Adeno-associated Virus 2-mediated High Efficiency Gene Transfer into Immature and Mature Subsets of Hematopoietic Progenitor Cells in Human Umbilical Cord Blood

By Shang Zhen Zhou,* Scott Cooper,* Li Ya Kang,* Luciano Ruggieri,* Shelly Heimfeld, Arun Srivastava,* and Hal E. Broxmeyer*

From the Division of Hematology/Oncology, Departments of *Medicine, 1Microbiology and Immunology, and 5Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202-5120; and 6CellPro, Inc., Bothell, Washington 98021

Summary

Recombinant adeno-associated virus 2 (AAV) virions were constructed containing a gene for resistance to neomycin (neo<sup>+</sup>), under the control of either the herpesvirus thymidine kinase (TK) gene promoter (vTK-Neo), or the human parvovirus B19 p6 promoter (vB19-Neo), as well as those containing an upstream erythroid cell–specific enhancer (HS-2) from the locus control region of the human β-globin gene cluster (vHS2-TK-Neo; vHS2-B19-Neo). These recombinant virions were used to infect either low density or highly enriched populations of CD<sub>34</sub><sup>+</sup> cells isolated from human umbilical cord blood. In clonogenic assays initiated with cells infected with the different recombinant AAV-Neo virions, equivalent high frequency transduction of the neo<sup>+</sup> gene into slow-cycling multipotential, erythroid, and granulocyte/macrophage (GM) progenitor cells, including those with high proliferative potential, was obtained without prestimulation with growth factors, indicating that these immature and mature hematopoietic progenitor cells were susceptible to infection by the recombinant AAV virions. Successful transduction did not require and was not enhanced by prestimulation of these cell populations with cytokines. The functional activity of the transduced neo gene was evident by the development of resistance to the drug G418, a neomycin analogue. Individual high and low proliferative colony-forming unit (CFU)-GM, burst-forming unit–erythroid, and CFU-granulocyte erythroid macrophage megakaryocyte colonies from mock-infected, or the recombinant virus-infected cultures were subjected to polymerase chain reaction analysis using a neo-specific synthetic oligonucleotide primer pair. A 276-bp DNA fragment that hybridized with a neo-specific DNA probe on Southern blots was only detected in those colonies cloned from the recombinant virus–infected cells, indicating stable integration of the transduced neo gene. These studies suggest that parvovirus-based vectors may prove to be a useful alternative to the more commonly used retroviral vectors for high efficiency gene transfer into slow or noncycling primitive hematopoietic progenitor cells, without the need for growth factor stimulation, which could potentially lead to differentiation of these cells before transplantation.

Parvoviruses are among the smallest of the DNA-containing viruses that infect a wide variety of vertebrates (1, 2). Two parvoviruses of human origin, the adeno-associated virus 2 (AAV)<sup>1</sup>, and the parvovirus B19, have been studied extensively (3, 4). AAV has thus far not been shown to be associated with any known human disease (5, 6). Although the wild-type (wt) AAV genome has been documented to integrate via its inverted terminal repeats (ITRs) into human chromosomal DNA in a site-specific manner (7–9), this is apparently not the case with the recombinant AAV genomes. Parvovirus B19, on the other hand, is now known to be the etiologic agent of a variety of clinical disorders in humans (10–15), and has been shown to have a remarkable tropism for human erythroid cells (16–20). We have described the construction of an AAV-B19 hybrid genome in which we com-

<sup>1</sup>Abbreviations used in this paper: AAV, adeno-associated virus 2; AAV-2H, human AAV-2; Ad2, human adenovirus 2; BFU-E, burst-forming unit erythroid; Epo, erythropoietin; LDCB, low density mononuclear cord blood; SLF, steel factor.
bined the remarkable features of these two human paroviruses, and have speculated that this hybrid parovirus may be a useful vector for gene transfer in human bone marrow cells (21).

AAV-based vectors, in particular, have recently attracted attention largely due to the fact that in contrast to retroviruses, the wt AAV is a nonpathogenic virus, and although the site-specific integration of the AAV genome into the human chromosome does not apparently lead to insertional mutagenesis (22), this remains to be established with the recombinant AAV. Furthermore, the relatively higher stability, viral titers and transduction efficiency of AAV add to the desirable features of these types of vectors (23–25). Indeed, using the recombinant AAV vector systems, regulated high level expression of the human γ-globin in the K562 erythroleukemia cell line in vitro has been accomplished (26). In addition, near-total suppression in vitro of HIV-1 gene expression and replication in established cell lines using a similar vector system has recently been documented (27). Despite these advances, data are lacking on the feasibility of using the parovirus-based vectors for transducing primary normal human diploid cells, specifically primitive human hematopoietic stem and progenitor cells, believed to be ideal targets for somatic cell gene therapy.

