Development of virus vectors for gene therapy of \( \beta \) chain hemoglobinopathies: flanking with a chromatin insulator reduces \( \gamma \)-globin gene silencing in vivo

David W. Emery, Evangelia Yannaki, Julie Tubb, Tamon Nishino, Qiliang Li, and George Stamatoyannopoulos

We have previously described the development of oncoretrovirus vectors for human \( \gamma \)-globin using a truncated \( \beta \)-globin promoter, modified \( \gamma \)-globin cassette, and \( \alpha \)-globin enhancer. However, one of these vectors is genetically unstable, and both vectors exhibit variable expression patterns in cultured cells, common characteristics of oncoretrovirus vectors for globin genes. To address these problems, we identified and removed the vector sequences responsible for genetic instability and flanked the resultant vector with the chicken \( \beta \)-globin HS4 chromatin insulator to protect expression from chromosomal position effects. After determining that flanking with the cHS4 element allowed higher, more uniform levels of \( \gamma \)-globin expression in MEL cell lines, we tested these vectors using a mouse bone marrow transduction and transplantation model. When present, the \( \gamma \)-globin cassettes from the uninsulated vectors were expressed in only 2% to 5% of red blood cells (RBCs) long term, indicating they are highly sensitive to epigenetic silencing. In contrast, when present the \( \gamma \)-globin cassette from the insulated vector was expressed in 49% ± 20% of RBCs long term. RNase protection analysis indicated that the insulated \( \gamma \)-globin cassette was expressed at 23% ± 16% per copy of mouse \( \alpha \)-globin in transduced RBCs. These results demonstrate that flanking a globin vector with the cHS4 insulator increases the likelihood of expression nearly 10-fold, which in turn allows for \( \gamma \)-globin expression approaching the therapeutic range for sickle cell anemia and \( \beta \) thalassemia. (Blood. 2002;100:2012-2019)

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Introduction

The \( \beta \) chain hemoglobinopathies \( \beta \) thalassemia and sickle cell anemia constitute the most common class of hereditary, monogenic disorders in the human population, affecting hundreds of thousands of persons worldwide.\(^1\) In \( \beta \) thalassemia, a lack of \( \beta \)-globin synthesis results in the precipitation of free \( \alpha \)-globin chains and the subsequent destruction of erythroid precursors in the marrow.\(^1\) In sickle cell anemia, a single amino acid substitution in the \( \beta \)-globin chain leads to globin chain polymerization, red cell sickling, and subsequent vascular occlusions and red cell destruction.\(^2\) Recent therapeutic interventions include the use of cytotoxic drugs to induce the synthesis of fetal \( \gamma \)-globin, which can bind up free \( \alpha \)-globin chains in \( \beta \)-thalassemia\(^1,4\) and can interfere with globin chain polymerization in sickle cell anemia.\(^5,7\) However, these agents have proven ineffective for the treatment of severe transfusion dependency \( \beta \)-thalassemia, and safety concerns remain about the lifelong administration of cytotoxic drugs in patients with sickle cell disease. Allogeneic bone marrow transplantation can cure patients with \( \beta \) chain hemoglobinopathies.\(^1,8,9\) However, this procedure is limited by the availability of HLA-identical donors and morbidity and mortality risks that increase as the clinical phenotype of these diseases worsens with age. For these reasons, we and others have pursued the development of gene therapy for the treatment of the \( \beta \) chain hemoglobinopathies.

