Adeno-Associated Virus Type 2-Mediated Transduction of Murine Hematopoietic Cells with Long-Term Repopulating Ability and Sustained Expression of a Human Globin Gene In Vivo

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Adeno-associated virus type 2 (AAV), a nonpathogenic human parvovirus, is gaining attention as a vector for potential use in human gene therapy. We and others have described AAV-mediated β -globin gene transfer and expression in established human and murine erythroleukemia cell lines in vitro. However, successful AAV-mediated globin gene transduction of hematopoietic stem cells and long-term expression in vivo in progeny cells have not been documented. We report here that infection of murine hematopoietic bone marrow cells ex vivo with a recombinant AAV vector containing the genomic copy of a normal human globin gene followed by transplantation of these cells into lethally irradiated congenic mice resulted in efficient gene transfer into hematopoietic cells with long-term repopulating ability as detected by the presence of the human globin gene sequences in bone marrow and spleen in primary recipient mice for at least 6 months. Long-term expression of the human globin gene was also detected in bone marrow, but not in spleen, in primary recipient mice. Furthermore, in secondary-transplant experiments, we were also able to document the presence as well as expression of the transduced human globin gene in mouse bone marrow for up to 3 months. These results provide further support for potential use of the AAV-based vector system in gene therapy of human hemoglobinopathies in general and sickle-cell anemia and β -thalassemia in particular.

Hereditary human hemoglobin diseases such as thalassemia and sickle-cell anemia comprise a major proportion of the genetic diseases worldwide. Potential long-term therapeutic strategies for these diseases involve replacement of the defective gene via allogeneic bone marrow transplantation or introduction of the functional gene into defective bone marrow cells (35). The molecular defects in sickle-cell anemia and β -thalassemia have been well characterized and seem amenable to genetic correction (33). β -Thalassemia syndromes result from a decrease or total absence of β -globin synthesis leading to poorly hemoglobinized erythrocytes causing severe anemia. Sickle-cell anemia is caused by a single nucleotide substitution that produces a mutant hemoglobin which is susceptible to polymerization resulting in altered erythrocyte morphology and membrane properties leading to vaso-occlusion (25). The structure of the human globin gene clusters has been well defined along with the locus control region (LCR) that governs globin gene expression (34).

Among the different modes developed for genetic correction of human hemoglobinopathies, virus-mediated transduction of primitive bone marrow stem/progenitor cells appears promising for achievement of long-term expression of globin genes. Attempts have been made to obtain transduction and expression of globin genes using retroviral vectors, both in vitro and in vivo (1, 2, 6, 8, 12, 13, 20), but the problem of structural integrity of the LCR-containing retroviral vectors has only

recently been overcome (5, 17, 26). However, the use of retroviral vectors may not necessarily be without complications, since these vectors may be associated with neoplastic events (7). Recently, recombinant adeno-associated virus (AAV) vectors have emerged as a useful alternative to the more commonly used retroviral and adenoviral vectors for gene transfer (22). AAV is a single-stranded DNA-containing parvovirus which has thus far not been shown to be associated with any known symptoms or pathology in humans (3, 4). The wild-type AAV genome has been shown to integrate in a site-specific manner into human chromosome 19q13.3-qter (14–16, 30). Neither wild-type AAV nor recombinant AAV is capable of autonomous replication without the help of a helper virus, such as adenovirus (22). Recombinant AAV vectors are devoid of the viral *rep* gene sequences, required to generate the replication (*Rep*) proteins that are necessary for AAV replication in the presence of a helper virus. In addition, recombinant AAV vectors can be efficiently generated without contaminating adenovirus since the capsid structures of adenovirus and AAV are distinctly separable in cesium chloride gradients (22). These remarkable features add to the desirability of AAV vectors for gene therapy purposes.