Human umbilical cord blood, which contains hematopoietic stem/progenitor cells at a frequency equal to or greater than that of adult bone marrow (28, 29), and with a high quality for proliferation (30–34) and self-renewal capacity, as estimated by replating colonies in vitro (30, 31, 35), has shown promise clinically as an alternative source of human transplantable and marrow-repopulating cells (36–43). Hematopoietic stem and progenitor cells in human cord blood may be ideal targets as recipients of potential gene therapy for hematopoietic disorders (44). We have recently demonstrated AAV-mediated gene transduction in rapid cycling hematopoietic progenitor cells in murine bone marrow (45).

In this report, we present data to suggest that it is feasible to achieve high efficiency gene transfer into slow or noncytolytic primary primitive and more mature human hematopoietic progenitor cells using the recombinant AAV-based vector system without first inducing a higher proliferative state with growth factor stimulation. These studies may have important implications for human gene therapy of hematopoietic disorders.

Materials and Methods

Cells, Viruses, and Plasmids. A clonal population of a human naso-pharyngeal carcinoma cell line, KB, was obtained from Dr. A. C. Antony (Indiana University School of Medicine) and maintained as monolayer cultures in Eagle's MEM supplemented with 10% fetal bovine serum. Human AAV-2 (AAV-2H), and human adenovirus 2 (Ad2) viral stocks were provided by Drs K. I. Berens (Cornell University Medical College, New York) and K. H. Fife (Indiana University School of Medicine), respectively, and grown in KB cells as previously described (46–48). Human cord blood, scheduled for discard, was collected from the placental umbilical vein of normal, healthy infants immediately after birth at Indiana University Hospitals (Indianapolis, IN).

Recombinant AAV (49, 50) and B19 (51) plasmids have been described, and were obtained from Dr. R. J. Samulski (University of Pittsburgh, Pittsburgh, PA) and Dr. P. Tattersall (Yale University School of Medicine, New Haven, CT), respectively. Two recombinant plasmids containing the neo gene have also been described (52, 53), and were obtained respectively from Drs. S. H. Larsen and R. H. Schloemer (Indiana University School of Medicine). A recombinant plasmid, pHS-2, containing the DNase I-hypersensitive site 2 from the human β-globin gene cluster (54) was provided by Dr. Tim Townes (University of Alabama, Birmingham, AL).

Cell Separation Techniques. Low density mononuclear cord blood (LDCB; <1.077 g/cm3) cells were obtained by density centrifugation using Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). LDCB cells were enriched for hematopoietic progenitor cell populations using CD34 column separation as follows. Nonadherent, low density, T lymphocyte-depleted cord blood cells (31) were incubated with biotinylated CD34 antibody, resuspended at a concentration of 100–200 × 10⁶ cells/ml, and processed through an avidin-immunoaffinity column (Cepheir TM System; CellPro, Inc., Bothell, WA). These cells, analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA) showed a CD34 purity range between 80 and 98%. Cell viability (trypan blue exclusion) was consistently >95%.

