Restriction of Expression of an Integrated Recombinant Retrovirus in Primary but Not Immortalized Murine Hematopoietic Stem Cells

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A recombinant retrovirus (DHFR*·SVADA) in which human adenosine deaminase (ADA) cDNA is transcribed from an internal SV40 promoter was used to infect murine hematopoietic stem and progenitor cells. Human ADA enzyme was not expressed in infected primary murine pluripotent stem cell–derived spleen or progenitor colonies (CFU-GM, CFU-Mix, BFU-E). In contrast, human ADA enzyme activity was readily detected in progenitor colonies derived from immortalized multipotent factor–dependent cells. The level of human enzyme was near endogenous murine enzyme levels and was equivalent in undifferentiated stem cells and differentiated myeloid, erythroid, and mixed colonies. These results indicate that cellular properties other than the stage of differentiation are important in determining the expression of foreign sequences introduced by retroviruses. Cell lines that are immortalized but still capable of induced differentiation may contain factors that abrogate blocks to expression that are manifested in primary hematopoietic stem cells.

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Molecular techniques to effect gene transfer into hematopoietic stem cells provide the opportunity to investigate expression of foreign sequences introduced into developmentally competent cells. Because of the paucity of hematopoietic stem cells in bone marrow, retrovirus vectors have been extensively explored as vehicles for gene transfer for biologic studies and potential somatic genetic therapy. Transduction of pluripotent hematopoietic murine stem cells has been achieved with a variety of recombinant retroviruses. In the earliest reports, expression of transferred sequences has appeared problematic. For example, low-level transcription from the retroviral long terminal repeat (LTR) compared with that seen in tissue culture cells has been evident in murine hematopoietic stem cells, specifically CFU-S. Also, an apparent progressive shutdown of expression of the transferred sequences may occur in reconstituted animals. Finally, retroviruses that transfer and express sequences efficiently in cell culture may fail to be expressed in primary hematopoietic cells.

The bases for these observations are not yet defined but may relate to idiosyncratic features of the retroviruses used, specific sequences contained within the viruses, or the nature of the target cells themselves. In several respects, hematopoietic stem cells may be analogous to embryonal carcinoma (EC) or teratocarcinoma cells, which maintain the capacity for self-renewal and multilineage differentiation. In undifferentiated EC cells, the Moloney murine leukemia virus (MoMuLV) LTR and the SV40 early-region promoter function poorly as transcriptional elements. In mature, end-stage cells the block to expression is reduced.

The contribution of the state of differentiation of hematopoietic progenitors and other factors such as the immortalized state to the anomalous expression from an integrated retrovirus is the subject of the studies reported here. We have compared the expression of a reporter sequence (adenosine deaminase [ADA] cDNA) transcribed from an internal SV40 promoter in primary murine hematopoietic cells and primitive growth factor–dependent murine stem cell lines, termed factor-dependent cell Patterson–Mix (FDCP-Mix). Because the FDCP-Mix stem cells are capable of multilineage differentiation in vitro, we have also compared the expression of this same sequence in the cell lines (which contain less than 1% mature cells) and the differentiated progeny derived from these cells. Our results suggest that distinct differences in expression of foreign genetic sequences exist between primary cells and immortalized cell lines.

MATERIALS AND METHODS

Cells and viruses. The virus producer lines used were Psi-AM–derived clones producing DHFR·SVADA at titers of 1 x 10^7 methotrexate-resistant colonies/mL when assayed on NIH/3T3 cells. The derivation and structure of DHFR·SVADA has been previously published. Cell lines were maintained in alpha minimum essential media (α-MEM) with 5% calf serum (CS) and 5% fetal calf serum (FCS) with antibiotics (all GIBCO, Grand Island, NY). FDCP-Mix cell lines were derived as previously described and maintained in Fischer’s modified medium for mouse leukemia cells (GIBCO) with 20% horse serum (Flow Laboratories, McLean, VA), 10% WEHI-3b–conditioned media as a source of growth factor, and antibiotics.