We (27, 28, 41) and others (9, 21, 36) have described successful transduction of human and murine hematopoietic cells with recombinant AAV vectors containing human globin genes in vitro. However, AAV-mediated stable transduction of hematopoietic stem cells and long-term expression of human globin genes in vivo have not been evaluated. In the present report, we document successful transduction of primitive murine repopulating hematopoietic progenitor cells and long-term in vivo expression of a human gamma globin gene in a serial bone marrow transplant model. Our data suggest that

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FIG. 1. Schematic representation of the recombinant vHS2- β p- γ -globin vector. Various structural components of the recombinant AAV-globin vector are indicated. High-titer, purified viral stocks were generated as previously described (19, 27, 28, 37, 40). ITR, inverted terminal repeat.

AAV-based vectors may prove useful for gene therapy of human hemoglobinopathies in general and sickle-cell anemia and β -thalassemia in particular.

MATERIALS AND METHODS

Plasmids and viruses. The plasmids containing genomic copies of the human β -globin and human γ -globin gene sequences were a kind gift of George Stamatyannopoulos, University of Washington, Seattle, and a plasmid containing the DNase I hypersensitive site 2 (HS-2) from the human β -globin gene cluster was provided by Tim Townes, University of Alabama at Birmingham. The AAV helper plasmid pAAV/Ad was a gift of Richard Samulski, University of North Carolina, Chapel Hill. The recombinant AAV containing the human β -globin promoter (β p)-driven human γ -globin gene along with the entire HS-2 enhancer element was first cloned as a recombinant plasmid, and then the recombinant genome was rescued and subsequently packaged into virions as described earlier (27, 28). The packaged vHS2- β p- γ -globin vector was purified through two rounds of cesium chloride equilibrium density gradients and heated at 56°C for 30 min to remove any contaminating proteins and adenovirus particles as previously described (19, 37). The titers of purified recombinant virus stocks were determined by quantitative DNA slot blots (31, 32). The extent of wild-type AAV contamination in the recombinant virus preparations was determined to be approximately 0.01%.

Isolation of murine LDBM cells, recombinant AAV infection, transplantation, and functional reconstitution of recipient mice. Transplant donor adult mice (6 to 8 weeks old) were drawn from our breeding colony of B6 hemoglobin-diffuse (*Hbb^d/Hbb^d*), glucose phosphate isomerase-1a (*Gpi-1^d/Gpi-1^d*) mice. These animals are congenic with C57BL/6J transplant recipients which are *Hbb^b/Hbb^b* (hemoglobin single) and *Gpi-1^b/Gpi-1^b*. Bone marrow donor mice were sacrificed by cervical dislocation, bone marrow cells were obtained from each femur and tibia, and low-density bone marrow mononuclear (LDBM) cells were isolated by centrifugation on Ficoll-Hypaque density gradients and counted as previously described (39). LDBM cells were mock infected or infected with 1 or 10 multiplicities of infection (MOI) of the vHS2- β p- γ -globin vector *ex vivo* for 2 h at 37°C. Following infection, cells were resuspended at $10^6/250 \mu\text{l}$ of Iscove's modified Dulbecco's medium (IMDM) and injected intravenously via the tail vein into C57BL/6J recipient animals that had been lethally conditioned with total body irradiation (TBI) using 11-Gy γ -irradiation (96 cGy/min in two doses divided by 4 h). In every instance, animals that received 11-Gy TBI and no infusion of donor cells died at 13 to 15 days after TBI. For secondary-transplant assays, C57BL/6J hosts were conditioned with TBI as above, and 3×10^6 LDBM cells from primary recipient animals were injected *i.v.* Peripheral blood was drawn from the tail vein of primary recipients at 1, 2, 4, and 6 months and from secondary recipients at 1, 2, and 3 months posttransplantation. Erythrocytes and leukocytes were isolated and analyzed for percent donor cell *Gpi-1^b* chimerism as previously described (38). Briefly, the isolated erythrocytes, lymphocytes, and granulocytes were pipetted into individual microcentrifuge tubes and lysed by freeze-thawing in distilled water. The cell lysates were applied to Titan III Zip Zone cellulose acetate plates with a Super Z 0.5-ml eight-well applicator (Helena, Beaumont, Tex.). The loaded gels were electrophoresed for 30 min at 300 V. *Gpi-1* isoenzyme activity was detected as previously described (38). Stained gels were scanned with a Rep clinical densitometer (Helena) for quantitation.

