Production of Genetically Stable High-Titer Retroviral Vectors That Carry a Human γ-Globin Gene Under the Control of the α-Globin Locus Control Region

By Sicong Ren, Bryan Y. Wong, Jihong Li, Xiang-Nong Luo, Peter M.C. Wong, and George F. Atweh

The ability to generate stable high-titer vectors that give rise to high levels of expression of transduced globin genes in erythroid cells is a prerequisite for effective retroviral-mediated globin gene therapy. The human β-globin gene with its immediate flanking sequences does not contain all the regulatory elements necessary for regulated high-level and position-independent expression in erythroid cells. The regulatory element known as the β-globin locus control region (BLCR) can provide a linked β-globin gene with these properties. However, addition of BLCR sequences to a retrovirus carrying a β-globin gene increases its genetic instability. We have developed a new generation of retroviral vectors in which a human γ-globin gene is placed under the control of the αLCR, the major regulatory element of the α-globin gene cluster. We demonstrate that these retroviruses are genetically stable in producer cell lines and can be produced at high titers that exceed 5 x 10^6 colony-forming units (CFU)/ml. In addition, we show that the transduced γ-globin gene can be expressed in the adult erythroid environment of mouse erythroleukemia (MEL) cells at a level comparable to that of a single endogenous βγ-globin gene. These retroviruses can also transduce primary murine bone marrow progenitor cells as efficiently as retroviruses that carry the neo' marker gene (neo') gene. This new generation of globin retroviral vectors may prove useful for gene therapy of human β-globin gene disorders such as sickle cell disease and β-thalassemia.

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SICKLE CELL DISEASE and β-thalassemia are single-gene disorders that are potentially curable by gene therapy. The former is a result of a mutation that alters the structure of β-globin chains, whereas the latter results from one of many mutations that decrease β-globin production. The molecular bases of these disorders have been thoroughly characterized, and the major regulatory elements of the β-globin gene cluster have been precisely localized. Nonetheless, translation of this knowledge into effective gene therapy for these disorders remains an elusive goal. In pioneering gene transfer experiments, Cone et al. demonstrated the feasibility of using retroviral vectors to transfer and express an intact human β-globin gene in mouse erythroleukemia (MEL) cells. The same group later demonstrated that retroviral vectors carrying a human β-globin gene can transduce murine hematopoietic stem cells and result in long-term expression in mouse red blood cells in vivo. The major shortcomings of these studies and several others that followed were the low titers of retroviral stocks used to infect the bone marrow, genetic instability of retroviruses that carry the β-globin gene, low efficiency of transduction of the hematopoietic stem cells, and low level of expression of the human β-globin gene in transduced erythroid cells. The discovery of the distant major regulatory element of the β-globin gene cluster known as the αLCR (α-globin locus control region) promised to facilitate globin gene therapy by increasing the level of expression of transduced genes in erythroid cells. Several studies have shown that retroviruses carrying some or all of the hypersensitive sites that make up the βLCR can give rise to high levels of expression of the human β-globin gene in transduced cells. However, these βLCR sequence elements appear highly recombinogenic and result in a dramatic increase in genetic instability of the retroviral constructs. This instability is associated with low-titer retroviral stocks that infect hematopoietic stem cells at a low efficiency. We have recently shown that a 255-bp core of the major regulatory element of the α-globin gene cluster known as HS-40, or αLCR, is capable of enhancing the activity of a heterologous promoter such as the viral SV40 promoter in an erythroid-specific manner. In the course of these studies, we observed that αLCR sequences are less recombinogenic in bacteria than ΒLCR sequences (S. Ren and G.F. Atweh, unpublished observations, August 1992). Based on these observations, we hypothesized that the αLCR core element may be capable of activating a β-like globin gene in retroviral vectors. We also hypothesized that the improved genetic stability of such retroviruses may allow their production at higher titers. In this report, we describe the production of high-titer stocks of retroviral vectors that carry the human α-globin gene under the control of the αLCR. We demonstrate that these retroviral vectors are genetically stable when they integrate in the genome of NIH 3T3 cells and MEL cells. More importantly, human γ-globin genes that are transduced by these retroviruses are expressed at high levels in MEL cells. We also show that these novel gene transfer vectors can infect primary murine bone marrow colony-forming units (CFU) as efficiently as the parental retroviral vectors that carry the neo' marker gene alone. It remains to be seen whether the lack of copy-number dependence that was observed in transgenic mice is relevant to the utility of this generation of retroviral vectors for the gene therapy of hemoglobin disorders.

