-labeled *Nco*I-*Hin*fI fragment of human ADA cDNA.

Prehybridization, hybridization, and posthybridization washes of filters were performed as described by the commercial supplier. Filters were exposed to X-ray film in the presence of a calcium tungstate intensifying screen at −70°C.

In situ ADA enzyme assay. ADA enzymatic activity of cell lysates or progenitor colonies was analyzed by a modification of the starch gel in situ enzyme assay (18). Electrophoretic separation of ADA isozymes was accomplished on cellulose-acetate strips (Helena Laboratories, Beaumont, Tex.). Individual progenitor-derived colonies were plucked from methylcellulose with a finely drawn Pasteur pipette while viewed under an inverted microscope and loaded onto cellulose-acetate strips presoaked in running buffer (Supra-Heme buffer; Helena Laboratories). Colonies were lysed by repeated freeze-thawing on dry ice. For CFU-S-derived spleen colonies, cell pellets were lysed by freeze-thawing in lysis buffer (5 mM K_2HPO_4, 1 mM EDTA). Lysates were cleared by centrifugation at 10,000 × *g*. After electrophoretic separation, enzyme activity was detected by reacting the cellulose-acetate strip with a substrate consisting of adenosine (Sigma) (2 mg/ml), xanthine oxidase (0.06 U/ml), nucleoside phosphorylase (both from Boehringer Mannheim) (15 μg/ml), dimethylthiazol diphenyltetrazolium bromide (1 mg/ml), and phenazine methosulfate (both from Sigma) (0.1 mg/ml) in phosphate buffer at 37°C for 10 min.

RESULTS

Establishment of recombinant PGK-ADA retrovirus producer clones. Retroviral plasmids (Fig. 1) were transfected into Psi-2 packaging cells, and producer clones were selected for overexpression of ADA enzyme by the method of Kaufman et al. (9). The level of human ADA enzyme generated in NIH 3T3 cells infected with producer clone supernatants and allowed to grow without selection correlated quite well with the titer of PGK-ADA retrovirus. With this screening method, producers were chosen for quantitative titering by selection with Xyl-A and dCF. Because the titers determined in this way were uniformly low, the virus titer obtained with this selection was compared with that obtained by methotrexate (MTX) selection with a producer clone (Psi-2 DHFR*-SVADA6) (23), which contains both the MTX-resistant (DHFR*) cDNA sequence and human ADA cDNA driven internally from an SV40 promoter. Titers obtained on NIH 3T3 cells with 0.25 μM MTX were 100-fold higher than those obtained with Xyl-A–dCF selection (5×10^5 MTX^r colonies per ml versus 5×10^3 dCF^r colonies per ml). This discrepancy may arise in part from differences in the threshold of expression needed to survive selection. Thus, the dCF^r titer may not represent the actual number of infectious PGK-ADA particles made by the producer clones. The highest-titer Psi-2 PGK-ADA clones produced 1×10^3 to 2×10^3 dCF^r colonies per ml. These clones were used to infect NIH 3T3 cells and primary murine bone marrow cells.

Analysis of RNA transcripts in PGK-ADA producer cells and infected NIH 3T3 cells. The three PGK-ADA retroviral vectors (Fig. 1A) differed in the orientation of the PGK-ADA transcriptional unit and the presence of the MoMuLV enhancer. To assess the transcriptional units of these vectors, we analyzed RNA from Psi-2 PGK-ADA producer clones and infected NIH 3T3 cells for the presence of ADA transcripts.

Cytoplasmic RNA obtained from producer Psi-2 clones of each virus, as well as NIH 3T3 cells infected and then selected with Xyl-A and dCF, was examined for human ADA sequence RNA transcripts by Northern blot analysis (Fig. 1B). The Psi-2 producer of PGK-ADA sense (with enhancer) (Fig. 1B, lane 1) contained two transcripts which corresponded to initiation from the MoMuLV (transcript A) and the internal *pgk* (transcript B) promoters. NIH 3T3 cells infected with virus from this producer clone contained both transcripts (lane 2). The Psi-2 producer of the ΔPGK-ADA sense (enhancerless) vector also contained MoMuLV and *pgk* promoter-initiated transcripts (Fig. 1B, lane 3, A and B), whereas NIH 3T3 cells infected with this virus contained only transcripts initiated from the *pgk* promoter (Fig. 1B, lane 4, B). Thus, RNA analysis of cells infected with the enhancerless ΔPGK-ADA virus demonstrated that deletion of the LTR enhancer effectively rendered the MoMuLV promoter inactive in infected cells. A single transcript (C) was observed in NIH 3T3 cells infected with the producer clone of ΔPGK-ADA antisense (Fig. 1B, lane 5). The size of the transcript was consistent with initiation at the *pgk* promoter, with termination and polyadenylation at the reverse-orientation polyadenylation site in the PGK-ADA antisense virus. Human ADA expression from both enhancerless vectors must have been derived from *pgk* promoter-initiated RNA.

