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Genetics

Expression of the human β-globin gene following retroviral-mediated transfer into multipotential hematopoietic progenitors of mice

(retroviral vectors/gene regulation/colony-forming units, spleen/stem cells/gene therapy)

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ABSTRACT **Efficient transfer of the β-globin gene into primitive hematopoietic progenitors was achieved with consistent and significant expression in the progeny of those cells. Retroviral vectors containing the intact genomic human β-globin gene and the neomycin (G418)-resistance (neo^R) gene were constructed. These gave titers of 10^6 or more neo^R colony-forming units/ml when packaged in ψ2 cells. Mouse bone marrow cells were infected by coculture with producer cells and injected into lethally irradiated animals. Several parameters were varied to enhance infection frequency of colony-forming units, spleen (CFU-S); overall 41% of 116 foci studied contained an intact proviral genome. The human β-globin gene was expressed in 31 of 35 CFU-S-derived spleen colonies that contained the intact vector genome at levels ranging from 1% to 5% of that of the mouse β-globin genes. Infected bone marrow cells were also injected into genetically anemic W/W^v recipients without prior irradiation. Human β-globin chains were detected in circulating erythrocytes by immunofluorescent staining with a specific monoclonal antibody. All animals injected with donor cells that had been cultured in G418 (1 mg/ml) for 48 hr after retroviral infection had circulating erythrocytes containing human β-globin chains between 3 and 8 weeks after transplantation.**

Retroviral vectors are useful for transferring genes efficiently into embryos and embryonic (1) and hematopoietic (2) stem cells. Genes can be transferred into embryos by microinjection (3) and into embryonic stem cells by DNA-mediated gene transfer (4), but totipotent hematopoietic stem and progenitor cells represent a very small minority of cells in hematopoietic tissues. Only highly efficient retroviral vectors have proved useful for transfer into such cells. In contrast, DNA viruses have not proven useful for stable gene transfer into primary hematopoietic cells (5). Gene transfer into hematopoietic stem and progenitor cells is a highly desirable goal. First, one can study the regulation of the transferred gene during hematopoietic differentiation in experimental animals. Second, the feasibility of gene replacement therapy in experimental animals, and later perhaps in man, can be estimated (2, 6). Third, hematopoietic lineage studies can be performed to track the fate of primitive hematopoietic cells following transplantation (7–9).

The human β-globin gene is regulated normally in mouse erythroleukemia (MEL) cells following retroviral-mediated gene transfer (10, 11). We have investigated whether similar vectors can lead to globin gene expression in primary hematopoietic precursor cells. There are a number of reports demonstrating expression of transferred genes in cell lines but not in multipotential hematopoietic progenitors following retroviral gene transfer. The human adenosine deaminase (*ADA*) gene has been inserted into retroviral vectors and found to express readily in transduced cell lines (12–14) but not in colony-forming units, spleen (CFU-S)-derived colonies (12, 14, 15). Recently, Lim *et al.* (16) succeeded in making single gene *ADA* vectors (without a dominant selectable marker gene) containing the *ADA* gene driven by the human phosphoglycerate kinase promoter that resulted in transfer and expression of the *ADA* gene in CFU-S colonies.

The present study reports experiments performed with high-titer retroviral vectors containing the intact genomic human β-globin gene with native transcriptional and processing signals. Efficient transfer of the intact β-globin gene into CFU-S was achieved. The β-globin gene was expressed in most CFU-S-derived colonies containing the intact gene. Genetically anemic W/W^v mice were reconstituted with donor cells by competitive repopulation without prior irradiation. This transplant model proved useful in serially monitoring the human β-globin in donor erythrocytes following transplantation.

METHODS

Cells and Viruses. Thymidine kinase-negative 3T3 cells, ψ2 cells, PA317 cells, and tetraploid MEL cells were grown as described (11). Viruses were titered as described before (11) but the S^+L^- helper virus assay was performed on D-56 cells (detects both ecotropic and amphotropic helper), kindly provided by Robert Bassin (National Institutes of Health). MEL cells were treated with tunicamycin (10) prior to infection with ecotropic viruses, by coculture with virus-producing cells.