MATERIALS AND METHODS

Production of retroviruses. The N2A double-copy retroviral vector, a derivative of the N2 vector, was used to generate the
globin gene transfer construct described in this report. This vector contains a selectable neo' gene flanked by two long terminal repeats (LTRs) from the Moloney murine leukemia virus.12 The human γ-globin gene (a 2.2-kb real fragment from position -410 to position +1761) was cloned by blunt-end ligation at the XhoI site of the N2A retroviral construct by electroporation as previously described.17 Twenty-four hours after transfection, cells were split 1:5 and replated in the presence of G418 400 μg/mL (active concentration). Individual colonies were isolated 2 weeks later using cloning cylinders, and expanded into producer cell lines. Retroviral titers of supernatants from these producer cell lines were assayed by slot-blot hybridization as previously described.18 Dilutions of two viral stocks of known titers (5 × 10^6 and 1 × 10^7 CFU/mL) were used as standards in the slot-blot assay. Titers of stocks from the highest-producer cell lines were confirmed by the widely used NIH 3T3 assay of transfer of G418 resistance as previously described.18

We have used a stringent vector rescue assay to test for the presence of replication-competent helper virus in the retroviral stocks. NIH 3T3 cells were infected with retroviral stocks and passaged for 1 month in the absence of G418 as previously described.15 Culture supernatants were harvested 1 month later and tested for the presence of retrovirus that can infect NIH 3T3 cells and transform them to G418-resistant cells.

Stability of retroviral sequences in transfected and infected cells. DNA was isolated from all producer cell lines for analysis of stability of the integrated plasmid DNA by Southern blotting. Ten micrograms of DNA were digested with Sall, separated on a 1% agarose gel, transferred to modified nylon membranes, and hybridized to a 255-bp αLCR probe. NIH 3T3 cells were infected with retroviruses as previously described.7 Individual NIH 3T3 colonies that became G418-resistant after retroviral infection were expanded and their DNA was isolated for analysis of stability of the integrated retroviral DNA by Southern blotting. Ten micrograms of DNA from each clone were digested with XbaI, transferred to modified nylon membranes, and hybridized to the 255-bp αLCR probe.9

Expression of the γ-globin gene in transduced MEL cells. Two pools of infected MEL cells were generated by cocultivation with producer cell lines and selection in G418 media.5 Five individual colonies were isolated from one of these pools by limiting dilution. The nature of the retroviral integration event in each clone was analyzed by Southern analysis using the restriction enzyme Xbal, which generates unique junctional fragments. Pools and clones were expanded and induced to differentiate by exposure to dimethyl sulfoxide (DMSO) as previously described.19 Expression of endogenous mouse βγ-globin genes and transduced human γ-globin genes in these cells was assayed by S1 mapping as previously described.19

In vitro transduction of primary murine hematopoietic progenitors. Murine bone marrow cells were obtained from femurs of adult BALB/c mice.30,31 Bone marrow cells (5 × 10^5) were resuspended in 1 mL of retroviral stocks supplemented with 5 μg/mL Polybrene and incubated overnight at 37°C in a humidified 5% CO2 incubator. The bone marrow cells were washed the next day and plated in 0.8% methylcellulose supplemented with 1% bovine serum albumin, 30% fetal calf serum, 0.1 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 2% spleen cell-conditioned medium, and 1 U/mL recombinant murine erythropoietin at a final cell concentration of 3 × 10^7/mL.32,33 Half the cells were plated in the presence of G418, and the other half were plated without it. Colonies that formed under both conditions were counted on day 12. Efficiency of retroviral transduction was assessed by comparing the number of colonies that formed in the presence and absence of G418.

RESULTS

A schematic map of the retroviral constructs is shown in Fig 1. The human 4γ-globin gene was cloned in the reversed transcriptional orientation relative to the 5′ LTR, and the αLCR core element was cloned in the 3′ LTR. When the N2A-γ retroviral vector integrates in the genome of an infected cell, the 3′ LTR (including the αLCR) will be duplicated to flank the γ-globin gene from both sides.14 We speculated that such an organization may have two advantages. First, expression of the γ-globin gene would be enhanced by two copies of the αLCR instead of one. Although there is no clear evidence that two copies of the αLCR will give rise to an additive enhancement of expression of a linked gene, we believed that it was at least theoretically possible that two copies may result in more enhancement than a single copy. Second, some LCR elements such as HS5 of the BLCR are believed to insulate a linked gene from the effect of surrounding sequences.24 If the αLCR turns out to have similar insulator activity, then flanking the γ-globin gene with aLCR sequences from both sides may minimize effects of the sites of integration on the activity of the γ-globin gene.