Expression of human ADA cDNA in primary marrow-derived progenitor colonies. Expression of retrovirally transferred human ADA cDNA was analyzed in primary marrow-derived progenitor colonies in vitro by analysis of functional ADA. Bone marrow cells infected by coculturing with PGK-ADA virus producer cells were plated in methylcellulose by the CFU-GEMM (granulocyte, erythroblast, megakaryocyte, macrophage) (5a) assay with and without Xyl-A and dCF. In some experiments, infected bone marrow cells were preselected in medium containing Xyl-A and dCF for 48 h before plating in methylcellulose. Individual progenitor colonies were removed beginning on day 14 and analyzed by in situ enzyme analysis for functional ADA enzyme. In some instances a fraction of a large colony was removed for enzyme analysis, and the remainder of the colony was suspended for the CFU-GEMM assay to generate secondary colonies (see below).

Table 1 summarizes the total number of colonies available for analysis and the frequency of colonies that were positive for human ADA enzyme activity after infection with each PGK-ADA retrovirus. The colonies were classified by growth with and without Xyl-A–dCF selection. The type of colonies obtained in these experiments included those de-

TABLE 1. ADA enzyme analysis of primary marrow-derived progenitor colonies

Vector	No. of human-enzyme-positive colonies/no. examined	
	Unselected	Selected (Xyl-A and dCF)
PGK-ADA sense	1/25	20/26
ΔPGK-ADA sense	2/33	6/9
ΔPGK-ADA antisense	2/13	3/5

rived from CFU-GM, CFU-MIX, and CFU-MIX with megakarocytes (see legend to Fig. 2). Although Xyl-A–dCF selection did not reduce the number of enzyme-negative colonies to zero, a substantial increase in the fraction of enzyme-positive colonies was observed (7% positive colonies unselected versus 70% selected; Table 1).

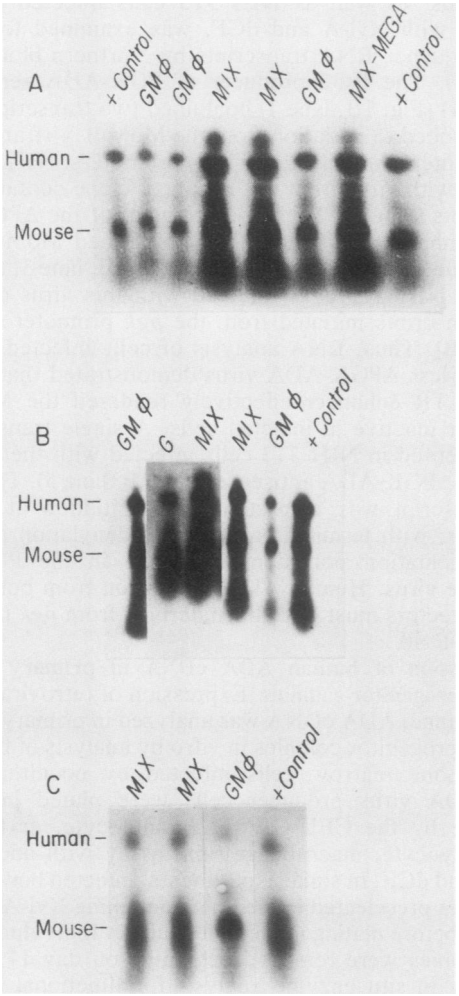

FIG. 2. In situ ADA enzyme analysis of primary murine marrow-derived progenitor colonies. The locations of the human and murine isozymes are shown on the left. Colony morphology is noted above each lane. The positive control lane is a lysate of the murine stem cell line FDCP-MIX infected with the recombinant retrovirus DHFR*-SVADA (23). (A) Progenitor colonies infected with ΔPGK-ADA sense; (B) progenitor colonies infected with PGK-ADA sense; (C) progenitor colonies infected with ΔPGK-ADA antisense. Abbreviations of CFU type: GMφ, granulocyte-macrophage; MIX, mixed myeloid-erythroid; MIX-MEGA, mixed myeloid-erythroid and megakaryocytic; G, granulocyte.