Hematopoietic Progenitor Cell Assays. LDCB or CD34 cord blood cells were either mock infected, or exposed to each recombinant AAV-Neo virus separately at a multiplicity-of-infection (moi) of 1 at 37°C for 2 h, washed with sterile PBS, pH 7.0, and suspended at various concentrations (usually 1–2.5 × 10⁶ LDCB, and 200–300 CD34 cells/ml) in 35-mm plastic tissue culture dishes (Costar Data Packaging, Cambridge, MA) containing either 1 U/ml of recombinant human erythropoietin (Epo), or Epo plus 100 U/ml of recombinant human IL-3 and 100 U/ml of recombinant human GM-CSF, or Epo plus IL-3 + GM-CSF and 50 ng/ml of recombinant human steel factor (SLF) in 1.1% methylcellulose in IMDM. Unless otherwise indicated, the cells were not prestimulated with growth factors before viral infections. In some experiments, cells were incubated with IL-3 + GM-CSF + SLF for 48 h before viral infections. The presence of SLF in cultures allows detection of earlier subsets of stem/progenitor cells (29–31). These cell-plating concentrations allowed clear detection of individual colonies with no overlap with other colonies. IL-3, GM-CSF, and SLF were kind gifts from Immuneex Corp. (Seattle, WA) and Epo was purchased from Amgen, Inc. (Thousand Oaks, CA). Some assays received 200–1,000 µg/ml of G418 (Genetecis; Sigma Chemical Co., St. Louis, MO) into dishes within 1 d after cells had been plated in the presence of growth factors. G418 was dissolved at a final effective concentration of 4 mg/ml in IMDM, the pH was adjusted to 7.2 with 1 N NaOH, filtered through 0.22-µm filters (Schleicher & Schuell, Keene, NH), and stored frozen in 5-ml aliquots at −20°C. The cultures were incubated at 37°C in a 100% humidified atmosphere of 5% CO₂ at lowered (5%) O₂. After 14 d, burst-forming unit-erythroid (BFU-E) and CFU-GM and mixed cell population using CD34 column separation as follows.

Construction of Recombinant Plasmids and Virions. The overall strategy for the construction of the recombinant AAV containing the herpesvirus thymidine kinase (TK) promoter–driven neo gene (vTK-Neo) from a recombinant plasmid, designated pWP-8A, has been described recently (25, 45), and is illustrated in Fig. 1 A. Similarly, a recombinant plasmid containing the HS-2 enhancer upstream of the TK promoter was constructed as shown in Fig. 1 B. Briefly, plasmid pWP-19 was cleaved with KpnI, and plasmid pHS-2 was cleaved with AatII and HincII to isolate the HS-2 enhancer ele-
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plasmid containing the B19 promoter (p6) was constructed as depicted in Fig. 1 C. Plasmid

virions. The strategy for construction of the recombinant AAV virions

Figure 1. Construction of the recombinant AAV-Neo plasmids and

pB19p6 to generate a recombinant plasmid, designated pBN-7.

A 1.5-kb DNA fragment containing the enhancer element was inserted upstream of the TK promoter to generate a recombinant AAV genome containing a gene for resistance to ampicillin (Ap). The neo gene driven by the human parvovirus B19 promoter (p6) was constructed as depicted in Fig. 1 C. Plasmid pYT104 was cleaved with EcoRI and XbaI, and a 277-bp DNA fragment containing the B19p6 promoter was cloned between EcoRI and XbaI sites of plasmid pUC19 by the standard methods described in Sambrook et al. (55) to generate a plasmid designated, pB19p6. A 1.5-kb DNA fragment containing the neo gene was isolated from plasmid pWu.G1 after digestion with BglII and BamHI, treated with polI/fK, and blunt-end-ligated with HincII-cleaved pB19p6 to generate a recombinant plasmid, designated pBN-7. This plasmid was linearized by digestion with EcoRI and ligated to EcoRI-cleaved plasmid pAS203 containing the AAV-ITRs (21). This resulted in a plasmid, designated pBN-8. Recombinant AAV virions (vB19-Neo) were generated as described above. Finally, a recombinant plasmid containing the B19p6-neo gene with an upstream HS-2 enhancer element was constructed as illustrated in Fig. 1 D. The HS-2 enhancer element was ligated at the EcoRI site to generate a plasmid designated pHS2-B19p6. The HS2-B19p6 DNA fragment was isolated by digesting this plasmid with AatII and HincII, and ligated with BglII-cleaved plasmid pWP-19 after treatment with PolI/fK to generate a plasmid designated pSZ-16. Recombinant AAV virions (vHS2-B19-Neo) were generated as described above. The viral titers were determined on quantitative DNA dot blots as previously described (17, 56).

PCR Amplification, and Southern Blot Analysis. Individual colonies (CFU-GM, BFU-E, and CFU-GEMM) from mock-infected, or recombinant AAV-neo virus–infected cultures were subjected to 35-cycle PCR amplification using a neo-specific synthetic oligonucleotide primer pair (5'-TGAAGACTTTGGCGCGGAAATG-3' and 5'-GGCGAAGAATCAGCAATG-3'). Amplified DNA products were electrophoresed on 1.5% agarose gels and analyzed on Southern blots using a neo DNA probe as previously described (25, 45, 53). Plasmids pWu.G1 and pUC19 were used as positive and negative controls, respectively.