Infection of FDCP-Mix cells with recombinant viruses. Infection was accomplished by cocultivation of 5 x 10^5 FDCP-Mix cells with confluent, irradiated (13 Gy) monolayers of Psi-AM producer lines in α-MEM, 5% CS, and 5% FCS with hexadimethrine bromide (Polybrene; Aldrich Chemical Co, New York; final concentration, 4 µg/mL) for 48 hours. Nonadherent cells were subsequently harvested, selected for 48 to 72 hours in 0.2 µmol/L methotrexate.

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Progenitor colony assay. Progenitor colonies were grown as previously described, with minor modifications. Briefly, 3 x 10^4 to 1 x 10^5 FDCP-Mix cells were plated in 1% α-MEM plus methylcellulose, 24% horse serum or human plasma, 1% bovine serum albumin (BSA; Boehringer Mannheim, Indianapolis), 1% penicillin-streptomycin, 10⁻² mol/L mercaptoethanol (Sigma Chemical Co, St Louis), and 10% WEHI-3b–conditioned medium as a source of colony-stimulating factor with or without 2 U/mL erythropoietin (Connaught Laboratories, Inc, Swiftwater, PA). Progenitor cocktails were plated in 1 mL of culture medium (in triplicates) in gridded 10 x 35-mm tissue culture plates (Lux, Harbor, ME) were infected and selected for 48 to 72 hours in 0.2 mol/L methotrexate and then either plated in methylcellulose (with or without methotrexate) or continued in liquid culture with methotrexate selection. Colonies were scored (>50 cells) at ten days and enzyme analysis performed at ten to 20 days. Freshly explanted murine bone marrow obtained from the hind limbs of C57/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were infected and selected in identical fashion and then plated in methylcellulose. The use of horse serum obviates the need for dialysis of the serum to reduce background colonies with methotrexate selection. Human plasma was used in some progenitor cultures to produce terminal differentiation of FDCP-Mix cells.

Southern and Northern analysis. Standard methods were used to prepare high–molecular weight genomic DNA and total cellular RNA from FDCP-Mix cells. DNA was digested with XbaI or XhoI (New England Biolabs, Boston). Digested DNA or RNA was electrophoresed through 1% agarose gel. The DNA was transferred to a Zetabind filter (AMF/Voit, Menidan, CT) in 10x standard sodium citrate (SSC) and hybridized to a 32P-labeled approximately 1.6-kb fragment of ADA cDNA. Prehybridization, hybridization, and posthybridization washes were carried out as described by the manufacturer. X-ray films were exposed at −70°C in the presence of a calcium tungstate intensifying screen.

In situ assay for ADA enzyme activity. Enzymatic activity of the cell lysate of colonies derived from normal marrow progenitor cells or from FDCP-Mix cells were examined by electrophoretic separation on cellulose acetate strips. Individual progenitor-derived colonies were plucked from the methylcellulose with a finely drawn Pasteur pipette, viewed by using an inverted microscope, and loaded onto cellulose acetate strips (Helena Laboratories, Beaumont, TX) presoaked in running buffer (Supra-Heme buffer, Helena). Colonies were lysed by repeated freezing and thawing on dry ice. For the FDCP-Mix cell lines, cell pellets were lysed by freezing and thawing in lysis buffer consisting of 5 mmol/L K,HPO₄ and 1 mmol/L 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 2 mg/mL adenosine (Sigma), 0.06 U/mL xanthine oxidase, 15 µg/mL nucleoside phosphorylase (both Boehringer Mannheim), 1.0 mg/mL dimethylthiazol diphenyltetrazolium bromide, and 0.1 mg/mL phenazine methosulfate (both Sigma) in phosphate buffer at 37°C for ten minutes.