Qualitative and semiquantitative PCR assays. DNA samples from bone marrow, spleen, and thymus tissues isolated from recipient mice that were mock infected and vHS2- β p- γ -globin-infected (at 1 or 10 MOI) cells from both primary and secondary transplantation groups were analyzed following PCR amplification. Thirty cycles of PCR were carried out with an oligonucleotide primer pair specific for the human globin gene sequences. The forward primer, 5'-GATGGTATGGGGCAAGAGA-3', was specific for the human β -globin promoter, and the reverse primer, 5'-GGGTTTCTCTCCAGATCT-3', was specific for the coding sequence of the human γ -globin gene. A 354-bp specific amplified DNA product was detected following hybridization with the human β -globin promoter probe on Southern blots. For semiquantitative PCR assays, equivalent amounts of DNA isolated from bone marrow cells of positive mice were subjected to a 30-cycle PCR amplification with two sets of primer pairs under identical conditions. The primers for amplifying the transduced human globin gene sequences were the same as described above, and the primers for the endogenous mouse β -actin gene were 5'-ACCTCAACACCCAGCCAT-3' and 5'-TCAGGCAGCTCATAGCTCTT-3'. Similarly to the transduced human

globin sequences, the amplification of the β -actin gene was also designed to yield a 354-bp DNA product. The PCRs were performed in the presence of 2 μCi of [α - ^{32}P]dCTP (specific activity, 800 Ci/mmol) in each reaction mix. Ten percent of the DNA products from the human globin gene and 15-fold dilutions of samples from the mouse β -actin gene amplification reactions were analyzed on 6% polyacrylamide gels and autoradiographed. The relative intensities of the corresponding bands were determined by scanning the autoradiograms with the Phosphor 3.0 program as previously described (27).

RT-PCR assays and quantitation of transduced gene expression. Total RNA was isolated from bone marrow, spleen, and thymus cells of mock-transduced and vHS2- β p- γ -globin-transduced and transplanted mice by using the Micro RNA isolation kit (Stratagene, La Jolla, Calif.). Ten micrograms of total RNA was reverse transcribed by random primers (Stratagene) at 37°C for 1 h, following which the cDNA was subjected to two rounds of PCR analysis. The primers for PCRs were designed to amplify the transduced human γ -globin gene transcripts and to distinguish them from any possible amplification of the viral DNA sequences. The first-round PCR was carried out with the oligonucleotide primer pair with the sequences 5'-ACAGAGGAGGACAAGGCTAC-3' and 5'-CAGGGGTGAATTCTTTGCGG-3' to yield an amplified DNA product of 366 bp. Five microliters of the product of first-round PCR was further subjected to a second-round amplification with nested primers that yielded an amplified DNA product of 243 bp. The forward and reverse primers for the nested PCR were 5'-TGGTGTCTACCCATGACC-3' and 5'-ACGGTCACCAGCACATTTTC-3'. The quality of the reverse-transcribed RNA was also determined by using the same cDNA template for one round of 30-cycle PCR using oligonucleotide primers specific for the amplification of the mouse β -actin mRNA transcripts. The sequences of the forward and reverse β -actin primers were 5'-TCCTCTTCCTCCCTGGAGAA-3' and 5'-GCTGATCCACATCTGCTGGA-3'. The level of expression of the transduced human γ -globin was quantified in comparison with the amount of endogenous mouse β ^{major}-globin mRNA transcripts. Semiquantitative PCR was performed with the reverse-transcribed cDNA essentially as described above. The primers used for amplifying the mouse β ^{major}-globin reverse transcription-PCR (RT-PCR) products were 5'-CCAGCGGTACTTTGATAGC-3' and 5'-AATCCTTGCCAAGGTGGTGG-3'. Following electrophoresis of the RT-PCR products, the relative levels of expression were measured as described above.