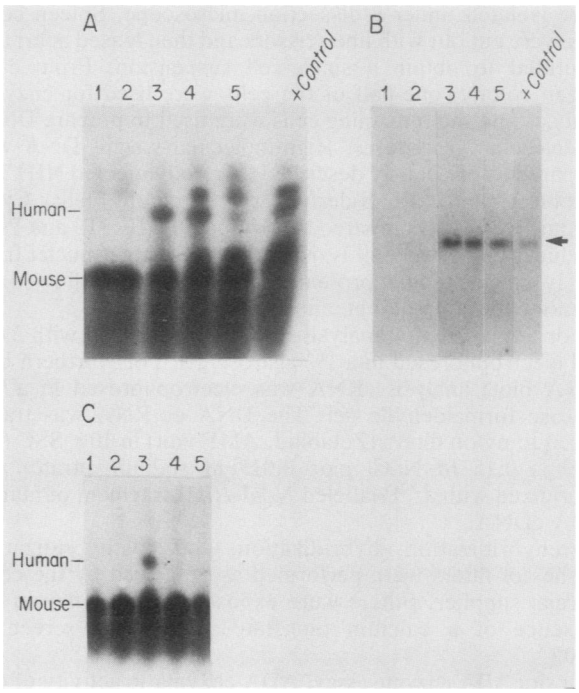

FIG. 3. In situ ADA enzyme analysis (A and C) and Southern blot analysis (B) of protein extracts and DNA obtained from CFU-S-derived spleen colonies. Migration points of human and murine isozymes are noted. (A and B) Spleen colonies derived after infection of primary murine cells with the ΔPGK-ADA sense virus. (C) Spleen colonies derived after infection with the PGK-ADA sense virus. Lane numbers in panel A correspond to the lane numbers in panel B. The positive control lane in A is the same as in Fig. 2. The positive control lane in panel B is DNA obtained from NIH 3T3 cells infected with limiting dilutions of ΔPGK-ADA sense virus and selected in Xyl-A–dCF as described in the text. DNA samples in panel B were digested to completion with *Xho*I (see text). The probe for Southern blot analysis was identical to that described for Fig. 1B. The ADA-hybridizing provirus is shown (arrow).

Effect of MoMuLV enhancer on human ADA expression in primary marrow-derived progenitor colonies. In primitive stem cells, such as teratocarcinoma cells, the MoMuLV enhancer has been implicated as a negative regulatory element (6, 19). The effect of the MoMuLV enhancer on ADA expression in primary hematopoietic stem cells has not been previously examined with otherwise identical retroviral constructs. We compared ADA expression in primary marrow-derived progenitor colonies infected with PGK-ADA vectors identical except for the presence (PGK-ADA sense) or absence (ΔPGK-ADA sense) of the MoMuLV enhancer sequence. Figure 2A shows in situ enzyme analysis of representative progenitor colonies infected with ΔPGK-ADA sense (enhancerless) virus. The human ADA enzyme migrated more slowly than the mouse enzyme, allowing a clear separation of the two enzymes. In some instances, an additional ADA band running slower than the primary isozyme band of each species gave rise to a doublet (Fig. 3A, lanes 4, 5, and control). This secondary band was never present in the absence of the primary ADA band. Expression of human ADA enzyme was at levels approximating the endogenous murine level. No difference was noted between colonies containing only myeloid (CFU-GM) and those

TABLE 2. ADA enzyme analysis of primary CFU-S-derived spleen colonies

Vector	No. of positive[a] colonies/ no. examined	
	Unselected	Selected[b]
PGK-ADA sense	3/71	5/19
ΔPGK-ADA sense	1/32	2/7
ΔPGK-ADA antisense	7/20	ND[c]

[a] DNA and human enzyme positive.
[b] Selected spleen colonies were derived by culturing infected bone marrow cells in Xyl-A–dCF for 48 h prior to injection into recipient animals.
[c] ND, Not done.