The levels of gene transfer and expression necessary for effective gene therapy of the \( \beta \)-chain hemoglobinopathies can be estimated from clinical and pathophysiological data on patients with naturally elevated fetal hemoglobin levels,\(^1,2\) bone marrow transplant recipients with mixed donor–recipient chimerism,\(^8,9\) and patients receiving hydroxyurea therapy. Such data suggest that it will be necessary to transfer the therapeutic gene into at least 15% to 20% of repopulating hematopoietic stem cells and that the transferred gene will have to be expressed in a pancellular fashion at 20% to 30% of total \( \alpha \)-globin to achieve curative levels. This has been extremely difficult to attain, in part because of the complex role that globin gene regulatory elements and intronic sequences play on vector expression and stability.\(^10-15\) High-level expression of the endogenous \( \beta \)-like globin genes requires the presence of the major regulatory elements of the \( \beta \)-globin locus control region (LCR).\(^16\) However, the use of LCR components in oncoretrovirus vectors for \( \beta \)-globin and \( \gamma \)-globin has been limited by size constraints and the effect of such sequences on vector stability and titer.\(^10,11,13\) As an alternative, we and others\(^14,15,17\) have turned to the use of the HS-40 regulatory element from the \( \alpha \)-globin locus, which does not adversely affect vector titer and stability. In a previous report\(^15\) we described the development of an oncoretrovirus vector, called HS40-6, in which the HS-40 enhancer was directly linked to an expression cassette for \( \gamma \)-globin using a truncated \( \beta \)-globin promoter and a large internal deletion of the \( \beta \)-globin locus. This vector was designed to be copied into the L3 retrotransposition target site and express \( \gamma \)-globin using a chimeric promoter in which the LTR was directly linked to an expression cassette for \( \gamma \)-globin using a truncated \( \beta \)-globin promoter and a large internal deletion of the \( \beta \)-globin locus.
5' LTR during formation of the provirus, effectively flanking the vector with 2 copies. These changes resulted in a genetically stable high-titer vector capable of high-level expression in transduced mouse erythroleukemia (MEL) cell lines and primary erythroid progenitor colonies. However, expression of this vector was found to be highly variable, especially in primary cell cultures. These results are consistent with those of other investigators and emphasize the relative sensitivity of globin gene vectors to the epigenetic effects of surrounding chromatin, which can lead to histone deacetylation and CpG methylation. Such epigenetic position effects often lead to expression variation and silencing, especially during terminal erythropoiesis when global expression patterns become more restricted and the chromatin condenses before nuclear exclusion.

To address the problems of position effects, we have been investigating a class of regulatory sequences called chromatin insulators. As recently reviewed, these elements, first described in Drosophila and more recently in several vertebrate species, help define the boundary between differentially regulated loci and serve to shield promoters from the influence of neighboring regulatory elements. We recently reported that a particular insulator element from the chicken β-globin LCR, called HS4, can protect the expression of an oncoretrovirus reporter vector from position effects. This was done by flanking the reporter vector with a 1.2-kilobase (kb) fragment containing the CHS4 element and by analyzing vector expression in cell lines, primary marrow progenitor cultures, and murine bone marrow transplantation assays. We found that the insulator had no effect on vector titer and stability and was able to protect 2 separate expression cassettes from negative position effects in vitro and in vivo. Similar studies in cell lines showed that this protection is associated with a dramatic decrease in the de novo methylation of sequences in the virus 5' LTR.

In the studies reported here, we sought to test whether the CHS4 element can insulate expression of oncoretrovirus vectors for human β-globin using a stringent mouse bone marrow transplantation and transplantation model in which globin vector silencing is particularly severe.

Materials and methods

Retrovirus vectors

All vectors are diagrammed in Figure 1. Construction of vector HS40-5 has been previously described. It contains an expression cassette consisting of a −127 β-globin promoter (RsuL-NcoI, 62060-62238), fused to a NcoI-RsuI coding fragment from the Aγ-globin gene 39483-41192, with a 714-base pair (bp) internal deletion (XhoI-HpaI, 39960-40674) of intron 2. This cassette was inserted between the BamHI (blunt) and StuI sites of LNSX in the opposite orientation with respect to virus transcription. A 262-bp fragment containing the α-globin HS-40 enhancer core (HS40) was inserted in the 5' LTR during provirus integration. All other vectors incorporate the HS-40 enhancer immediately adjacent to the β-globin promoter. The γ-globin cassette extends 277 bp 3' of exon 3 in vectors HS40-5 and -9, and 470 bp 3' of exon 3 in vectors HS40-6, -10, and -11. Vectors HS40-10 and HS40-11 also contain a 58-bp deletion within the extended 3' region. Vector HS40-11 has a 1.2-kb fragment (graded box) containing the CHS4 chromatin insulator integrated in the double-copy position of the 3' LTR.