RESULTS

Construction of the recombinant AAV globin vector. The AAV vector used in the study, vHS2- β p- γ -globin, schematically depicted in Fig. 1, was generated first as a recombinant plasmid containing the genomic copy of the human γ -globin gene sequences under the control of human β -globin promoter and the HS-2 enhancer, known to augment the level of expression of the globin genes both *in vitro* and *in vivo* (24, 26, 34). This chimeric gene was used for the following two reasons. First, by using the β p- and the γ -globin-specific oligonucleotide primer pair, unambiguous detection of the transduced sequences could easily be accomplished in murine cells. And second, expression from the human β p in murine cells has previously been documented (1, 2, 12, 13, 17, 20, 26). The recombinant plasmid was tested for efficiency in rescuing the chimeric globin gene from the vector and was subsequently packaged with the help of a nonrescuable helper plasmid that provided the necessary AAV proteins *in trans* and adenovirus as a helper virus in 293 cells (27, 28, 37, 40, 41). The recombinant AAV vector stocks were subsequently purified on cesium chloride equilibrium density gradients followed by DNase I digestion and heat inactivation at 56°C to render them free of any input plasmid DNA and adenovirus (27, 28, 37). The absence of any contaminating adenovirus was also confirmed

TABLE 1. Donor type Gpi isoenzyme in peripheral blood cells of primary and secondary bone marrow transplant recipient mice

Donor cells	% Donor Gpi the following no. of mo posttransplantation ^a :				
	1	2	3	4	6
Primary recipients	78 ± 8	89 ± 6	ND	100	100
Secondary recipients	71 ± 9	91 ± 7	100	ND	ND

^a The data are means ± standard deviations. ND, not determined.

by testing the virus preparation for cytopathic effect on 293 cells (data not shown).

AAV-mediated ex vivo transduction and functional reconstitution of murine hematopoietic cells. The vHS2-βp-^Λγ-globin virus was used to infect murine LDBM cells obtained from *B6.Hbb^d/Hbb^d-Gpi-1^a/Gpi-1^a* mice at MOI of 1 and 10 ex vivo as described in Materials and Methods. The cells were transplanted into six lethally irradiated C57BL/6J mice (three for each MOI). Following transplantation, the animals were bled to sequentially determine the level of engraftment measured as percent donor type Gpi-1^a present in peripheral blood cells. All transplanted conditioned animals survived. Animals from the control group that did not receive any donor marrow following irradiation did not survive, indicating that the radiation dose (1,100 rads) was sufficient to destroy endogenous hematopoietic stem/progenitor cells. A progressive increase in percent donor type Gpi-1^a was observed in the peripheral blood of recipient animals for the first 4 months posttransplantation. These data are shown in Table 1. After the donor contribution stabilized at 4 months, no change in the level of donor reconstitution was observed (Table 1). In all recipient animals, full peripheral blood reconstitution with donor type erythrocytes was matched by complete reconstitution of peripheral blood granulocyte and B- and T-lymphocyte populations with donor type cells (data not shown).

Transduction and long-term expression of the human globin gene in primary recipients. Six months posttransplantation, mice from both groups (1 and 10 MOI) along with the mock-transduced controls were sacrificed and cells from bone marrow, spleen, and thymus tissues were analyzed by PCR amplification of DNA sequences specific for the transduced human globin gene. PCR assays were performed with a primer pair consisting of sequences corresponding to the human β-globin promoter as the forward primer and to the coding sequences of the human ^Λγ-globin as the reverse primer. DNA products

were analyzed on Southern blots using a human ^Λγ-globin-specific DNA probe. These results are shown in Fig. 2. It is interesting to note that animals that were transplanted with the vHS2-βp-^Λγ-globin-transduced bone marrow cells (two animals at 1 MOI and three animals at 10 MOI) showed the presence of the transduced human globin sequences in the bone marrow, and except for one animal in the 1 MOI group, the remaining animals that were transplanted with the recombinant AAV-transduced cells also showed the presence of the human globin sequences in the spleen. However, thymus tissues from none of these animals showed the presence of the transduced gene. The transplanted animals with mock-transduced cells did not show the presence of the human globin gene, as expected, confirming the specificity of detection of the transduced sequences. The results from semiquantitative PCR analyses indicated that the copy number of the transduced human ^Λγ-globin gene ranged from approximately (7 ± 0.4)% for 1 MOI to approximately (8 ± 1)% for 10 MOI compared with that of the mouse endogenous β-actin gene (Fig. 3).

