Retroviral Vector Design for Long-Term Expression in Murine Hematopoietic Cells In Vivo

By Pamela H. Correll, Susan Calilla, and Stefan Karlsson

A series of retroviral vectors containing the human glucocerebrosidase (GC) cDNA driven by various promoters have been constructed in an attempt to discover which vector design can most efficiently transduce murine hematopoietic stem cells (HSCs) and drive expression of the transferred gene in hematopoietic cells of mice reconstituted with the transduced stem cells. The simplest vector, LG, in which the GC gene is driven by the viral LTR, was the most efficient vector at infecting HSCs, with an average viral copy number in hematopoietic tissues of 3 copies/cell in recipient mice. In general, the viral vectors that contained any additional promoters or enhancers to drive expression of either the GC gene or a selectable marker gene (NeoR) had lower titers and/or transduced HSCs at a lower efficiency. This was seen most markedly when the human phosphoglycerate (PGK) promoter was used to drive the human GC cDNA. Despite repeated attempts to obtain a high titer producer clone, this virus consistently produced low titers and subsequently resulted in the lowest proviral copy numbers in long-term reconstituted mice. Only the viral LTR and the PGK promoter were capable of driving significant levels of human GC RNA in hematopoietic cells of long-term reconstituted mice, with a much lower level of RNA generated by an internal herpes TK or SV40 immediate early promoter. Insertion of the internal transcription unit in the opposite orientation relative to the viral LTRs had a detrimental effect on gene expression. The levels of RNA generated by a hybrid LTR containing the myeloproliferative sarcoma virus enhancer were higher in bone marrow-derived macrophages than in nonadherent cells of the bone marrow when compared with the LG vector. The presence of an internal promoter to drive expression of the human GC cDNA did not seem to have a detrimental effect on expression levels from the viral LTR. In fact, in the presence of an internal TK or PGK promoter expression from the LTR was increased despite the presence of lower proviral copy numbers. Insertion of a second gene (NeoR) into the vector had a negative impact on long-term expression in hematopoietic cells in vivo; however, this seems to be due solely to the lower transduction efficiency of this vector. Overall, the highest levels of GC activity in macrophages of long-term reconstituted mice were generated by the LG vector; however, these levels were variable. Vectors containing an internal SV40, TK, or PGK promoter produced consistent levels of GC activity in these cells, but because of the lower transduction efficiency obtained in the presence of these promoters, simple vectors containing a single gene driven by the viral LTR currently remain the most promising viruses for gene therapy of human hematopoietic disorders. This is a US government work. There are no restrictions on its use.

Retroviral vectors have been used for the last decade to transfer genes into mammalian cells for the purpose of developing gene therapy for inherited disorders, cancer, and acquired immunodeficiency syndrome (AIDS). The majority of research to date has focused on bone marrow (BM) as a target tissue for retroviral gene therapy. Hematopoietic stem cells (HSCs) are able to persist throughout life by undergoing proliferation to produce daughter stem cells (self-renewal) and are also able to differentiate to form all cells of the hematopoietic system. Erythrocytes, lymphocytes (B and T), granulocytes, monocytes/macrophages, and platelets, all mature hematopoietic cells derived from these pluripotent stem cells, are replaced continuously throughout life. Transfer of the appropriate gene into HSCs could be used for treatment of any disorder that affects one or more of these cell lineages.

Retroviral vectors have been used successfully to transfer a number of genes into murine HSCs; however, persistent, long-term expression in the progeny of these cells in vivo has been historically difficult to obtain. Vectors that produce high levels of expression in immortalized fibroblasts or hematopoietic cell lines do not necessarily result in expression in differentiated progeny of infected HSCs in vivo. It has been unclear whether the low levels of expression in the majority of these studies were due to insufficient transcription or low transduction of HSCs. In addition, it is impossible to directly compare the results from these studies because of differences in methods of infection, transduction efficiency, and vector design (genes being transferred, regulatory elements present, direction of transcription, and vector backbone used).