In short, vector plasmid was used to transfect the amphotropic packaging line PA317 by CaPO4 precipitation, and after 48 hours virus supernatant was collected and used to transduce the ecotropic packaging line GP+E86.
After an additional 24 hours, the transduced cells were plated at low dilution with 0.5 mg/mL active G418 (Gibco BRL), and individual drug-resistant colonies were isolated after 7 to 10 days. Virus titers were determined by serial dilution and transfer of G418 resistance to naive NIH3T3 cells as previously described.14 Clones with the highest titers were further analyzed by Southern blot analysis for intact provirus (methods described below) and for the presence of replication-competent virus by a standard marker-rescue assay.14 Vector-containing supernatant was collected from semiconfluent plates after 48-hour culture at 33°C and was passed through a 0.44-μm filter.

**Retrovirus vector transductions**

MEL585 cells were transduced by 24-hour culture in vector-containing supernatant plus 8 μg/mL polybrene (hexadimethrine bromide; Sigma Chemical, St Louis, MO) at 1-2 × 10⁶ cells/mL. The cells were then washed and plated at limiting dilution in 96-well, flat-bottomed dishes with 0.6 ng/mL active G418. Mouse bone marrow progenitors were transduced as previously described.18 In short, marrow was harvested from the femora of 6- to 12-week-old B6 × D2 F1 female donors treated 2 days previously with 150 mg/kg 5-fluorouracil (Adrucil; Pharmacia, Kalamazoo, MI) intraperitoneally. Cells were preinduced at 1 × 10⁶ cells/mL in Iscove modified Dulbecco medium (IMDM; Gibco/BRL) containing 10% defined FBS (Invitrogen, Purchase, NY), L-glutamine, sodium pyruvate, nonessential amino acids, antibiotics, 5% interleukin-3 culture supplement (IL-3; Collaborative Biomedical Products, Bedford, MA), 100 ng/mL recombinant human IL-6 (Sandoz Pharmaceuticals, Hanover, NJ), and 50 ng/mL recombinant mouse stem cell factor (SCF; PeproTech, Rocky Hill, NJ). After 48-hour culture at 37°C in 5% CO₂, the marrow cells were transferred to irradiated (15 Gy), subconfluent producer cells at a density of 5-10 × 10⁶ cells per 10-cm plate in 10 mL media further supplemented with 8 μg/mL polybrene. After an additional 48-hour culture, the nonadherent bone marrow cells were carefully collected on ice, washed in cold Hanks buffered saline solution (HBSS; Gibco BRL), and transplanted into irradiated (1050 Gy) syngeneic recipients at a dose of 5-10 × 10⁶ cells per animal.

**Progenitor colony assay**

Based on an established protocol,37 marrow cells were suspended at 1-2 × 10⁶ cells/mL in plating medium consisting of IMDM, 50% defined FBS, 1% wt/vol bovine serum albumin, L-glutamine, 10⁻⁴ M β-mercaptoethanol, antibiotics, and 0.9% methylcellulose. Myeloid progenitors (colony-forming cells, CFCs) were induced to form granulocyte–macrophage colonies by the addition of 5% IL-3 and were scored after 7 to 10 days of incubation at 37°C, 5% CO₂. Selection was carried out with 0.8 mg/mL active G418. Untransduced marrow was routinely included as a control to ensure that G418 selection was complete.

**Southern blot analysis**

Genomic DNA was isolated by standard methods38 and was quantified by spectrophotometry. Approximately 10 μg was digested with KpnI, which cuts once in each virus LTR, separated on 0.8% agarose gels, and blotted onto nylon filters. The blots were probed with a radiolabeled 923-bp PstI fragment for neo and were compared with samples from vector producer cells with known copies of provirus. To control for loading, the blots were stripped and reprobed with a radiolabeled 583-bp EcoRI-HindIII fragment (coordinates 18300-18883; GenBank MMBGXCD) from a noncoding region of the mouse β-globin loci, which is specific for a 3941-bp KpnI fragment. Signal intensities were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**RNase protection analysis**