We next wished to determine whether the transduced human γ-globin gene sequences were transcriptionally active in bone marrow and spleen cells in primary recipient mice. Total RNA was isolated from bone marrow, spleen, and thymus tissues and subjected to RT-PCR analyses. Primers for PCR, following RT, were designed in such a way so as to eliminate amplification of the mouse endogenous globin mRNA as well as to distinguish amplification products from the transduced viral sequences. The RT reactions were performed with random primers to facilitate subsequent amplification with primers specific for the mouse β-actin gene as well as to ensure the quality and structural integrity of RNA preparations as described in Materials and Methods. The results of expression analysis by RT-PCR, shown in Fig. 4A, indicated the presence of the human ^Λγ-globin transcripts only in bone marrow cells of each animal transplanted with the recombinant AAV at 1 or 10 MOI, whereas no expression was detected in cells from spleen and thymus tissues from these animals. However, the quality of RNA from each of the cell types from all animals was confirmed to be good by using the mouse β-actin gene transcript-specific primer pair (Fig. 4B). No RT-PCR product was generated in the absence of RT (Fig. 4).

Subsequent to our observation that expression of the transduced human globin gene sequences was limited to bone marrow cells of the recipient mice, we performed semiquantitative RT-PCR assays to determine the level of expression of the transduced human ^Λγ-globin gene relative to that of the en-

FIG. 2. Southern blot analysis for the presence of the transduced human globin gene-specific sequences in hematopoietic cells from primary recipient mice. Bone marrow, spleen, and thymus tissues obtained from mice transplanted with mock-infected ($n = 3$; MOI = 0), vHS2-βp-^Λγ-globin virus-infected ($n = 2$; MOI = 1), or vHS2-βp-^Λγ-globin virus-infected ($n = 3$; MOI = 10) LDBM cells were obtained 6 months posttransplantation, subjected to PCR amplification, and analyzed on Southern blots as described in Materials and Methods. Positive and negative controls were the same as those described previously (27).

FIG. 3. Transduction efficiency of the human globin sequences in bone marrow cells from primary recipient mice. Equivalent amounts of total genomic DNA isolated from bone marrow samples obtained from primary recipient mice transplanted with mock-infected (MOI, 0) and vHS2- β p- γ -globin virus-infected (moi, 1 or 10) cells were used to amplify DNA products specific for the human β p- γ -globin gene and the mouse β -actin gene sequences, respectively, and analyzed on 6% polyacrylamide gels. The transduction efficiency in bone marrow cells from each animal is indicated below the blot and was determined as described in Materials and Methods.

ogenous mouse β^{major} -globin gene. The level of expression was approximately $(4 \pm 0.8)\%$ for 1 MOI and approximately $(6 \pm 0.9)\%$ for 10 MOI, compared with that of the endogenous mouse β^{major} -globin gene (Fig. 5).

Expression of the transduced human globin gene in secondary transplant recipients. In order to examine the potential of AAV-mediated stable transduction and long-term expression of the human γ -globin gene in murine hematopoietic cells, a portion of the bone marrow cells from primary recipients was also transplanted into secondary recipients. Approximately $3 \times$

10^6 LDBM cells, obtained 6 months posttransplantation from a primary recipient, were transplanted into six lethally irradiated congenic mice along with appropriate controls. The secondary recipients were maintained for 3 months posttransplantation prior to the analyses, at which time all were fully reconstituted with donor cells (Table 1). As described for the primary recipients, cells were obtained from bone marrow, spleen, and thymus tissues and subjected to PCR and RT-PCR analyses. Of the six animals transplanted with cells obtained from transduced and transplanted primary recipients, one animal showed the presence of the transduced human γ -globin sequences in both bone marrow and spleen cells (Fig. 6A). The results of the semiquantitative PCR analysis indicated that the transduced gene copy number was reduced to approximately 0.5% compared with the endogenous mouse β -actin gene (Fig. 6B). Interestingly, however, the expression of the transduced human globin gene was still detected in the secondary recipient and was restricted to bone marrow cells as in primary transplant recipients (Fig. 7A). Again, the quality of RNA from these cells was confirmed to be good by using the mouse β -actin gene transcript-specific primer pair (Fig. 7B), and no RT-PCR product was generated in the absence of RT (Fig. 7). The level of expression of the transduced human γ -globin gene was determined by semiquantitative RT-PCR assays to be approximately 0.4% compared with that of the endogenous mouse β^{major} -globin gene (Fig. 7C).