In situ hybridization. In situ hybridization was performed by modification of a previously published technique. Cell lines or pooled colonies were washed and resuspended in MEM at 10⁶ cells/mL and cytospin centrifuged onto clean glass slides. The cells were fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for five minutes and stored in 70% alcohol at 4°C until used for hybridization. Slide preparations were rehydrated in PBS and incubated in 1 µg/mL of proteinase K (Bethesda Research Laboratories, Gaithersburg, MD) in 0.1 mol/L Tris and 50 mmol/L EDTA at 37°C for five minutes. The proteolytic reaction was terminated by immersion in 4% paraformaldehyde in PBS for one minute. The cells were rinsed in PBS, placed in 0.2 mol/L Tris and 0.1 mol/L glycine for ten minutes at room temperature followed by acetylation in freshly prepared 0.25% acetyl anhydride in 0.1 mol/L triethanolamine buffer. The slides were prehybridized in 50% denatured formamide and 2 x SSC (0.3 mol/L sodium citrate buffer) at 37°C for 15 minutes. For hybridization, the probe, salmon sperm DNA, and yeast tRNA were suspended in 100% formamide and heated to 80°C to denature the DNA heteroduplexes. The DNA was then mixed with hybridization buffer for a final concentration of 50% formamide, 2 x SSC, 1% BSA, and 300 µg/mL salmon sperm DNA and tRNA. Twenty microliters of the hybridization mix was applied to the prehybridized cells, covered with a clean glass coverslip, and hybridized in a moist chamber at 37°C for 12 to 16 hours. The probe consisted of a NcoI-Hinfl fragment of ADA cDNA labeled with 32P-cytochrome c (CTP) by the random hexamer priming technique. Control probes consisted of similarly labeled and sized λ DNA sequences. Activity of 5 x 10⁶ cpm of probe was added to each slide.

After hybridization, the slides were washed in 50% formamide and 2 x SSC at 37°C for 30 minutes followed by a similar wash in 50% formamide and 1 x SSC. The slides were dehydrated in graded alcohols, air-dried, and autoradiographed with NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled water. After exposure for five days at 4°C, the slides were developed in Kodak D-19 and stained with Wright’s stain (Harelco, Gibbstown, NJ).

RESULTS

Infection of FDCP-Mix cells with DHFR*-SVADA. An amphotropic producer of DHFR*-SVADA (titer, 1 x 10⁴) was used to infect FDCP-Mix cells in a 48-hour cocultivation. In this retrovirus, dihydrofolate reductase (DHFR) is expressed from the MoMuLV LTR and transfers resistance to methotrexate. Human ADA cDNA is expressed from the SV40 early promoter. After infection, FDCP-Mix cells were selected for 48 to 72 hours in 0.2 µmol/L methotrexate and then either plated in methylcellulose (with or without methotrexate) or continued in liquid culture with methotrexate. FDCP-Mix cells plated at a low cell density gave rise to progenitor-derived colonies of morphologically normal myeloid or erythroid cells (see the next section). Inclusion of methotrexate dramatically decreased the growth of progenitors in control cultures but had minimal effects on infected cells (Table 1). By comparison, infection of normal bone marrow with an ecotropic producer of DHFR*-SVADA...
Southern blot analysis of XbaI- (lanes 1 and 2) and XhoI-digested (lanes 3 to 5) genomic DNA. The provirus genome is shown below with the location of relevant restriction enzyme sites. Lanes 1 and 4 contain DNA from infected and selected NIH/3T3 cells, lanes 2 and 5 from infected and selected FDCP-Mix cells, and lane 3 from uninfected FDCP-Mix cells. The probe is a 32P-labeled, Ncol-Hinfl ADA cDNA fragment.

DNA from FDCP-Mix cells infected with DHFR*-SVADA were analyzed by Southern blot analysis to document integration and structure of the provirus. DNA was digested either within each LTR to ascertain the presence of an intact provirus structure or internally to establish the

Fig 1.

Fig 2. Northern blot analysis of RNA from infected and selected NIH/3T3 fibroblasts (lane 1, left) and infected and selected FDCP-Mix cells (lane 2, left). Ethidium bromide in the same RNA samples is shown on right. The probe used as in Fig 1.

Fig 3. ADA expression in (A) primary murine hematopoietic progenitor-derived colonies, (B) uninduced FDCP-Mix stem cell lines, and (C) FDCP-Mix-derived progenitor colonies.
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integrity of the SV40–ADA cDNA transcriptional unit. Methotrexate-selected FDCP-Mix cells contained an intact provirus (Fig 1, lane 2) and an unrarranged SV40–ADA cDNA unit (Fig 1, lane 5).