Twelve or more colonies were isolated from each of three transfected packaging cell lines and expanded into producer cell lines. Retroviral titers of supernatants from the different producer cell lines are summarized in Table 1. Retroviral titers were generally higher in GP + envAM12- and GP + E86-derived producer cell lines than in PA317-derived producer cell lines. We observed an excellent correlation

### Table 1. Titters of Retroviral Stocks

<table>
<thead>
<tr>
<th>Packaging Cells</th>
<th>Host Range</th>
<th>Titers &gt; 1 × 10^6</th>
<th>Highest Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP + envAM12</td>
<td>Amphotropic</td>
<td>3/9</td>
<td>7 × 10^4</td>
</tr>
<tr>
<td>PA317</td>
<td>Amphotropic</td>
<td>0/10</td>
<td>1 × 10^4</td>
</tr>
<tr>
<td>GP + E86</td>
<td>Ectropic</td>
<td>3/9</td>
<td>5 × 10^4</td>
</tr>
</tbody>
</table>
between retroviral titers measured by slot-blot assay and by assay of NIH 3T3 transfer of G418 resistance. For example, the highest titer was determined to be \(7 \times 10^6\) CFU/mL by slot-blot hybridization and \(6 \times 10^6\) CFU/mL by the G418 resistance assay. This suggests that cloning of the \(\alpha\)LCR and the \(\gamma\)-globin gene in the retroviral genome does not result in a high proportion of noninfectious virions. The high-titer retroviral stock (6 to \(7 \times 10^6\) CFU/mL) that was derived from the amphotropic GP + envAM12 producer cell line was used exclusively in the retroviral transduction experiments described in this report. No replication-competent helper viruses were detected in this retroviral stock by a stringent vector rescue assay.13,15

The stability of integration of the retroviral constructs in producer cell lines was investigated by Southern blotting. Figure 2 shows a representative autoradiograph from a Southern blotting experiment in which DNAs from GP + envAM12-generated producer cell lines were probed with the \(\alpha\)LCR fragment. In nine of 12 cell lines, the expected 4.3-kb \(SalI\) fragment was seen, whereas no hybridizing fragments were seen in the remaining three cell lines. This suggests that rearrangements of the integrated sequences in these three cell lines resulted in deletion of the \(\alpha\)LCR element. Stable integration was seen in 75% of GP + envAM12-derived producer cell lines, 90% of PA317-derived producer cell lines, and 70% of GP + E86-derived producer cell lines.

We also assessed stability of the integration of retroviral sequences in transduced NIH 3T3 cells. DNA was harvested from clonal isolates of infected cells and analyzed by Southern blotting using \(XhoI\), an enzyme with recognition sites in the 5' and 3' LTRs (Fig 1). When genomic DNA of a transduced cell is digested with \(XhoI\) and probed with an \(\alpha\)LCR probe, two hybridizing fragments are expected: a constant 2.2-kb fragment, and a variable fragment that represents junctional sequences at the sites of chromosomal integration. Figure 3 shows a representative autoradiograph of a Southern blotting experiment in which six of eight clones show the expected constant fragment and different-sized variable fragments. Although the variable fragments in lanes 4 and 6 appear the same, the corresponding clones could not have resulted from the same integration event, since they were derived from different transduction experiments. Therefore, 75% of these unique clones show unrearranged integration of the viral genome, while 25% show sequence rearrangements.

We generated two different pools of transduced MEL cells and isolated five different clonal cell lines. All pools and cell lines were expanded and induced to differentiate by exposure to DMSO. RNA was isolated from uninduced and DMSO-induced cells and analyzed for mouse and human globin gene expression by quantitative S1 mapping. A 5' end-labeled probe that detects human \(\gamma\)-globin mRNA and...
Fig 4. Expression of transduced y-globin genes in MEL cells. The two autoradiographs represent S1 assays for quantifying mouse β<sup>mu</sup>-globin and human y-globin mRNA in infected MEL cells. U, RNA from uninduced cells; I, RNA from DMSO-induced cells. (A) RNA isolated from a pool of infected MEL cells in induced and uninduced states. (B) RNA isolated from 5 individual clones of infected MEL cells in induced and uninduced states.

Table 2. Variability of Expression of Transduced Human y-Globin Genes in Independent MEL Cell Clones

<table>
<thead>
<tr>
<th>Clone No.</th>
<th>γ/β&lt;sup&gt;mu&lt;/sup&gt;-Globin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
</tr>
<tr>
<td>1</td>
<td>1,100%</td>
</tr>
<tr>
<td>2</td>
<td>14%</td>
</tr>
<tr>
<td>3</td>
<td>235%</td>
</tr>
<tr>
<td>4</td>
<td>210%</td>
</tr>
<tr>
<td>5</td>
<td>174%</td>
</tr>
</tbody>
</table>