Vector Construction. The β-globin retroviral vector was constructed by removing the 5′ upstream β-globin region used in our previous retroviral plasmid containing a 3.4-kilobase (kb) human β-globin gene (11) and replacing it with a shorter β-globin sequence from −270 (*Ssp* I site, converted to *Xho* I through an intermediate construct). An enhancer plus vector was made by inserting a 250-base-pair (bp) *Pst* I fragment, after addition of *Sal* I linkers, into the *Xho* I site 5′ to the β-globin gene. In the genome this fragment is located beginning 550 bp 3′ to the end of the human β-globin gene and contains an "enhancer activity" as defined by its ability to increase function of the human γ-gene promoter in transgenic mice (17, 18).

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Abbreviations: CFU-S, colony-forming units, spleen; neo^R, neomycin-resistance gene; IL, interleukin; FUra, 5-fluorouracil; MEL, mouse erythroleukemia.

Bone Marrow Infection, Transplantation, and CFU-S Assay. Bone marrow infection was done largely as described (8). Briefly, bone marrow cells were harvested from the hind limbs of C57BL/6J female donor mice. In some experiments, bone marrow was used from donor mice treated with 150 mg of 5-fluorouracil (FUra) per kg given intravenously 2 days prior to bone marrow harvest. Prestimulation was performed by incubating cells (1×10^6 per ml) in medium containing 10% or 15% WEHI 3D-conditioned medium [a source of interleukin 3 (IL-3)] alone or 15% 5637-conditioned medium [a source of interleukin 1α (IL-1α)] and 20 units of purified IL-3 per ml (a gift of J. Ihle, National Cancer Institute–Frederick Cancer Research Facility). Infection was subsequently performed by cocultivating the bone marrow cells with the virus producer cells for 24–48 hr in the presence of 8 μg of Polybrene per ml and 15% WEHI 3D-conditioned medium. When appropriate, 5637-conditioned medium and purified IL-3 were added so that the culture conditions were identical as during the prestimulation period for each experiment. Following infection the bone marrow cells were either transplanted into recipient mice or selected in medium containing 1 mg of G418 per ml and WEHI 3D-conditioned medium for 48 hr before injection. For the CFU-S assay, WBB6F1 mice were irradiated with 850 rads (1 rad = 0.01 Gy) of total body radiation and transplanted with 1–5 $\times 10^5$ cells to obtain individual spleen colonies and with 10^6 cells to obtain spleens confluent with CFU-S-derived colonies. Mice were sacrificed on day 12 or 14 and individual colonies were dissected for DNA and/or RNA extraction. W/W^v mice were injected with 10^6–10^7 viable bone marrow cells for long-term reconstitution.

Animals. C57BL/6J, WBB6F1-$\pm/\pm$, and WBB6F1-W/W^v mice were obtained from the production facility of The Jackson Laboratory. WBB6F1-W/W^v mice were initially obtained as a gift from Jane E. Barker (The Jackson Laboratory).

DNA and RNA Analysis. Southern blot analysis was done by using standard techniques. An RNase protection assay (19, 20) was performed by using probes cloned into pTZ18R and pTZ19R (Pharmacia) that were labeled to high activity with T7 polymerase; alternatively, fragments were cloned into a plasmid containing the SP6 promoter (21). RNA·RNA hybridization was carried out in 80% formamide overnight at 45°C and RNase digestion was for 30 min at room temperature. In certain experiments S1 nuclease analysis was performed as described (11).

Detection of Globin Chains. Human globin chains were detected by immunofluorescent staining using anti-human β-chain antibodies as described (22, 23). Separation of mouse hemoglobin was performed by cellulose acetate electrophoresis using applicators and gel tanks from Helena Laboratories (Beaumont, TX) as described (24).