Results

Sensitivity of Cord Blood Progenitors to G418. Before determining the efficiency of AAV-mediated transduction of cord blood progenitors, the sensitivity of the different progenitor cell classes to inhibition by G418 was assessed. As shown in Fig. 2, the different progenitor cell classes differed in their sensitivity to inhibition by G418. Within the CFU-GM compartment, those cells responsive to stimulation by the combination of Epo + IL-3 + GM-CSF + SLF (believed to be a more immature subset of CFU-GM) were less sensitive to inhibition by G418 than were the more mature subsets of CFU-GM (those responsive to stimulation by Epo + IL-3 + GM-CSF). Within the BFU-E compartment, the same age-related sensitivity to G418 was noted such that the subset of BFU-E responsive to stimulation by Epo + IL-3 + GM-CSF was less sensitive to inhibition by the drug than the more mature subset of BFU-E responsive to stimulation by Epo alone. As previously noted (29), when cord blood cells are plated in the presence of Epo + SLF + IL-3 and GM-CSF, CFU-GEMM but not BFU-E colonies are detected (data not shown). Next to the subset of CFU-GM responsive to stimulation by the combination of Epo + IL-3 + GM-CSF + SLF, the CFU-GEMM responsive to this same combination of growth factors was the least sensitive to inhibition by G418. Based on the information shown in Fig. 2, active concentrations of 600 and 800 μg/ml of G418 were chosen to estimate G418-resistant progenitors of AAV-transduced cells.

Recombinant AAV-mediated Transduction of LDCB Cells, and Stable Integration of the neo Gene. Equivalent numbers of mock-infected, or the recombinant AAV-Neo virus–infected cells were assayed for primitive as well as more mature hematopoietic progenitor cells in semi-solid media in the presence of G418 as described in Materials and Methods. These data, derived from experiments with four different cord blood samples, either in the absence (Fig. 3 A), or the presence (Fig. 3 B) of SLF, are depicted in Fig. 3. As is evident, whereas ~12% of the CFU-GM colonies derived from mock-infected cultures survived the G418-treatment at a final active concentration of 600 μg/ml of the drug, the percent survival of the CFU-GM colonies cloned from the recombinant AAV-Neo virions was as follows: 25% with vTK-Neo, 38% with vHS2-TK-Neo, 39% with vB19-Neo, and 27% with vHS2-
Figure 2. Comparative analysis of the sensitivity of cord blood progenitors to G418 inhibition of colony formation. 10^4 LDCB cells were plated +/- G418 in the presence of either Epo, Epo + IL3 + GM-CSF, or Epo + IL3 + GM-CSF + SLF, and the sensitivity of CFU-GM-, BFU-E-, and CFU-GEMM-derived colonies to G418 assessed. Results are based on an average of three experiments each with mean control (-G418) colony numbers for CFU-GM (Epo + GM-CSF), CFU-GM (Epo + IL3 + GM-CSF + SLF), BFU-E (Epo), BFU-E (Epo + IL3 + GM-CSF), and CFU-GEMM (Epo + IL3 + GM-CSF + SLF), respectively, ranging from 9 to 50, 16 to 40, 9 to 33, 22 to 63, and 36 to 72 in the individual experiments.

Figure 3. Transduction of the neo^ gene in LDCB progenitor cells and hematopoietic progenitor cell assays. Approximately 2.5 x 10^4 LDCB cells, after mock infection (open bar), or infection with the recombinant AAV-Neo virions (closed bars) at a moi of 1, were plated in methylcellulose either in the absence (A) or in the presence (B) of SLF, and evaluated using a neo-specific oligonucleotide primer pair in a PCR amplification system. These data from one representative experiment are presented in Fig. 4. As can be seen, a 276-bp DNA fragment that hybridized with a neo-specific DNA probe on Southern blots could be detected in CFU-GM, BFU-E, and CFU-GEMM colonies cloned from the B19-Neo, respectively. A similar pattern with the BFU-E colonies was also observed. In the presence of SLF, ~15% of the CFU-GM colonies from mock-infected cultures were resistant at a final active concentration of 800 ~g/ml of G418, and 37-49% of these colonies cloned from the various recombinant virus-infected cultures were resistant to G418. Under these assay conditions, no BFU-E colonies were obtained, but the pattern of the CFU-GEMM colony formation was similar to that of the CFU-GM colony formation. For all the data in Fig. 3, colony formation by cells transduced with the different recombinant AAV-Neo vectors was similar (p >0.05), but significantly different than the mock-transduced control group (p <0.05).