Total cytoplasmic RNA was prepared from MEL585 cells after 3 days of induction or from peripheral blood cells using a commercially available kit (Promega, Madison, WI), and concentrations were determined by UV spectrophotometry. Globin gene transcripts were quantified by RNase protection as previously described39 using the following probes: pT7 mouse α128 linearized with HindIII to give a 128-bp protected fragment within exon 1 of the mouse α-globin gene; and pT7α170 linearized with BstEII to give a 170-bp protected fragment within exon 2 of the human β-globin gene. A total of 500 ng RNA was hybridized overnight at 48°C with 10⁶ cpm of each radiolabeled probe. A pilot experiment confirmed that the probe was in excess under these conditions. After digestion with RNase A and T1, the protected fragments were separated on 6% polyacrylamide–8 M urea gels, and autoradiography was performed without intensifying screens. Signal intensities were quantified by PhosphorImager, and expression levels of the human β-globin transgenes were calculated as a percentage per copy of mouse α-globin.

**Immunofluorescence staining and flow cytometry analysis**

Blood smears were analyzed by immunofluorescence staining as previously described40 using a mouse anti-γ monoclonal antibody followed by a secondary anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC). For flow cytometry analysis, approximately 10⁴ MEL585 cells induced for 4 days or 3 μL peripheral red blood cells (RBCs) collected in heparin were pelleted by centrifugation, resuspended in 1 mL HBSS with 4% formaldehyde and were fixed for 30 minutes at room temperature. Cells were then permeabilized by serial washes in cold acetone as previously described,14 washed once in cold HBSS–2% bovine serum albumin (BSA), and stained with an antibody to hemoglobin F (HbF) directly conjugated to FITC (PerkinElmer Wallac, Norton, OH) for 30 minutes on ice. Cells were then again washed and analyzed by flow cytometry on a FACSscan (Becton Dickinson, San Jose, CA) using CellQuest software. The percentage of γ-globin–positive cells in the experimental samples was determined by subtracting the amount of background staining within the established gate (typically 1%-2%) from RBCs of mock-transduced control mice.

**Table 1. Vector characterization**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Titer</th>
<th>Stability</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS40-5</td>
<td>2 × 10⁶</td>
<td>12 of 12</td>
<td>203 ± 56</td>
</tr>
<tr>
<td>HS40-6</td>
<td>1 × 10⁶</td>
<td>7 of 12</td>
<td>194 ± 53*</td>
</tr>
<tr>
<td>HS40-9</td>
<td>3 × 10⁵</td>
<td>11 of 12</td>
<td>82 ± 54†</td>
</tr>
<tr>
<td>HS40-10</td>
<td>1 × 10⁶</td>
<td>11 of 11</td>
<td>293 ± 186</td>
</tr>
<tr>
<td>HS40-11</td>
<td>3 × 10⁵</td>
<td>8 of 8</td>
<td>545 ± 185‡</td>
</tr>
</tbody>
</table>

Titer was determined by transfer of G418 resistance to NIH3T3 cells. Stability indicates fraction of transduced MEL cell clones with intact provirus. Expression of γ-globin protein in transduced MEL clones containing intact provirus from Figure 2 was determined by flow cytometry; reported as average ± SD mfu. HS40-5 and HS40-6 were reported previously.14,15

*Includes clones with intact provirus only.
†P < .001 versus HS40-5 and HS40-6.
‡P < .01 versus HS40-10.