RESULTS

High-Titer Ecotropic β-Globin Vectors. The β-globin retroviral plasmids were transfected into PA317 and ψ2 cells as described (11). Eleven of 17 virus-producing clones contained the intact β-globin proviral genome (Fig. 1) as determined by Southern blot analyses; their titers ranged from 3×10^4 to 3×10^6 colony-forming units (cfu)/ml on 3T3 cells. A producer clone having a titer of 1–2 $\times 10^6$ cfu/ml was selected for further use. Similar screening was performed with the enhancer plus vector, leading to identification of a producer clone having an intact proviral genome with a titer of 1×10^6 cfu/ml.

A diagram of the β-globin provirus and its transcripts is shown in Fig. 1. MEL cells were infected, selected in 1 mg of G418 per ml, and induced with hexamethylenebisacetamide, and RNA was isolated. An RNase protection assay

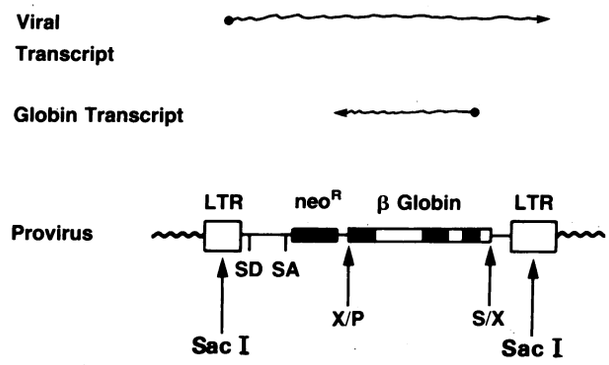

FIG. 1. Diagram of the β-globin provirus (below) and its transcripts (above). LTR, long terminal repeat; SD and SA, splice sites, donor and acceptor, respectively, that generate spliced viral transcripts. The dark boxes indicate coding sequences of the neomycin-resistance (neo^R) and the human β-globin genes. The genomic β-globin gene is reversed and therefore generates globin RNA that is transcribed in an orientation opposite to that of the viral transcript. *Sac* I sites in the retroviral LTRs, the *Xho* I site (X) in the retroviral genome, the *Pst* I site (P) at the 3′ end, and the *Ssp* I site (S) at the 5′ end of the β-globin gene are shown. These sites were joined by using linkers.

demonstrated regulated expression of the human β-globin gene in MEL cells (data not shown) that was comparable to data previously reported with the virus containing the longer globin gene fragment (11).

Gene Transfer and Expression in CFU-S-Derived Colonies. DNA extracted from individual spleen colonies was cut with *Sac* I, an enzyme that cuts in the long terminal repeats of the provirus, releasing a 5.7-kb fragment when the genome is intact (Fig. 2). The percentage of foci containing an intact proviral genome varied with various methods of infection (Table 1). A total of 116 foci were analyzed in which 47 (41%) contained an intact proviral genome. Preselection increased the proportion of positive CFU-S-derived colonies, although the number of CFU-S per 10^5 cells was reduced to 20% of control. Pretreatment of donor animals with FUra or pre-

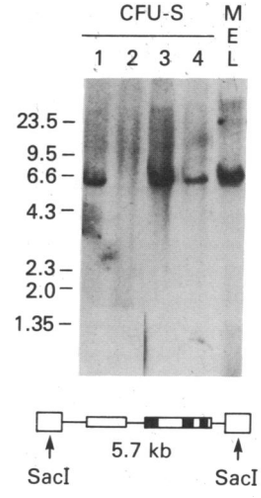

FIG. 2. Southern blot analysis of DNA from individual CFU-S colonies. MEL cell DNA containing one copy of the β-globin proviral DNA is shown as a positive control. The DNA was cut with the enzyme *Sac* I, which generates a 5.7-kb fragment as shown below the autoradiogram. The probe used was a 1.68-kb *Bam*HI–*Pst* I fragment from the human β-globin gene. Equal amounts (10 μg) of DNA were loaded in each lane. The variation in signal intensity from one lane to another presumably reflects variable contamination of cells derived from the focus by adjacent spleen cells that lack the provirus. Molecular weights are given as $M_r \times 10^{-3}$.