We also wished to document the physical presence of the recombinant AAV-Neo virus-mediated transduction of the neo^ gene, presumably stably integrated with the chromosomal DNA, in CFU-GM, BFU-E, and CFU-GEMM colonies. Five individual colonies each for CFU-GM, BFU-E, and CFU-GEMM analysis were picked from mock-infected, or recombinant AAV-Neo virus–infected cultures, either in the absence (Fig. 3 A), or the presence (Fig. 3 B), of SLF, and evaluated using a neo-specific oligonucleotide primer pair in a PCR amplification system. These data from one representative experiment are presented in Fig. 4. As can be seen, a 276-bp DNA fragment that hybridized with a neo-specific DNA probe on Southern blots could be detected in CFU-GM, BFU-E, and CFU-GEMM colonies cloned from the
Figure 4. Southern blot analysis of the PCR-amplified neo gene-specific DNA fragments from myeloid and erythroid colonies. Day 14 CFU-GM and BFU-E or CFU-GM and CFU-GEMM colonies derived either in the absence (A), or in the presence (B) of SLF, from mock-infected (lanes 1–5), vTK-Neo virus-infected (lanes 6–10), vHS2-TK-Neo virus-infected (lanes 11–15), vB19-Neo virus-infected (lanes 16–20), and vHS2-B19-Neo virus-infected (lanes 21–25) cultures were individually picked and used in PCR amplification reactions as described in Materials and Methods. Plasmids pUC19 and pTwu.G1 were used as negative and positive controls, respectively. The PCR products were analyzed on Southern blots using a neo-specific DNA probe. (Arrows) 276-bp neo-specific DNA fragment.

primitive as well as more mature hematopoietic progenitor cells infected by the recombinant AAV-Neo virus. In none of the colonies derived from mock-infected cells, could a neo-specific DNA product be amplified by PCR under identical conditions. Taken together, these data suggest that both immature and mature subsets of hematopoietic progenitor cells in cord blood cells can be successively transduced, and that high efficiency transduction of human hematopoietic progenitor cells is indeed possible using the recombinant AAV-based virions.

Lack of Requirement for Prestimulation with Growth Factors for AAV-mediated Transduction. We next wished to examine the effect of prestimulation of cord blood progenitor cells with various growth factors before transduction with the recombinant AAV virions. In this experiment, three separate cord blood samples were either mock infected or infected at time zero (T = 0), with the vTK-Neo virions or allowed to incubate the presence of IL-3 + GM-CSF + SLF at 37°C for 48 h (T = 48), followed by mock infection with the vTK-Neo virions under identical conditions. Progenitor cell assays were carried out as described above. These data are shown in Fig. 5. In each case, the colonies grown from virally infected cells were significantly enhanced compared with the mock-infected cells (p < 0.05). As is evident, whereas the ex-
tent of the CFU-GM colony formation without the growth factor pretreatment ranged between 33 and 86% among the three cord blood samples, it was not significantly different from that when cells were pretreated with growth factors for 48 h, which ranged between 26 and 62% \((p >0.05)\). Similar patterns were obtained for the BFU-E and the CFU-GEMM colonies. We conclude from these data that unlike in retroviral-mediated transduction, pretreatment with exogenously added growth factors is not a prerequisite for successful, high efficiency transduction by the AAV-based vector system.

Successful Transduction of CD34+ Cells. It was next of interest to examine whether highly enriched CD34+ hematopoietic cord blood progenitor cells could also be transduced with the recombinant AAV Neo virions in the absence of drug selection. Clonogenic assays were carried out under a variety of conditions that yielded progeny cell colonies from primitive subsets of hematopoietic progenitors as described in Materials and Methods. For each of the assay conditions, eight to nine individual CFU-GM and CFU-GEMM colonies were picked and subjected to PCR amplification as described above. The representative data for one of the sets in which the assays were carried out in the presence of Epo + IL-3 + GM-CSF + SLF are shown in Fig. 6.

As is evident, whereas none of the colonies from mock-infected cultures demonstrated a neo-hybridizing DNA fragment, such a fragment could be readily amplified in 33–75% of the colonies cloned from the recombinant AAV Neo virions. Since subsets of hematopoietic progenitor cells in cord blood are in relatively slow cycle \((57)\) compared with those in adult human bone marrow, and since these cells were not preexposed to growth factors in vitro to enhance their proliferative status before viral infections, these results suggest that the relatively slow or noncycling hematopoietic progenitor cell populations in cord blood are susceptible to infection by the recombinant AAV virions.