**Results**

**Vector development**

Chromatin insulators are thought to work best when used in pairs to flank a gene of interest.26 At a minimum, such an arrangement allows the flanked gene to be insulated from silencing epigenetic effects of chromatin surrounding the integrated provirus on both sides. In our previous studies with the cHS4 chromatin insulator in oncoretrovirus vectors, we achieved this flanking configuration by placing a 1.2-kb fragment containing the cHS4 core element in the U3 region of the 5’ LTR, from which it is copied into the 5’ LTR during the formation of provirus.27 We sought to test the cHS4 element in oncoretrovirus vectors for human γ-globin using a similar configuration. As summarized in Table 1, we have previously determined that vectors HS40-5 and HS40-6 are capable of generating high virus titers and expressing γ-globin at relatively high levels in MEL cell lines,14,15 as determined by flow cytometry.
However, vector HS40-5 already has a regulatory element, the α-globin HS-40 enhancer, inserted in the double-copy position of the U3 region (Figure 1). In addition, as summarized in Table 1, we had previously determined that vector HS40-6 is prone to a high degree of genetic recombination. As an alternative, we combined the internal enhancer–promoter combination from vector HS40-6 with the truncated γ-globin coding sequence from vector HS40-5 to generate vector HS40-9, diagrammed in Figure 1. This vector was capable of generating high virus titers and was genetically stable (Table 1). However, the level of γ-globin expression from this vector was much lower (82 ± 54 mean fluorescence units, mfu) than that from the parental vectors HS40-5 (203 ± 56 mfu) and HS40-6 (194 ± 53 mfu) in MEL cells (Table 1, Figure 2). These results suggested that sequences located between the RsaI and HindIII sites 3′ of the γ-globin polyadenylation signal are responsible for the genetic instability and the elevated expression observed for vector HS40-6. Polymerase chain reaction analysis of recombined HS40-6 provirus indicated that sequences in this region were recombining with sequences in exon 3 of the γ-globin gene. Close inspection revealed a stretch of partial sequence homology between these regions of recombination. A 58-bp stretch containing much of the partially homologous sequence located between the 3′ RsaI and HindIII sites of the γ-globin cassette was deleted to generate vector HS40-10. This vector was genetically stable and capable of high-level γ-globin expression (293 ± 186 mfu) in MEL cells (Table 1, Figure 2).

We then flanked this vector with a 1.2-kb fragment containing the cHS4 chromatin insulator using a double-copy configuration to generate vector HS40-11 (Figure 1). Flanking with the cHS4 element reduced the optimal titer of this vector a moderate 3-fold to 3 × 10⁴ colony-forming units per milliliter (Table 1). However, the insulating element had no adverse effects on vector stability and increased the average level of γ-globin expression in MEL cells to 545 ± 185 mfu (Table 1, Figure 2). This represents a nearly 2-fold increase in the level of expression and a nearly 2-fold decrease in the relative variation in expression. Analysis by RNase protection confirmed that the γ-globin cassette in the insulated vector HS40-11 was expressed in MEL cells at 66% ± 36% per copy of endogenous mouse α-globin. This is in contrast to 46% ± 20% and 49% ± 40% previously reported for vectors HS40-5 and HS40-6, respectively.¹⁴,¹⁵

**Likelihood of vector expression in vivo**

To further test the insulating activity of the cHS4 element on expression of the reengineered γ-globin cassette, we turned to a mouse bone marrow transduction and transplantation assay in which globin vector silencing has been reported to be particularly severe.¹⁸⁻²⁰ For this purpose, we transduced marrow with vectors HS40-5, HS40-10, and HS40-11 and transplanted syngeneic recipients following myeloablative irradiation. Serial blood samples were then collected, and the fraction of RBCs expressing γ-globin protein was determined by immunofluorescence staining and flow cytometry (Figure 5, for example). The fraction of RBC expressing γ-globin in the mice treated with vector HS40-5 remained uniformly low throughout the analysis, averaging only 1.4% ± 1.1% at the time of death at 6 to 7 months after transplantation (Figure 3). Results with the reengineered vector HS40-10 were only modestly better, with the fraction of RBCs expressing γ-globin at the latest time point averaging only 2.0% ± 1.9%. In the case of the insulated vector HS40-11, the fraction of RBCs expressing γ-globin continued to increase to 10.8% ± 8.3% at 2 to 3 months and to 13.2% ± 11.6% at 5 to 7 months after transplantation. This initial rise in the fraction of RBCs expressing the γ-globin cassette between 1 and 2 to 3 months after transplantation can most easily be explained by the kinetics of red cells in mice, in which circulating RBCs survive approximately 40 days.⁴²

To determine whether these differences simply reflected a difference in the level of gene transfer between these vectors, the relative level of provirus in the hematopoietic cells of individual mice was determined by quantitative Southern blotting and was used to estimate the fraction of cells that contained provirus. These levels ranged from 60% ± 13% for vector HS40-5, 61% ± 26% for vector HS40-10, and 25% ± 17% for vector HS40-11. We then normalized the level of γ-expressing RBCs to the level of provirus-containing cells to estimate the percentage of expressing