Table 1. Infection of mouse multipotential progenitors (CFU-S) with globin retroviral vectors

Exp.	FUra*	Prestimulation† WEHI 3D	Prestimulation† WEHI 3D + 5637	Preselection‡	CFU-S No./total	CFU-S %
1	−	−	−	−	2/12	17
2	+	+	−	−	6/22	27
3	+	−	+	−	26/62	33
4	+	−	−	−	5/9	56
5	−	−	−	+	3/4	75
6	+	−	−	+	5/7	71

*150 mg of FUra per kg was given intravenously to donor animals 48 hr before harvest of bone marrow.
†Bone marrow cells were incubated for 48 hr in medium containing 15% WEHI 3D-conditioned medium alone or 15% 5637-conditioned medium and purified IL-3 (20 units/ml) in addition before infection by coculture.
‡Infected cells were cultured in medium containing 1 mg of G418 per ml for 48 hr before reinjection.

stimulation of marrow cells *in vitro* with growth factors appeared also to enhance infection frequency (Table 1).

Expression was initially studied in RNA extracted from seven intact spleens containing several foci; data obtained on analysis of two are shown in Fig. 3*A*. A semiquantitative estimate of the relative level of expression of the integrated human β-globin gene was obtained by probing separate aliquots of the RNA samples with either a mouse β-globin RNA or human β-globin RNA probe. The signal intensity of the mouse probe fragment protected by 20 ng of spleen RNA was comparable to the intensity of the human probe fragment protected by 10 μg of RNA, suggesting that the single human β-globin gene was expressed at <1% the level of the four mouse β-globin genes.

Thirty-one of 35 individual foci containing the β-globin, studied by an RNase protection assay, contained human β-globin mRNA (Fig. 3*C* and data not shown). RNA from each was probed with the human and mouse β-globin probe. The concentration of human β-globin mRNA averaged 1% of that of mouse β-globin mRNA, with a range of 0.5–5%. The hybridizations were done under conditions of probe excess, and quantitation was achieved by densitometric scanning of the signal produced by the appropriate protected fragment. Thirteen foci were derived from progenitors infected with the enhancer plus vector; there was no difference between the average level of expression of the β-globin gene in these foci compared to the other 18 that contained the vector without the enhancer.

Detection of β-Globin in Circulating Erythrocytes. Human β-globin was detected in the circulating erythrocytes of recipient mice within 2–3 weeks of injection of infected bone marrow cells. These experiments were performed in the genetically anemic W/W^v strain. C57BL/6J cells competitively repopulate the hematopoietic tissues in W/W^v animals.

