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 (βLCR) can provide a linked β-globin gene with these properties. However, addition of βLCR 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 β^stop^-globin gene. These retroviruses can also transduce primary murine bone marrow progenitor cells as efficiently as retroviruses that carry the neomycin resistance (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 βLCR (β-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 HIS-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 γ-globin gene under the control of the αLCR. We demonstrate that these retrovectors 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
stable high-titer globin retroviral vectors

DNA by Southern blotting. Ten clones were digested with restriction sites are marked. SD, splice donor; SA, splice acceptor.

Fig 1. Schematic illustration of the retroviral constructs. Thin lines represent plasmid sequences, and thick lines represent chromosomal sequences. Arrows indicate direction of transcription. Relevant restriction sites are marked. SD, splice donor; SA, splice acceptor.

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. The human "γ-globin gene (a 2.2-kb Real fragment from position -410 to position +1761) was cloned by blunt-end ligation to generate the N2A-γ retroviral construct (Fig 1).

Three different packaging cell lines were used to generate retroviral stocks of the N2A-γ retroviral vector. Two of these packaging cells produce amphotropic retroviruses (GP + envAM12 and PA317) and the third produces ecotropic retroviruses (GP + E86). All three packaging cell lines had been specifically designed to make production of replication-competent helper virus extremely unlikely. These packaging cells were transfected with the N2A-γ retroviral construct by electroporation as previously described. Twenty-four hours after transfection, cells were split 1:5 and replated in the presence of G418 to generate the N2A-γ retroviral construct (Fig 1).

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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 hybridization 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 XbaI, an enzyme with recognition sites in the 5' and 3' LTRs (Fig 1). When genomic DNA of a transduced cell is digested with XbaI 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 unarranged 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 γ-globin genes in MEL cells. The two autoradiographs represent S1 assays for quantifying mouse β\textsuperscript{maj} and human γ-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 γ-Globin Genes in Independent MEL Cell Clones

<table>
<thead>
<tr>
<th>Clone No.</th>
<th>γ/β\textsuperscript{maj}-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></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2A-γ</td>
<td>9.2% ± 2.1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The first advantage is improved genetic stability. It was recognized early in the course of development of globin gene transfer technology that inclusion of a human β-globin gene in retroviral vectors results in genetic instability.\(^6\)\(^,\)\(^\text{1,4,5}\)\) The transcriptional orientation of the β-globin gene appeared to play a significant role in this instability.\(^1\)\(^,\)\(^\text{4,5}\)\) When the β-globin gene was cloned downstream of the 5' LTR in the same transcriptional orientation, essentially all producer cell lines showed rearrangements of the integrated viral sequences.\(^2\)\(^,\)\(^\text{5}\)\) When the β-globin gene was cloned in the reversed transcriptional orientation, it was possible to generate producer cell lines with unarranged viral sequences, but titers of viral stocks from these producer cells were generally less than 10\(^5\) CFU/mL.\(^2\)\(^,\)\(^\text{5}\)\) Interestingly, when a human β-globin minigene that lacks the two intervening sequences was used to generate the retroviral vectors, the retroviruses were stable and could be produced at titers greater than 10\(^6\) CFU/mL.\(^2\)\(^,\)\(^\text{5}\)\) However, the β-globin minigene was completely inactive in transduced erythroid cells.\(^4\)\(^,\)\(^\text{5}\)\) These studies suggested that the intervening sequences that are indispensable for β-globin expression may be responsible for genetic instability in retroviruses that carry an intact β-globin gene.\(^4\)\(^,\)\(^\text{5}\)\) This was confirmed by a more recent study in which deletion of some IVS-II sequences of the human β-globin gene allowed production of more stable retroviruses at reasonably high titers of 4 \(\times\) 10\(^5\) CFU/mL.\(^6\)\) Although the level of expression of transduced β-globin genes was high in transduced cells, significant variability resulted from position-of-integration effects.\(^6\) The level of expression of human β-globin gene per transduced copy ranged from 54% to 108% relative to the mouse β\(^\text{maj}\)-globin gene.\(^6\)\) Another factor that adversely affects the stability of globin vectors is the presence of one or more hypersensitive sites of the βLCR in the retroviral constructs.\(^5\)\)\(^,\)\(^\text{6,7}\)\) Unfortunately, it has not yet been possible to localize sequences within the βLCR that are responsible for this instability. When a 36-bp core sequence of HS2 was included in a β-globin retroviral vector, the overall stability appeared similar to that of vectors that contain the β-globin gene without βLCR sequences.\(^5\)\) However, the activity of this truncated regulatory element was limited to twofold enhancement.\(^6\) More studies are needed to determine whether it would be possible to remove the recombinogenic sequences of the βLCR without sacrificing its enhancer activity.