Expression of foreign sequences in colonies derived from transduced FDCP-Mix cells or from transduced normal marrow progenitor cells. Northern blot analysis of FDCP-Mix cells infected with DHFR–SVADA and selected in methotrexate showed two RNA transcripts hybridizing to an ADA probe (Fig 2, lane 2). The larger and more abundant transcript (Fig 2, a) corresponds in size to that expected from initiation at the Moloney LTR promoter. In contrast to CFU-S–derived primary spleen colonies, the level of the LTR-initiated transcript in FDCP-Mix cells is similar to that seen in infected NIH/3T3 (Fig 2, lane 1). The level of LTR-initiated message in primary CFU-S–derived spleen colonies is below the level of detection by Northern blot analysis. Based on the level of transcript detected by more sensitive S1-nuclease protection experiments in these spleen colonies, we estimate that the amount of RNA detected in FDCP-Mix cells (Fig 2, lane 2) is at least 50-fold higher than in primary CFU-S–derived spleen colonies. In addition, a second RNA transcript corresponding in size to that expected from initiation at the SV40 promoter (Fig 2, b) is also detected. Again this is in contrast to data obtained and previously reported from primary CFU-S infected with DHFR–SVADA that contain no RNA transcripts initiated at the SV40 promoter. The relative abundance of the two transcripts is similar in NIH/3T3 fibroblast and FDCP-Mix cells. Therefore FDCP-Mix cells contain significantly more RNA initiated from both the LTR promoter and the internal SV40 promoter than do primary CFU-S–derived spleen colonies and, presumably, progenitor-derived primary colonies (see the following material). As expected from this RNA analysis, CFU-S–derived primary spleen colonies containing intact DHFR–SVADA provirus do not express human ADA enzyme. In addition, analysis of over 200 normal marrow-derived progenitor cell colonies (CFU-MIX, BFU-E, and CFU-GM) infected with DHFR–SVADA has failed to reveal human ADA enzyme, even after preselection or continuous selection with methotrexate. Figure 3A shows an enzyme analysis on ten methotrexate-resistant, primary marrow-derived colonies of myeloid, erythroid, and mixed lineages.

Analysis of infected and selected FDCP-Mix cells showed human ADA expression at a level roughly equal to the endogenous murine expression by an in situ cellulose acetate gel assay (Fig 3B). This result has been confirmed in a variety of different FDCP-Mix cell lines (FDCP-Mix 9, 15, 17). Furthermore, there appears to be no difference in expression of the transferred cDNA between cell lines after selection for transduced cells. Cells remained morphologically undifferentiated after transduction and selection (Fig 4A). Cell cycle time and plating efficiency also remained unchanged.

Methotrexate-resistant progenitor colonies derived from the FDCP-Mix cells were individually analyzed for human ADA expression. Colony morphology allowed a distinction between colonies containing predominantly myeloid (CFU-GM–derived) colonies (Fig 4B), erythroid (BFU-E–derived) colonies, or a mixture of both (CFU-MIX–derived) colonies. Wright-Giemsa and benzidine stain confirmed colony morphology showing granulocytic differentiation in myeloid colonies and benzidine-positive cells in BFU-E and CFU-MIX colonies (Fig 4C). Analysis of individual progenitor-derived colonies revealed human ADA expression at or near endogenous murine expression in methotrexate-resistant colonies. Figure 3C shows enzyme analysis on nine FDCP-MIX–derived progenitor colonies. Human enzyme was detected in 100% of methotrexate-resistant colonies examined by in situ gel analysis. Although some variation in expression is seen between individual colonies, the expression is remarkably uniform. There is no difference in the level of expression between erythroid v myeloid colonies (compare

Fig 4. (A) Morphology of FDCP-Mix cell line after infection and selection. (B) FDCP-Mix–derived progenitor colony grown in methylcellulose containing methotrexate. (C) Benzidene stain of a mixed myeloid and erythroid FDCP-derived colony grown in methylcellulose. The arrow denotes benzidine-positive cells.
lenses BFU-E to lanes CFU-GM in Fig 2C). No difference in expression is seen between the undifferentiated FDCP-Mix stem cell line and differentiated progeny contained in colonies derived from them. This result was confirmed by in situ hybridization analysis for human ADA RNA transcripts in methotrexate-resistant, FDCP-Mix-derived colonies. Individual colonies contain both differentiated (granulocytes) and undifferentiated (blast) cells. No difference in expression was seen in undifferentiated v differentiated cells contained in the same colony by in situ RNA hybridization (Fig 5), which implies that levels of human ADA are similar in both cell types. The presence of human enzyme in progenitor colonies plated after only 48 to 72 hours' selection demonstrates that the expression seen in FDCP-Mix cells is not due to the growth of rare clones of cells that are not restricted for expression of DHFR (and therefore methotrexate resistant).