containing myeloid and erythroid (CFU-MIX) cells. Figure 2B shows an in situ enzyme analysis of representative primary marrow-derived progenitor colonies infected with the PGK-ADA sense (with enhancer) virus. No significant difference was seen in the level of human ADA expression in those colonies versus that in colonies derived from progenitor cells infected with the enhancerless ΔPGK-ADA virus (Fig. 2A). Analysis of progenitor colonies infected with ΔPGK-ADA antisense (Fig. 2C) showed human ADA enzyme at levels slightly lower than the endogenous murine ADA levels. ADA expression with this vector, as with the ΔPGK-ADA sense vector, was dependent on expression from the internal *pgk* promoter. Therefore, the difference in the level of expression between ΔPGK-ADA antisense and ΔPGK-ADA sense, if any, is not due to the presence or absence of the LTR promoter. These data were confirmed by ADA enzyme analysis of CFU-S-derived spleen colonies in vivo (see below).

Integration of PGK-ADA proviruses and expression of human ADA in CFU-S-derived colonies. Since other retroviruses containing human ADA cDNA have failed to produce functional enzyme in vivo (15, 23, 24), we examined expression in CFU-S-derived spleen colonies in vivo. Bone marrow cells cocultured for 24 h on monolayers of Psi-2 producer cells of each of the three vectors (PGK-ADA sense, ΔPGK-ADA sense, and ΔPGK-ADA antisense) were injected into lethally irradiated animals either without selection (1×10^5 to 3×10^5 cells per animal) or after 48 h of preselection in Xyl-A and dCF (5×10^5 to 8×10^5 cells per animal). Single and discrete colonies were dissected beginning 14 days after transplantation, and the cell suspensions were divided for enzyme assay and Southern analysis. A total of 123 colonies were obtained from transplantation with unselected bone marrow cells (Table 2). DNA obtained from these colonies was digested with *Xho*I, which cleaves at the borders of the PGK-ADA cassette (Fig. 1A). Eleven of 123 (9%) colonies were found to be positive for unrearranged PGK-ADA transcriptional sequence. Every spleen colony found to contain the PGK-ADA provirus by Southern analysis expressed detectable human ADA activity by enzyme analysis (see below). Conversely, no human ADA activity was noted in spleen colonies from which the DNA obtained showed no PGK-ADA sequence.

Twenty-six CFU-S-derived spleen colonies were obtained from animals injected with cells preselected for 48 h prior to transplantation. DNA from seven (27%) of these colonies demonstrated the presence of an unrearranged provirus. Human ADA enzyme activity was detected in all seven colonies. Examples of results from preselected cells are shown in Fig. 3. Figure 3A shows the ADA enzyme activity (by in situ gel analysis) of spleen colonies obtained from infection with the ΔPGK-ADA sense virus and shows two human-enzyme-negative (lanes 1 and 2) and three human-enzyme-positive (lanes 3 to 5) spleen colonies. Southern blot analysis of DNA obtained from these same colonies is shown in Fig. 3B. Lanes 1 and 2 show no hybridization to human ADA sequences, while lanes 3 to 5 show an unrearranged PGK-ADA transcriptional unit (at arrow) compared with infected NIH 3T3 cells (control lane). Southern blot analysis of DNA obtained from spleen colonies infected with PGK-ADA sense and ΔPGK-ADA antisense showed the same correlation between presence of the provirus by Southern blot analysis (not shown) and enzyme activity.

Figure 3C shows an example of the ADA enzyme activity in a spleen colony obtained from infection with PGK-ADA sense-containing virus. Lanes 1, 2, 4, and 5 contain cells from DNA-negative spleen colonies, while lane 3 contains cells from a DNA-positive spleen colony.

Human ADA enzyme activity in this and other DNA-positive spleen colonies generated from infection with the PGK-ADA sense (with enhancer) vector was detected and was similar to the level of human ADA enzyme activity in spleen colonies generated from the ΔPGK-ADA sense (enhancerless) vector (Fig. 3A). Similar results were obtained with spleen colonies generated from infection with the ΔPGK-ADA antisense vector (data not shown).