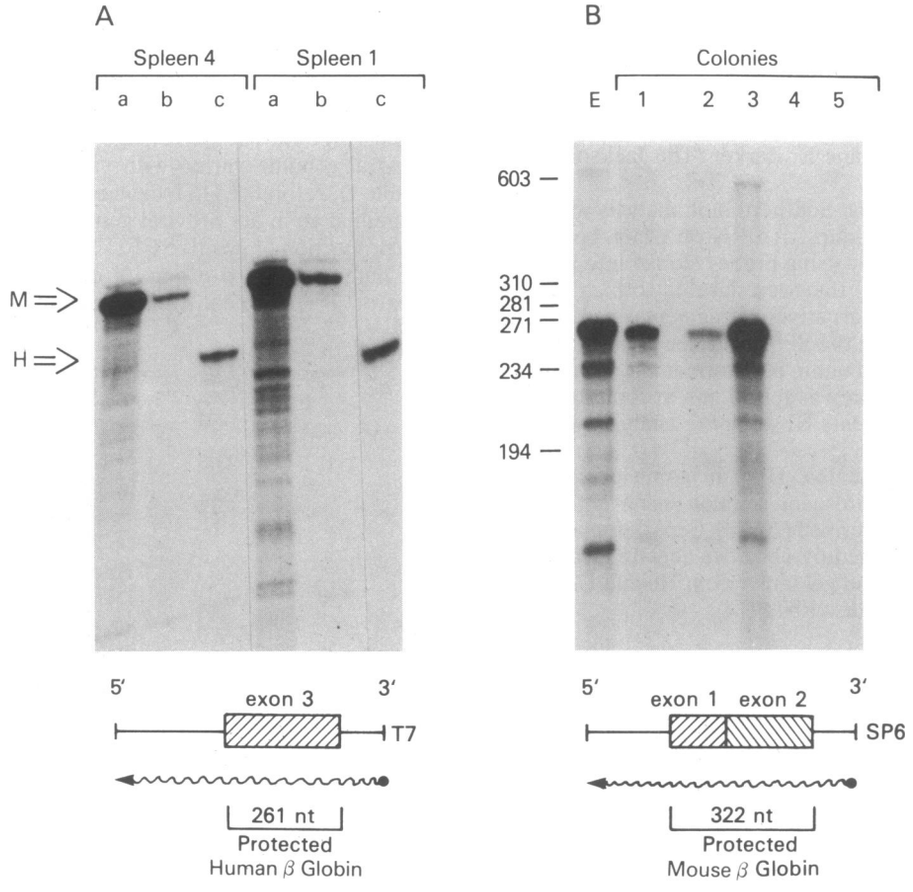

FIG. 3. Expression of human β-globin RNA in CFU-S-derived spleen colonies as detected by RNase protection assays. The probes used are outlined below the autoradiographs and the sizes of fragments protected by human β-globin mRNA [261 nucleotides (nt)] and mouse β-globin mRNA (322 nucleotides) are indicated. T7 and SP6 indicate the T7 and SP6 promoters. (*A*) Comparison of steady-state mouse β-globin (M) and human β-globin (H) mRNA levels in two spleen samples containing many CFU-S-derived colonies. Samples in lanes a and b contained 0.2 and 0.02 μg of RNA, respectively, and were probed with the mouse probe. Samples in lanes c contained 10 μg of RNA and were probed with the human probe. (*B*) RNA from individual colonies; 19.4, 5.9, and 24.3 μg of RNA were analyzed from colonies 1, 2, and 3, respectively, and 20 μg of RNA was analyzed from colonies 4 and 5. Colonies 4 and 5 did not contain the intact proviral genome. Molecular weights are given as $M_r \times 10^{-3}$.

Radiation is not necessary; the animals remained healthy so that serial sampling was feasible (25). A difference in hemoglobin phenotype between donor and recipient cells allowed reconstitution of the recipient with donor cells to be monitored. Characteristically, positive erythrocytes were detected by immunofluorescent analysis at 3 weeks, reached a peak frequency at ≈5 weeks, and diminished thereafter so that by 12 weeks only a few animals had 1–2% of erythrocytes positive for human β-chain (Figs. 4 and 5).

Selection in G418 increased the percentage of recipient animals that had positive erythrocytes from 46% without selection to 100% with selection (Table 2). All of the animals receiving nonpreselected donor cells had complete reconstitution with donor hemoglobin within 6–8 weeks of transplantation. The animals that received preselected cells had only transient engraftment reaching 20–25% donor hemoglobin at 6 weeks, but by 12 weeks all animals had reverted to the W/W^v hemoglobin phenotype. Despite dramatic differences in reconstitution between the unselected (Fig. 5*A*) and the selected (Fig. 5*B*) groups, no significant difference was seen in the human β-globin production profile with time between the two groups. Four animals reconstituted with nonselected cells were sacrificed at 12 weeks and DNA was obtained from spleen and bone marrow for Southern blot analysis. Proviral sequences were not detected in any of these samples.

DISCUSSION

Our results demonstrate that the human β-globin genomic gene with intact transcriptional control sequences is able to function consistently in the context of retroviral regulatory elements in primary hematopoietic cells. The vast majority of CFU-S containing an intact proviral genome gave rise to progeny erythroblasts containing human β-globin mRNA and protein. Prior reports of expression of genes transferred with retroviral vectors into tissue culture cells, but lack thereof in primary hematopoietic cells, possibly reflected the nature of the transcriptional control elements used. Our results are consistent with the recent report of globin gene expression in the erythroid cells of long-term reconstituted mice in which eight animals exhibited levels of β-globin gene expression that ranged from 0.4% to 4% that of the mouse globin genes (26). Tissue specificity of gene expression was demonstrated in that study. Taken together the data indicate that consistent and regulated expression of the human β-globin gene can be achieved in primary hematopoietic cells after retroviral-mediated transfer.