DISCUSSION

We report here the first successful recombinant AAV-mediated transduction as well as long-term in vivo expression of a human γ -globin gene in a mouse bone marrow serial transplant model. Gamma-globin forms an integral part of fetal hemoglobin, which is the predominant type of hemoglobin during the fetal stage in humans (33). Further, in disease such as sickle-cell anemia, the presence of fetal hemoglobin in increased amounts has been reported to ameliorate severe pathological consequences in affected individuals (35). The ability of recombinant AAV vectors to successfully transduce long-term repopulating hematopoietic progenitor cells has potential implications in virus-mediated gene therapy for diseases affecting the human hematopoietic system (10, 11, 18, 40).

FIG. 4. Expression of the transduced human globin gene in hematopoietic cells from primary recipient mice. Equivalent amounts of total RNA isolated from bone marrow samples obtained from primary recipient mice transplanted with mock-infected (MOI, 0) or vHS2- β p- γ -globin virus-infected (MOI, 1 or 10) cells were used to amplify DNA products specific for the human β p- γ -globin gene (A) and the mouse β -actin gene (B) transcripts, respectively, in the presence (+) or absence (-) of reverse transcriptase (RT) and analyzed on Southern blots as described in Materials and Methods.

FIG. 5. Level of expression of the transduced human globin gene in bone marrow cells from primary recipient mice. Equivalent amounts of total RNA isolated from bone marrow samples obtained from primary recipient mice transplanted with mock-infected (MOI, 0) or vHS2- β p- γ -globin virus-infected (MOI, 1 or 10) cells were used to amplify DNA products specific for the human β p- γ -globin gene and the mouse endogenous β ^{major}-globin gene transcripts, respectively, in the presence (+) or absence (–) of reverse transcriptase (RT) and analyzed on 6% polyacrylamide gels. The level of expression of the transduced human γ -globin gene in bone marrow cells from each animal is indicated below the blot and was determined as described in Materials and Methods.

Recombinant AAV-mediated transduction and expression of globin genes under the control of their own promoters in immortalized and primary hematopoietic cells in vitro have recently been documented (9, 21, 28, 36). The recombinant AAV vector that we used in the present study contained the human β -globin promoter to drive the expression of the human γ -globin gene sequences as well as the HS-2 element known to enhance expression of the human β -globin gene.

It was interesting to observe the presence of the transduced human γ -globin sequences in bone marrow cells of all animals and in spleen cells of all but one of the animals in the primary transplant group and in one animal in the secondary transplant

FIG. 6. Transduction efficiency of the human globin gene sequences in hematopoietic cells from secondary recipient mice. Equivalent amounts of total genomic DNA isolated from bone marrow samples obtained from secondary recipient mice transplanted with bone marrow cells obtained from vHS2- β p- γ -globin virus-infected (MOI, 10) cells were used to amplify DNA products specific for the human β p- γ -globin gene (A). Semiquantitative PCR analysis of DNA products specific for the human β p- γ -globin gene and the mouse β -actin gene sequences (B) was also performed as described in the legend to Fig. 3. The transduction efficiency of the human γ -globin gene in bone marrow cells from the positive animal is indicated below the blot and was determined as described in Materials and Methods.