Stability of human ADA expression in secondary progenitor colonies. Expression of some sequences transferred by recombinant retrovirus vectors appears to diminish in vivo when analyzed by replating progenitor colonies from long-term reconstituted mice (4, 10). As yet, it has not been possible in our studies to examine the stability of ADA expression in vivo (see below). However, we examined the stability of ADA expression in progenitor replating experiments in vitro.

Six large progenitor-derived colonies that initially scored

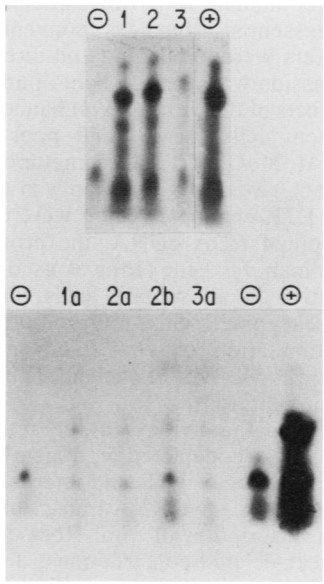

FIG. 4. In situ ADA enzyme analysis of primary (top) and secondary (bottom) progenitor colonies. Three primary progenitor colonies (lanes 1, 2, and 3) derived from infection with the ΔPGK-ADA sense virus were divided for enzyme analysis (top), and the remainder were suspended and replated for secondary progenitor colonies (bottom). Symbols: −, negative control lanes showing only murine isozyme; +, positive control lane (same as in Fig. 2) showing murine and human isozymes. The secondary colonies are numbered according to the primary colony from which each was derived.

positive for human enzyme activity were replated in the CFU-GEMM assay. Three of these yielded secondary colonies sufficiently large for further enzyme analysis. Figure 4 (lanes 1 through 3) shows human ADA enzyme activity in a fraction of each of these primary colonies and also shows one negative control colony. ADA enzyme activity was found in a fraction of each of these three primary colonies (Fig. 4, lanes 1, 2, and 3), and one colony was negative for human enzyme activity (Fig. 4). Figure 4 (lanes 1a, 2a, 2b, and 3a) shows examples of enzyme activity from the corresponding secondary colonies derived from primary colonies shown in lanes 1, 2, and 3. All secondary progenitor colonies demonstrated the same level of human ADA enzyme compared with endogenous murine enzyme activity as the primary colonies. Secondary colonies from negative primary colonies remained negative for human ADA activity (data not shown).

DISCUSSION

The expression of foreign genetic sequences introduced into primary murine hematopoietic stem cells by retroviral vectors appears to be under different regulatory controls than expression in cultured murine cell lines (15, 23; D. A. Williams et al., submitted for publication). This difference does not appear to be related to the primitive nature or differentiation characteristics of the transduced cell, since we have recently found a similar disparity between expression of introduced ADA cDNA in primary hematopoietic stem cells and primitive multipotent immortalized hematopoietic stem cell lines (Williams et al., submitted). Since the contribution of specific sequences contained in various retrovirus vectors in anomalous expression in vivo has not been defined, we constructed simplified retrovirus vectors carrying human ADA cDNA. Several considerations dictated the design of the recombinant retroviruses depicted in Fig. 1A. To remove nonessential sequences, conventional dominant selectable markers were eliminated and direct selection for ADA overexpression was used. In light of uncertainty about the potential effects of the MoMuLV enhancer on expression in primitive stem cells (6, 19) and poor transcriptional activity of the MoMuLV LTR in hematopoietic stem cells (23), recombinant viruses differing only in the presence or absence of the LTR enhancer region were constructed. To direct expression of ADA cDNA, the promoter of the X chromosome human *pgk* gene (16) was used. This promoter is normally active in all somatic cells, conforms to the GC-rich "housekeeping" class of promoters, and is not sensitive to stimulation of viral enhancers in transient expression assays (A. M. Michelson and S. H. Orkin, manuscript in preparation).

The transcriptional activities of the retrovirus vectors shown in Fig. 1A are depicted in Fig. 1B. In the Psi-2 producer lines, both the 5′ LTR and internal *pgk* promoters are active, giving rise to two RNA transcripts in these cells. After reverse transcription of viral RNA in cells infected with the enhancer-deleted constructions, duplication of the 3′ LTR occurs and abolishes transcriptional competence of the 5′ LTR, resulting in transcription only from the internal *pgk* promoter. To further ensure that human ADA expression was derived only from the internal *pgk* promoter, we constructed the ΔPGK-ADA antisense vector, in which the *pgk* promoter and ADA cDNA were reversed in orientation relative to the 5′ LTR. The 3′-flanking region of the human ADA gene was added to provide appropriate signals for polyadenylation for RNA transcripts. A single ADA-hybridizing RNA transcript was seen in cells infected with this antisense vector (Fig. 1B). These studies demonstrated inactivation of transcription from the MoMuLV promoter in cells infected with vectors containing the enhancer-deleted LTR.