Two significant problems remain: the level of expression is relatively low and transfer into repopulating stem cells has been an infrequent event with the globin gene vectors used to date. Many factors may contribute to the relatively low level of gene expression. Human globin genes are expressed at

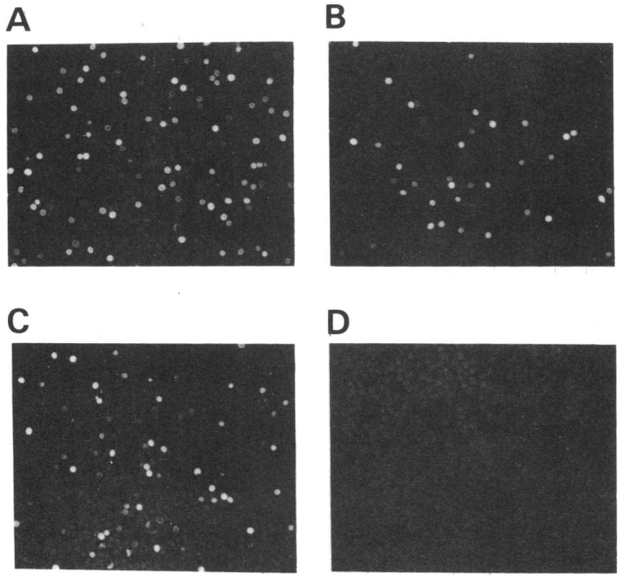

FIG. 4. Immunofluorescent staining with a monoclonal anti-human β-globin antibody of peripheral blood of mice transplanted with cells infected with the globin retrovirus. (*A* and *B*) Blood samples from a mouse injected with G418-selected cells obtained 4 and 8 weeks following transplantation, respectively. (*C*) Blood sample from another mouse injected with unselected cells obtained 4 weeks following transplantation. (*D*) Blood sample from a mouse injected with cells cocultivated with ψ2 cells (negative control).