FIG. 7. Level of expression of the transduced human globin gene in bone marrow cells from secondary recipient mice. Equivalent amounts of total RNA samples isolated from bone marrow samples obtained from secondary recipient mice transplanted with bone marrow cells from mock-infected (MOI, 0) or vHS2- β - γ -globin virus-infected (MOI, 10) cells from primary recipient mice were used to amplify DNA products specific for the human β - γ -globin gene (A) and the mouse endogenous β -actin gene (B) transcripts, respectively, in the presence (+) or absence (-) of reverse transcriptase (RT) and analyzed on Southern blots. Semiquantitative RT-PCR analysis of DNA products specific for the human β - γ -globin gene and the mouse endogenous β -actin gene transcripts (C) was also performed as described in the legend to Fig. 5. The relative level of expression of the transduced human γ -globin gene in bone marrow cells from the secondary transplant recipient animal is indicated below the blot and was determined as described in Materials and Methods.

group. However, expression of the transduced gene was observed only in bone marrow cells. It is well known that, in the murine system, spleen is also an organ of hematopoiesis along with bone marrow, unlike in humans, where bone marrow remains the principal organ of hematopoiesis. In this context, it is noteworthy that expression from the human β -globin promoter, which normally occurs only in bone marrow cells in humans, was also restricted to murine bone marrow cells. Our inability to document the presence of the transduced gene in the thymus in recipient animals restrains us from interpreting our results as indicating transduction of a multilineage hematopoietic stem cell. However, our observation that the progeny of transduced donor cells were present in secondary recipient marrow suggests that a primitive hematopoietic progenitor was transduced.

The results of the semiquantitative PCR indicated a transduction efficiency of approximately 8% in the bone marrow cells for the human γ -globin gene, compared with the mouse endogenous single-copy β -actin gene. Whether the transduced vector DNA sequences were stably integrated *in vivo* remains to be examined. However, in view of the presence of the vector DNA sequences for a period of 6 months in primary recipients and an additional period of 3 months in secondary recipients, it is less likely that the transduced AAV genomes persisted in episomal forms (10).

The results of semiquantitative RT-PCR analyses of RNA from bone marrow cells from the positive animals, on the other hand, yielded more definitive information on the level of expression of the transduced gene. We were able to document that the levels of expression of the transduced human γ -globin

gene were approximately 4% with 1 MOI and 6% with 10 MOI of the recombinant AAV vector compared with that of the endogenous mouse β -major-globin gene. Although a strict correlation cannot be made relating the percentage of the transduced gene to the level of expression, presumably because all of the AAV-transduced sequences may not be functional, it is interesting to note that even with a transduction efficiency of less than 10% it is still possible to achieve a significant level of expression of the transduced gene. It has been reported that a near-total correction of pathological manifestations of hemoglobinopathies such as β -thalassemia and sickle-cell anemia could be achieved with an expression level of about 20 to 40% of the normal γ -globin gene in these patients (23, 29). Thus, AAV-mediated γ -globin gene transfer and expression hold promise for potential treatment of β -thalassemia and sickle-cell anemia.

It was also of significant interest that expression of the transduced human globin gene was limited to bone marrow cells in the secondary transplant, even though the presence of these sequences could also be documented in spleen cells. Thus, it appears that the pattern of expression from the human β -globin promoter, and the HS-2 element-mediated enhancement of expression in human bone marrow cells, is recapitulated in murine bone marrow cells. However, since only one of six secondarily transplanted mice showed the presence of the transduced gene, we postulate that a further dilution of transduced cells during bone marrow reconstitution in secondary recipients had occurred. The initial transduction efficiency in the present study was determined to be approximately 10%, presumably because these experiments were carried out with LDBM cells. More recently, we have determined that, by sorting the LDBM cells with lineage-specific cell surface markers, it is indeed possible to obtain transduction efficiency exceeding 90 to 95% with 10 MOI of the recombinant AAV (27). In future experiments, the use of an enriched murine hematopoietic stem cell population and high-titer, purified recombinant AAV will allow us to determine whether expression of the transduced human globin gene is restricted to the murine erythroid cell lineage *in vivo*.

Finally, recent studies from our laboratory have also established that erythroid cell-specific expression can be achieved with the p6 promoter from a yet another human parvovirus, designated B19, in the context of a recombinant AAV genome (37). The recombinant AAV-B19 hybrid vectors promise to prove useful in gene therapy of human hemoglobinopathies in general and β -thalassemia and sickle-cell anemia in particular. Current efforts in our laboratory are focused on evaluating the safety and the efficacy of AAV-globin vectors in a β -thalassemic murine model as well as in nonhuman primates prior to their potential use in human gene therapy.

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