![Figure 2. Expression of γ-globin in MEL cell clones.](image)

![Figure 3. Fraction of RBCs expressing γ-globin following bone marrow transplantation.](image)
Level of vector expression in vivo

Although flanking with the cHS4 fragment allowed the γ-globin transgene to be expressed in a higher fraction of RBCs, the level of expression remained variable. As seen in Figure 5A, direct immunofluorescence staining of peripheral blood smears revealed a small fraction of RBCs with a bright pattern of staining and a larger fraction of RBCs with a dull pattern of staining. This variation was even more evident when analyzed by flow cytometry. As seen in Figure 5B, the RBC populations considered to be positive for γ-globin expression were distributed over nearly 2 logs of fluorescence intensity. There was also a pronounced skewing of this population to the lowest intensity of expression. As a positive control for these studies, we used a transgenic mouse line containing an intact human γ-globin gene linked to a μLCR enhancer.43 As seen at the top of Figure 5B, expression of this μLCR-γ cassette was also highly variable, with the transgene only expressed in approximately two thirds of peripheral RBCs and a distribution of expression similar to that observed for vector HS40-11. To more accurately quantify the level of γ-globin expression in the recipient mice, we compared the level of γ-globin RNA to the level of endogenous mouse α-globin RNA in peripheral blood samples by RNase-protection. As seen in Table 2, the analyzed mice that received vector HS40-11 expressed γ-globin at 3.5% ± 3.1% per copy of mouse α-globin, compared with 37.7% ± 4.3% for the μLCR-γ transgenic control. Results for this transgenic control are within the previously reported range of 11.2% to 40.0% per copy of mouse α-globin (2.8% to 10% vs total α-globin).43 However, when the fraction of RBCs that actually express the γ-globin cassette was taken into account (an average 16.3% ± 13.3% for the HS40-11 mice and 62.0% ± 10.8% for the


**Table 2. RNA expression analysis in mice receiving transplants**

<table>
<thead>
<tr>
<th>Gene</th>
<th>$\gamma$/a $\times 100^\dagger$</th>
<th>% $\gamma$ (+) RBC $\ddagger$</th>
<th>$\gamma$/a per $\gamma$ (+) RBC</th>
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<tr>
<td>$\mu$LCR-$\gamma$</td>
<td>42.6</td>
<td>66.2</td>
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<td></td>
<td>34.4</td>
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<td></td>
<td>36.1</td>
<td>70.1</td>
<td>51.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>37.7 ± 4.3</td>
<td>62.0 ± 10.8</td>
<td>61.6 ± 9.1</td>
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<tr>
<td>HS40-11</td>
<td>0.6</td>
<td>4.8</td>
<td>12.5</td>
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<td></td>
<td>1.2</td>
<td>2.4</td>
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<td>5.6</td>
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<tr>
<td>Mean ± SD</td>
<td>3.5 ± 3.1</td>
<td>16.3 ± 13.3</td>
<td>23.3 ± 16.0</td>
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</tbody>
</table>

$^\dagger$Ratio of $\gamma$-globin mRNA versus $\alpha$-globin mRNA on a per-copy basis determined by RNase protection.

$^\ddagger$Percentage of RBCs expressing $\gamma$-globin determined by flow cytometry as per Figure 5B.

|‡Control mice containing $\mu$LCR-$\gamma$ transgene as per Figure 5B. |

$\mu$LCR-$\gamma$ control mice), we calculated that vector HS40-11 expressed $\gamma$-globin at an average 23.3% ± 16.0% per copy of endogenous mouse $\alpha$-globin, compared with 61.6% ± 9.1% for the $\mu$LCR-$\gamma$ transgenic control. This correlates to 5.8% ± 4.0% of total endogenous mouse $\alpha$-globin for vector HS40-11 and 15.4% ± 2.3% of total endogenous mouse $\alpha$-globin for the $\mu$LCR-$\gamma$ transgenic control.