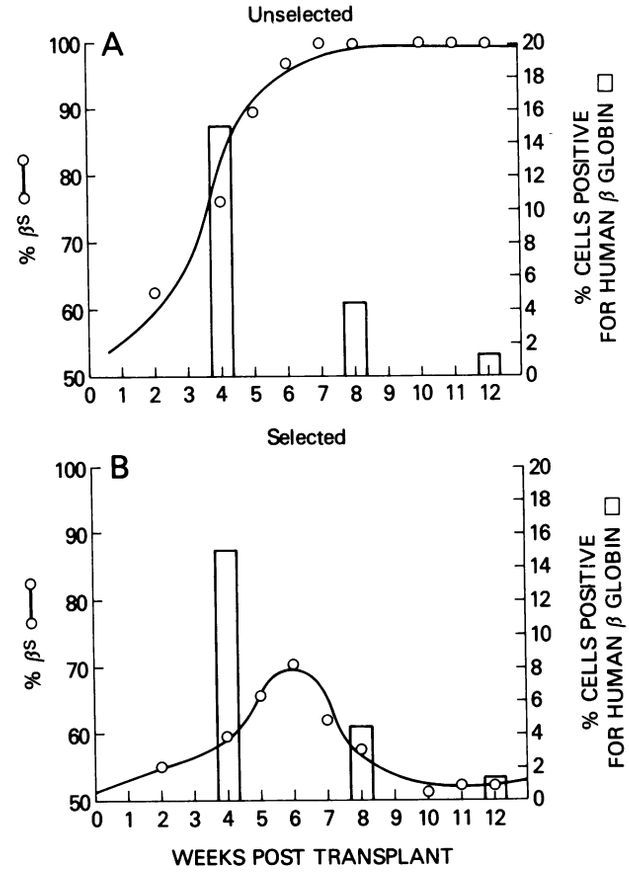

FIG. 5. Time course of human β-globin chain production in transplanted mice. The donor mice are homozygous for the β^s gene, but the recipients contain β^s, β^{major}, and β^{minor} hemoglobins. A recipient with 100% β^s has 100% reconstitution of donor bone marrow. The solid lines show the % donor hemoglobin (β^s) and the bars indicate the % cells stained with the anti-human β-globin monoclonal antibody. (*A*) The mouse whose cells are shown in Fig. 4*C* (injected with unselected cells). (*B*) The mouse whose cells are shown in Fig. 4 *A* and *B* (injected with G418-selected cells).

Table 2. Human β-globin production in W/W^v mice transplanted with infected bone marrow cells

Exp.	FUra	Preselection	Antibody-positive erythrocytes at 5–6 weeks: Animals with positive erythrocytes, no./total	% positive cells: Average	% positive cells: Range
1	–	–	5/11	8	1–18
2	+	–	3/4	5	3–10
3	–	+	12/12	6	1–20
4	+	+	6/6	6	3–15

See Table 1 footnotes * and ‡ for description of FUra pretreatment and preselection conditions, respectively.

lower levels than mouse genes when introduced by DNA transfection (27) or as part of intact chromosomes (28) into MEL cells *in vitro*. Germ-line transfer by microinjection of DNA fragments into fertilized oocytes also results in a low level of expression in transgenic animals (29). Expression levels of genes introduced as DNA fragments may depend on their integration position in a chromosome. Beyond these considerations it seems likely that the retroviral vectors tested to date do not contain all of the regulatory elements necessary for efficient globin gene expression. One such element, the "enhancer" 3′ to the β-globin gene, did not increase expression when included in the vector (this study; ref. 26). Perhaps this element is most important for developmentally specific regulation (e.g., fetal vs. adult expression). Yet to be tested are sequences at the 5′ and 3′ ends of the domain that may function as locus-activating elements in ensuring tissue-specific expression of globin genes (30). Recently these elements have been shown to result in consistent high-level expression of the human β-globin gene in transgenic animals independent of chromosomal position (31).

Our results illustrate that culture conditions satisfactory for retroviral infection of CFU-S do not ensure infection of self-renewing stem cells. The transient production of erythrocytes containing human β-globin chains early during reconstitution (Figs. 4 and 5) most likely reflects contribution of CFU-S-derived cells to the circulating erythrocyte mass. This interpretation is supported by the fact that G418 preselection increases the proportion of spleen foci containing the provirus (Table 1) and also increases the proportion of transplanted animals having erythrocytes containing human β-globin chains at 5–6 weeks (Table 2). In contrast, preselection in G418 appeared to completely eliminate repopulating stem cells, as there was no evidence of long-term reconstitution by donor cells in recipients of preselected cells (Fig. 5*B*). Animals reconstituted with infected donor cells that had not been exposed to G418 had only rare erythrocytes containing human β-globin chain at 12–16 weeks, and the hematopoietic cells of these animals lacked an integrated proviral genome on Southern blot analysis. Despite the presence of CFU-S containing the integrated provirus in the donor marrow inoculin, as reflected by erythrocytes containing human β-globin chain at 5–6 weeks, the stem cells that reconstituted these animals had not been successfully infected. CFU-S lack self-renewal potential and are capable of contributing to circulating erythrocytes only briefly after transplantation. These multipotential progenitors (CFU-S) are clearly distinct from repopulating stem cells.

Our difficulty in achieving stem cell infection is consistent with the experience of others. Dzierzak *et al.* (26) reported stem cell transfer of the β-globin gene in only 8 of 104 animals studied, and only a small percentage of hematopoietic cells in these animals contains an integrated proviral genome. Reports of successful infection and gene transfer into repopulating stem cells with other retroviral vectors (7–9) suggest that proper conditions can be achieved, although the important variables that influence the efficiency of gene transfer have not been defined. Several combinations of hematopoietic growth factors, particularly those that may act directly on early stem cells (32), must now be tested for their ability to facilitate this objective.

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