**DISCUSSION**

Introduction of foreign genetic sequences into hematopoietic stem cells allows the study of expression of the introduced sequences during differentiation in vivo and in vitro. Although we and other investigators have shown expression of foreign genetic sequences in cells derived from primary pluripotent hematopoietic stem cells, the level of expression appears to be quantitatively reduced compared with expression in cell lines when using some retroviral constructs. We have previously reported the failure of a particular retrovirus vector, DHFR*-SVADA, to adequately express human ADA in CFU-S-derived primary spleen colonies in spite of its ability to express it efficiently in tissue culture lines of several origins including lymphocytic cell lines. This lack of ADA expression in primary CFU-S-derived spleen colonies has been seen by other investigators using similar retroviruses with different selectable markers and promoters. Because the spleen colonies derived from CFU-S contain mainly maturing myeloid cells, this suggests that a block in expression occurs during differentiation and development of the transduced cells. This may be related to the primitive nature of the CFU-S and sequences within the specific retroviral construct used to infect these cells. Such blocks to expression have been seen also in primitive teratocarcinoma cells (EC) when using the MoMuLV LTR promoter, the SV40 early promoter, and the polyoma virus promoter. Our laboratory has recently reported the expression of human ADA in primary hematopoietic stem cells in vivo by using simplified retrovirus vectors. These vectors differ from DHFR*-SVADA in that they contain no selectable marker other than ADA (such as DHFR or Neomycin resistance). In addition, ADA is transcribed off the human X-chromosome phosphoglycerate kinase (PGK) promoter. The PGK promoter is not dependent on enhancer sequences for expression (Michelson and Orkin, unpublished results).

Using immortalized murine multipotent hematopoietic stem cell lines we have directly examined the role of differentiated state of the transduced cell on expression of exogenous DNA sequences. Factor-dependent cells derived from long-term marrow cultures maintain the capacity to differentiate along myeloid and erythroid lineages and form mixed myeloid/erythroid colonies in vitro. In addition, some clones maintain the ability to form spleen colonies and reconstitute the hematopoietic system when injected into lethally irradiated murine recipients in vivo. Although we have been unable to generate spleen colonies derived from transduced FDCP-Mix cells, these stem cells are at least as primitive as the CFU-MIX, CFU-GM, and BFU-E progenitors of primary murine bone marrow. The results reported here demonstrate that these primitive stem cells are permissive for the expression of introduced human ADA cDNA initiated from the SV40 promoter. Also there is no demonstrable difference in expression between the differentiated progeny and the primitive stem cells.

We have used the DHFR*-SVADA retrovirus as a probe to investigate subtle differences between primary hematopoietic cells and immortalized FDCP-Mix counterparts. The FDCP-Mix cell lines (and other cultured cell lines) may contain factors that abrogate blocks to expression that are manifested in primary cells. Altered regulation of gene expression may be an intrinsic part of the establishment of the immortalized state. The FDCP-Mix line may ultimately provide a system to study these features of eukaryotic gene expression.

Although it is tempting to speculate that the anomalous expression of the DHFR*-SVADA retrovirus in primary hematopoietic cells is specifically referable to the SV40
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promoter (or enhancer), this is unlikely to be the case. In particular, a variety of other promoters (eg, the endogenous ADA promoter, thymidine kinase, and metallothionein) have been used unsuccessfully in the same basic configuration as the SV40 promoter in the DHFR*-SVADA virus to express the ADA cDNA in primary hematopoietic cells in this and other laboratories. In addition, replacement of the SV40 promoter with the CMV promoter results in human ADA expression in FDCP-Mix-derived progenitor colonies and undifferentiated cells but no expression in infected CFU-S-derived primary spleen colonies or methotrexate-resistant, primary progenitor-derived colonies (Williams and Lim, unpublished results). Therefore, the precise determinants of the provirus unit that are recognized differentially in primary and immortalized murine hematopoietic cells are not as yet clear.

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