**Discussion**

In the studies presented here we sought to test whether the cHS4 chromatin insulator could be used to prevent $\gamma$-globin gene silencing from oncoretrovirus vectors. While generating vectors for this purpose, we identified a potential alternative 3' RNA processing signal for the human $\beta$-$\gamma$-globin locus. Our previous studies suggested that sequences located between 277 and 470 bases 3' of the $\gamma$-globin exon 3 were responsible for the high degree of genetic instability of vector HS40-6.12 Molecular analysis revealed 2 distinct functions related to this region. The source of genetic instability was further mapped to a 58-bp segment starting at 342 bases 3' of exon 3. This region contains a stretch of partial homology to sequences in the $\gamma$-globin exon 3 with which it was found to recombine. It has been long established that oncoretrovirus vectors can recombine at such sites of homology, providing a ready explanation for the source of instability.13,14 The studies also revealed an activity independent of this recombining region necessary for optimal expression of the $\gamma$-globin expression cassette. This region is not included in the fully processed $\gamma$-globin transcript and does not contain known enhancer activity, leading us to hypothesize that it may be involved in the efficient processing of nascent transcripts. Close inspection revealed a consensus sequence for a potential alternative RNA cleavage and polyadenylation site that would be disrupted in vectors HS40-5 and HS40-9 and that would be present in vectors HS40-6, HS40-10, and HS40-11.16 A BLAST search of the public NCBI-expressed sequence tag (EST) library revealed several cDNA sequences from human fetal liver consistent with the use of this site as a functional RNA cleavage and polyadenylation site for the endogenous human $\beta$-$\gamma$-globin loci. Use of this putative alternative 3' end would allow the addition of extended pyrimidine-rich tracks in the 3' untranscribed region, similar to those recently reported to stabilize $\beta$-globin transcripts.47 The role of this region in $\gamma$-globin transcript processing and expression regulation is being evaluated.

The first suggestion that flanking with the cHS4 chromatin insulator would increase the expression of the reoptimized $\gamma$-globin cassette came from the MEL cell studies. In these studies, the transduced MEL cell clones were derived under G418 selection. Studies by others suggest that such selection can lead to a bias for clones with provirus already integrated at relatively open chromatin locations and prevent the analysis of clones in which the provirus has been completely silenced.29 Even with such a bias, on average the insulated vector was expressed at a higher and more uniform level than the equivalent uninsulated vector (Figure 2). This is especially important in light of the high degree of variegation seen with globin expression cassettes in this cell line.48 More important, a high degree of insulation was also observed in a mouse bone marrow transduction and transplantation model in which globin vector silencing is particularly severe18-22 and expression during terminal erythroid differentiation is progressively limited.23,24 Longitudinal studies presented in Figure 3 indicate that the fraction of RBCs that expressed $\gamma$-globin from the insulated vector continued to increase over time. Although the level of provirus present at the earlier time points was not assessed, this continual increase in expression suggests that the cHS4 element can functionally prevent the temporal silencing observed for the uninsulated vectors. Functional insulation with the cHS4 element was also evidenced by the 9.6-fold increase (from 5.1% ± 7.2% to 48.9% ± 19.9%) in the concordance between the frequency of hematopoietic cells calculated to contain provirus and the frequency of peripheral red blood cells expressing $\gamma$-globin at the latest time tested (Figure 4). These results compare favorably with the results of our previous studies with the cHS4 chromatin insulator and oncoretrovirus vectors in mice. In this case, flanking a dual-reporter vector increased the probability of expression approximately 7-fold for a green fluorescence protein (GFP) cassette transcribed from the virus LTR (4%-29% in WBCs) and a neo cassette transcribed from an internal pgk promoter (11%-73% in bone marrow progenitors).27 Although the degree of insulation reported here is substantial, it is incomplete. Based on our calculations, approximately half the integrated provirus was still silent in vivo. Further, there remained a high degree of variation in the amount of $\gamma$-globin expressed between transduced MEL cell clones (Figure 2) and $\gamma$-expressing RBCs (Figure 5). Presumably this reflects a continued, albeit reduced, sensitivity of integrated provirus to the effects of surrounding chromatin. Options for improving the degree of insulation currently under consideration include the use of multiple copies of a smaller fragment containing the cHS4 core element49 or other sources of chromatin insulators, such as the HS5 element from the human $\beta$-globin LCR.50