Table 3. In Vitro Transduction of Murine Bone Marrow Progenitors

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Retroviral Construct</th>
<th>Colonies Without G418</th>
<th>Colonies With G418</th>
<th>Efficiency of Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N2</td>
<td>252</td>
<td>36</td>
<td>14.3%</td>
</tr>
<tr>
<td>2</td>
<td>N2A-γ</td>
<td>275</td>
<td>32</td>
<td>11.6%</td>
</tr>
<tr>
<td>2</td>
<td>N2</td>
<td>257</td>
<td>24</td>
<td>9.3%</td>
</tr>
<tr>
<td>3</td>
<td>N2A-γ</td>
<td>245</td>
<td>20</td>
<td>8.2%</td>
</tr>
<tr>
<td>3</td>
<td>N2</td>
<td>188</td>
<td>15</td>
<td>8.8%</td>
</tr>
<tr>
<td>3</td>
<td>N2A-γ</td>
<td>128</td>
<td>10</td>
<td>7.8%</td>
</tr>
<tr>
<td>Mean ± SD (1-3)</td>
<td>N2</td>
<td>10.6% ± 3.3%</td>
<td>9.2% ± 2.1%</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (1-3)</td>
<td>N2A-γ</td>
<td>10.6% ± 3.3%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The activity of this truncated regulatory gene per transduced copy ranged from 54% to 108% relative to that of a single copy of the endogenous mouse \(\beta\)-globin gene without \(\lambda\)LCR sequences. This was confirmed by a more recent study in which deletion of some IVS-II sequences of the human \(\beta\)-globin gene allowed production of more stable retroviruses at reasonably high titers of \(4 \times 10^6\) CFU/mL. However, the \(\beta\)-globin minigene was completely inactive in transduced erythroid cells. This study suggested that the intervening sequences that are dispensable for \(\beta\)-globin expression may be responsible for genetic instability in retroviruses that carry an intact \(\beta\)-globin gene.

Another factor that adversely affects the stability of globin vectors is the presence of one or more hypersensitive sites of the \(\lambda\)LCR in the retroviral constructs. Unfortunately, it has not yet been possible to localize sequences within the \(\lambda\)LCR that are responsible for this instability. When a 36-bp core sequence of HS2 was included in a \(\beta\)-globin retroviral vector, the overall stability appeared similar to that of vectors that contain the \(\beta\)-globin gene without \(\lambda\)LCR sequences. However, the activity of this truncated regulatory element was limited to twofold enhancement.

We have made two modifications in the design of our globin retroviral vectors that resulted in a significant improvement in their genetic stability. The first is the use of a fetal \(\gamma\)-globin gene instead of an adult \(\beta\)-globin gene. We elected to use the fetal globin gene because \(\gamma\)-globin chains are known to be more effective inhibitors of sickling than \(\beta\)-globin chains. Thus, a retrovirus that produces high levels of \(\gamma\)-globin chains should be more effective in gene therapy for sickle cell disease than a retrovirus that produces \(\beta\)-globin chains. A high level of expression of \(\gamma\)-globin chains should also be useful in gene therapy for \(\beta\)-thalassemia, since individuals that are homozygous for the deletion type of hereditary persistence of fetal hemoglobin are completely asymptomatic as a result of the compensatory increase in the production of \(\gamma\)-globin chains.

We have also demonstrated that the N2A-\(\gamma\) retrovirus can infect primary murine hematopoietic progenitors by in vitro methylcellulose assays. It should be emphasized that the...
aim of these experiments was not to determine the maximal efficiency of gene transfer that can be achieved with these retroviruses under the most optimal transduction protocols. Previous studies had shown that manipulations such as pre-treatment of donor bone marrow with 5-fluorouracil and in vitro exposure of donor bone marrow cells to growth factors such as interleukin-3 and -6 and stem-cell factor result in higher transduction efficiencies. In addition, repeated exposure of bone marrow cells to retroviruses in long-term marrow culture can significantly increase the efficiency of transduction of hematopoietic progenitor cells. We did not perform any of these manipulations, since our primary aim was to compare the efficiency of transduction by N2A-γ retroviruses under the most optimal transduction protocols. Efficiency of gene transfer that can be achieved with these retroviruses under the most optimal transduction protocols. The expression of the α-globin gene in the parental N2 retrovirus. Since neo' retroviral vectors have served as gold standards for bone marrow transduction experiments, the presence of the γ-globin gene and the αLCR in N2A-γ does not appear to compromise retroviral stability, titers, or ability to transduce primary hematopoietic cells. These promising results need to be extended to long-term bone marrow reconstitution experiments in which viral transductions are performed under optimized conditions. These studies may also allow us to determine the relevance of the lack of copy-number dependence in transgenic mice to the enhancement of the transduced γ-globin gene by the αLCR in erythroid cells in vivo. We hope that this new generation of globin retroviral vectors will allow us to move one step closer to effective gene therapy for sickle cell disease and β-thalassemia.

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