Discussion

Although retroviral vectors have been used in human gene therapy \((58)\), the fear remains that the use of retroviruses may be associated with the development of neoplastic events \((59)\). Since several DNA virus-based vectors, such as herpesvirus and adenovirus, may also suffer from a similar problem, the search for alternative vectors remains an important effort. It is interesting to note that a unique group of single-stranded DNA-containing viruses, the paroviruses, have to date not been shown to be associated with any malignant disease \((4–6)\). In fact, the wt AAV has been shown to possess antionconeogenic properties in vitro as well as in vivo \((60–63)\). Since AAV is nonpathogenic, it has emerged as a potentially useful alternative vector system \((23–25)\), although it remains to be established whether the recombinant AAV is also nonpathogenic. However, since ~90% of the human population is seropositive for AAV \((5)\), accidental infection by recombinant AAV virions in vivo may not be problematic.

Employing the recombinant AAV vector system, a number of recent studies \((26, 27, 64)\) have reported successful transduction of exogenous sequences in established cell lines that are frequently aneuploid. In our studies, we chose to focus on infection of primary human diploid cells since normal diploid cells are both the most likely target of a natural AAV infection \((47, 48)\), and are the likely recipients of potential gene therapy with this vector system \((22)\). In this report, we examined the efficacy of the AAV-based vector system for transduction of a population of human umbilical cord blood cells that contains hematopoietic stem and progenitor cells \((28–35)\). High efficiency transduction of CD34+ cells and expression of the transduced neo gene in the progeny of these primitive and more mature hematopoietic cells suggest that the AAV vector system might prove useful in a gene therapy setting.

In this context, it is noteworthy that high efficiency transduction of CD34+ cells from human umbilical cord blood cells has been obtained with retroviral vectors \((44, 65)\). In those experiments, cord blood cells were first exposed to potent combinations of growth factors to place the progenitors into a higher cell cycle state before exposing the cells to the retroviral vectors. In contrast, the data presented herein demonstrate high efficiency transduction into slow or noncycling immature and mature human hematopoietic progenitors from cord blood in the absence of growth factor stimulation using the recombinant AAV-based vector system without further enhancement, even when the cells are pretreated with growth factors. For retroviruses, at least one round of cell division seems to be required before random integration of a transduced gene \((66)\). AAV-mediated transduction may thus be potentially advantageous, compared with retrovirus-mediated transduction, in not requiring preincubation of cells in the presence of growth factors for stable gene integration. Incubation of cells with growth factors may lead to differentiation of some of the more immature cells responsible for long-term engraftment. This potential problem may be obviated by the use of AAV for gene transduction.

It is intriguing that in our present studies we were unable
to obtain lineage-specific expression of the neo<sup>R</sup> gene driven by the B19p6 promoter, given the remarkable erythroid cell tropism of the parvovirus B19 (16–21, 67). In our previous infection studies with a recombinant AAV-B19 hybrid virus, expression from this promoter was largely restricted to cells in the erythroid lineage, and to a lesser extent, in the megakaryocytic lineage (21, 56). After infections with the recombinant viruses, we also did not detect expression from the B19p6 promoter in nonerythroid human cells (our unpublished data). Although Liu et al. (68) have presented evidence of expression from this promoter in established cell lines in vitro, those studies were carried out with plasmid transfections. We speculate that our observed results may be due to the application of a selective pressure. Further studies will be needed to establish the underlying molecular mechanism of the erythroid cell-specific expression from the B19p6 promoter during nonselective viral infections. Similar strategies may nonetheless be employed by using tissue-specific promoters to limit expression of a transduced gene in a given cell type (69). Further in vivo studies with nonhuman primate model systems are warranted to evaluate the safety as well as the efficacy of the AAV-based vector system before its potential use in human gene therapy.

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Address correspondence to H. E. Broxmeyer and A. Srivastava, Walther Oncology Center, Indiana University School of Medicine, IB-501, 975 W. Walnut Street, Indianapolis, IN 46202-5120. S.-Z. Zhou is currently at Avigen, Inc., 1201 Harbor Way Parkway, #1000, Alameda, CA 94501.

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