By increasing the probability of expression for the transferred vector using the flanking insulators, it was possible to assess the level of expression of the $\gamma$-globin cassette in the peripheral RBCs of the mice receiving transduced marrow. The average level of expression observed by RNase protection analysis, 23.3% ± 16.0% per copy of $\alpha$-globin (5.8% ± 4.0% per total $\alpha$-globin) would probably afford a moderate therapeutic benefit if achieved in patients with $\beta$-thalassemia major or sickle cell anemia. However, this is still below the requisite 20% to 30% per total $\alpha$-globin thought to be necessary to cure these diseases. Because of the relatively low level of gene transfer obtained with vector HS40-11 in mice and the still modest level of gene expression from this vector compared with that of endogenous globin genes, it was not

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possible to accurately measure the absolute level of γ-globin protein expressed by this vector using conventional quantitative methods. Several options are under consideration to increase the level of expression from this vector. These include the use of enhancers such as those from the β-globin LCR or hereditary persistence of fetal hemoglobin (HPFH) recombinants51-53 or elements thought to improve transcript stability, such as those from the human α-globin 3' UT region or the woodchuck hepatitis virus posttranscriptional response element.54,55 Before use in clinical trials, this vector would also have to be further modified to remove the neo gene, which could elicit an immune response in the absence of appropriate conditioning.56 It is possible that deletion of the neo coding sequence alone or in combination with elements of the LCR (generating a self-inactivating or SIN vector) would also increase the level of γ-globin expression.

Chromatin insulators may not offer the only means to overcome the problems of expression silencing for globin gene vectors. One alternative approach involves replacing the promoter for the globin gene cassette with a promoter from other genes known to be expressed at high levels in RBCs. In one promising application of this approach, Sabatino et al57,58 demonstrate that fusion of a minimal ankyrin promoter to a γ-globin gene allowed for expression in a copy number-dependent fashion in transgenic mice and at low levels in virtually all peripheral RBCs following retrovirus vector-mediated transduction of bone marrow in mice, indicating that this cassette is relatively resistant to silencing and position effects. Another alternative approach to addressing the problem of vector silencing involves the use of selection schemes to enrich for stem or progenitor cells that have provirus integrated at transcriptionally permissive sites. In one application, Kalberer et al22 transduced mouse marrow with an oncotropic vector for human β-globin and a GFP reporter gene and preselected GFP-expressing cells before transplantation by flow cytometry.22 As a result of preselection, they observed that the fraction of RBCs expressing GFP remained constant for up to 9.5 months after transplantation, and all mice expressed human β-globin in at least some peripheral RBCs. However, the levels of human β-globin expression were highly variable, and the published analysis did not include a serial assessment of human β-globin expression over time or a correlation between the fraction of RBCs expressing human β-globin and the level of provirus for individual animals. Thus, the effects of preselection on silencing of the therapeutic β-globin cassette in their study are difficult to assess.

As a third approach to overcoming silencing of globin gene vectors, several groups have investigated the use of elements from the human β-globin LCR with potential enhancing and chromatin-opening functions.10,11,13 Until recently, this approach has only been partially effective, in part because the regulatory elements that could be tested were limited by size and the effects of these elements on the genetic stability and titer of conventional oncoretrovirus vectors. However, 2 groups recently reported the development of lentivirus-based vectors for human β-globin with extended LCR elements that are genetically stable (presumably because of the stabilizing influence of the virus rev-response element) and capable of expressing functional levels of β-globin in mouse β-chain hemoglobinopathy models.51,52 Compared with vectors containing minimal LCR elements, these optimized vectors express human β-globin in a higher fraction of RBCs and at a more constant level over time. However, vector expression was still found to be subject to chromosomal position in one of these studies.52

In summary, we present here the further refinement of an oncoretrovirus vector for human γ-globin and the ability of the chs4 chromatin insulator to protect this vector from silencing position effects in a critical bone marrow transplantation model. Although the level of γ-globin expression from this vector approaches a potentially therapeutic range, further modifications to improve expression and to remove the potentially immunogenic neo gene will be necessary before use in clinical trials.

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References


Development of virus vectors for gene therapy of β chain hemoglobinopathies: flanking with a chromatin insulator reduces γ-globin gene silencing in vivo

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