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 γ-globin gene instead of an adult β-globin gene. We elected to use the fetal globin gene because γ-globin chains are known to be more effective inhibitors of sickling than β-globin chains.\(^2\)\(^,\)\(^\text{5}\)\) Thus, a retrovirus that produces high levels of γ-globin chains should be more effective in gene therapy for sickle cell disease than a retrovirus that produces β-globin chains. A high level of expression of γ-globin chains should also be useful in gene therapy for β-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 γ-globin chains.\(^2\)\(^,\)\(^\text{6}\)\) We hoped that the γ-globin gene would be more stable in retroviruses than the β-globin gene, since the intervening sequences of the two genes are completely divergent.\(^2\)\(^,\)\(^\text{8}\)\)\(^\text{9}\)\) Although the γ-globin gene has not been previously used in retroviral vectors, its use in adeno-associated viruses did not appear to result in genetic instability of the vector sequences.\(^3\)\(^\text{10}\)\)

The second modification that we introduced in the design of N2A-γ retroviral vectors is the use of αLCR instead of βLCR to enhance expression of the γ-globin gene. In previous experiments from our laboratory that involved construction of plasmids that carry βLCR elements,\(^6\) we noted a high frequency of plasmid sequence rearrangements in the DH5α (recA1) bacterial strain and a much lower frequency of rearrangements in the recombinase-minus (recA) background of the HB101 strain (S. Ren and G.F. Atweh, unpublished observations, August 1992). We did not observe an increased frequency of sequence rearrangements when we performed similar cloning experiments to generate recombinant plasmids that carry the αLCR.\(^6\)\) Even though the molecular basis of the genetic instability of βLCR sequences is still not known, we speculated that the same mechanism(s) of genetic instability in bacteria may also contribute to genetic instability in retroviral vectors. Based on this speculation, we predicted that use of the αLCR will not compromise genetic stability of retroviral vectors that carry the human γ-globin gene. This prediction was borne out by production of the stable high-titer stocks of the N2A-γ retrovirus that are described in this report. Although we were very pleased with these results, we would like to caution that these observations do not prove that the mechanisms of genetic instability of the βLCR in bacteria are the same as those in eukaryotic cells.

A second advantage of our retroviral globin vectors is the high-level expression of the transduced γ-globin gene in erythroid cells. The level of expression of transduced γ-globin gene was found to be equivalent to that of a single copy of the endogenous mouse β\(^\text{maj}\)-globin gene in a pool of infected MEL cells. When expression was analyzed in five clonal isolates of transduced MEL cells, γ-globin expression was observed to vary significantly in the uninduced state. Interestingly, when MEL cells were induced with DMSO, the level of expression of human γ-globin gene became more uniform in the different clonal isolates. Previous studies had shown limited activity of the αLCR in uninduced MEL cells, which increased markedly with induction.\(^3\)\(^\text{10}\)\) We propose that the variability in expression of the γ-globin gene in uninduced MEL cells may be a result of chromosomal position of integration effects that are neutralized when MEL cells are induced and the αLCR is activated. The experiments described in this report do not allow us to conclude whether the two copies of the αLCR are necessary for insulating the γ-globin gene from the effects of surrounding sequences. Future studies should analyze the effects of position of integration on globin gene expression in retroviruses that integrate a single copy instead of two copies of the αLCR.

We have also demonstrated that the N2A-γ 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 mice 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-g versus a prototypic neo-containing retrovirus (N2) under simple transduction conditions. We found that the efficiency of transduction of primary hematopoietic progenitors by the N2A-g retrovirus was similar to the efficiency of transduction by the parental N2 retrovirus. Since neo retroviral vectors have served as gold standards for bone marrow transduction experiments, the presence of the g-globin gene and the aLCR in N2A-g 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 g-globin gene by the aLCR 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 g-thalassemia.

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