Recombinant retroviruses of each PGK-ADA vector produced by Psi-2 packaging cells were used to transfer human ADA cDNA into primary murine hematopoietic cells. The transferred human ADA cDNA expressed functional human ADA enzyme at easily detectable levels in vivo and at levels comparable to endogenous murine ADA enzyme levels in progenitor colonies in vitro. The expression of human ADA in vivo is in contrast to the lack of ADA expression that our laboratory and other investigators have reported previously (15, 23, 24). Retrovirus constructs containing a variety of dominant selectable markers (hypoxanthine phosphoribosyltransferase, neomycin phosphotransferase, and DHFR) and internal promoters (human ADA, herpesvirus thymidine kinase, mouse metallothionein, SV40 early-region, cytomegalovirus, and adenovirus E1A promoters) have consistently expressed ADA in cell lines but failed to express ADA in primary murine hematopoietic cells in vivo (15, 23, 24; unpublished results). However, the *pgk* promoter alone does not appear to be responsible for the difference in ADA expression in vivo reported here. We have detected human ADA enzyme in primary murine marrow-derived progenitor colonies infected with a recombinant retrovirus vector in which the *pgk* promoter has been replaced by the SV40 early-region promoter (unpublished data). The contribution of specific sequences to the lack of expression of ADA in vivo with other retroviruses remains unclear, but unknown aspects of vector design may be important in affecting expression in primary murine hematopoietic cells.

The expression of human ADA in CFU-S-derived colonies appears to be more variable, and on average lower, than that observed in progenitor-derived colonies. This difference may be due to variable dilution by host stromal cells in isolated CFU-S colonies. Alternatively, the difference in expression may reflect fundamental biological differences between CFU-S stem cells and the progenitors which give rise to in vitro colonies. However, the in vitro progenitor colonies studied in these experiments were derived from bone marrow cells harvested from animals which had been pretreated with 5-FU. Other investigators have shown that in vitro progenitor colonies derived from 5-FU-resistant marrow cells are capable of giving rise to secondary CFU-S-derived colonies when injected into animals (7). We examined the self-renewal ability of the progenitors in this study by replating some of the colonies in vitro. The secondary colonies obtained continued to demonstrate human ADA expression relative to murine ADA expression similar to the primary progenitor colonies. This observation also indicates that expression of exogenous DNA was stable during further self-renewal and differentiation.

An important and unresolved issue relates to the expression of transferred ADA in hematopoietic stem cells and the progeny of these cells in long-term reconstituted animals. We have not been successful yet in addressing the stability of expression in vivo directly due to the relatively low titers of our producer clones and the failure of 48 h of Xyl-A–dCF selection in vitro prior to transplantation to kill nontransduced cells. Nevertheless, the stability of expression in secondary progenitor colonies is encouraging for the prospects for transferring and expressing foreign sequences in hematopoietic stem cells in vivo.

Inclusion of an easily assayable marker sequence such as

ADA cDNA offers the advantage of surveying systematically the effects of specific sequences on expression in primary hematopoietic cells infected with retrovirus vectors. For instance, by direct comparison of PGK-ADA vectors differing only in the presence or absence of the MoMuLV enhancer in the retroviral LTR, we found no evidence of negative effects of the MoMuLV LTR enhancer on *pgk*-directed ADA expression in primary murine hematopoietic cells in vivo. We and other investigators (12, 22) have previously postulated that the MoMuLV enhancer may play a negative role in expression of retrovirus-transferred genetic sequences in murine hematopoietic cells in vivo. In contrast to previous work (12), we have examined the role of the MoMuLV enhancer directly by comparing expression of transferred ADA cDNA in otherwise identical vectors.

Further modification of the PGK-ADA vectors may allow the design of optimal vectors for efficient transfer of the human ADA cDNA and expression of human enzyme activity in primary murine and human hematopoietic stem cells.

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