In an attempt to determine what vector design would be most useful to drive expression of a transferred gene in hematopoietic cells, a series of retroviral vectors containing the human glucocerebrosidase (GC) cDNA under the transcriptional regulation of a variety of promoters and enhancers has been constructed. GC deficiency, commonly known as Gaucher's disease, is a leading candidate for HSC gene therapy. It is an autosomal recessive lysosomal storage disorder characterized by an accumulation of GC in macrophages of the BM, spleen, liver, lung, and brain. We previously transferred the human GC gene into murine hematopoietic cells by an internal herpes TK or SV40 immediate early promoter. Insertion of the internal transcription unit in the opposite orientation relative to the viral LTRs had a detrimental effect on gene expression. The levels of RNA generated by a hybrid LTR containing the myeloproliferative sarcoma virus enhancer were higher in bone marrow-derived macrophages than in nonadherent cells of the bone marrow when compared with the LG vector. The presence of an internal promoter to drive expression of the human GC cDNA did not seem to have a detrimental effect on expression levels from the viral LTR. In fact, in the presence of an internal TK or PGK promoter expression from the LTR was increased despite the presence of lower proviral copy numbers. Insertion of a second gene (NeoR) into the vector had a negative impact on long-term expression in hematopoietic cells in vivo; however, this seems to be due solely to the lower transduction efficiency of this vector. Overall, the highest levels of GC activity in macrophages of long-term reconstituted mice were generated by the LG vector; however, these levels were variable. Vectors containing an internal SV40, TK, or PGK promoter produced consistent levels of GC activity in these cells, but because of the lower transduction efficiency obtained in the presence of these promoters, simple vectors containing a single gene driven by the viral LTR currently remain the most promising viruses for gene therapy of human hematopoietic disorders. This is a US government work. There are no restrictions on its use.

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potentially therapeutic levels of human GC in the majority of macrophages from long-term reconstituted mice has been shown. In this report, we directly compare the transduction efficiency of several retroviral vectors in murine HSCs and the ability of these viruses to stably express human GC in macrophages derived from the transduced stem cells. In comparing these vectors, we are asking several fundamental questions about retroviral vector design and its effect on transduction efficiency and expression. How does the inclusion of an internal promoter to the viral construct affect its ability to infect HSCs and drive expression of the transferred gene, and how do different promoters compare with respect to these issues? What happens when the internal transcription unit is placed in the opposite orientation with respect to the viral LTRs? Does the addition of a selectable marker gene affect the ability of the virus to infect HSCs or the ability of the viral LTR to direct expression of the transferred gene in hematopoietic cells? Finally, does the choice of retroviral enhancer have an impact on the ability of the virus to transduce HSCs and/or direct expression of the transferred gene in the differentiated progeny of these cells? The answers to these questions have universal importance when designing retroviral vectors for gene therapy of any disorder involving the hematopoietic system.

MATERIALS AND METHODS

Retroviral Vectors

All vectors described in this study are based on the LN series of retroviral vectors. Construction of the LG and LGSN vectors has been described elsewhere. All other vectors were constructed from a plasmid (G1) in which the Neo gene from LN was removed and replaced with a multiple cloning site (Genetic Therapy Inc, Gaithersburg, MD). A 2.3-kb fragment of the human GC cDNA was subcloned into the EcoRI site of the polylinker in bluescript sk(−) (Stratagene, LaJolla, CA), with the 5' end of the gene towards the SacI site and the 3' end of the gene towards the KpnI site (SKGCS). A polyadenylation signal was added to the 3' end of the GC cDNA by ligation of the Nhe I/HindIII fragment of PMCINeoPolA into the EcoRV site in SKGCS (SKGCNPolA).

All internal promoters used were subcloned into the polylinker of bluescript sk(−) with the 3' end of the promoter oriented towards the SacI site. The 520-bp EcoRIBamHI fragment of PGK-puc19 (provided by Dr S.H. Orkin, Children's Hospital, Boston, MA) containing the human phosphoglycerate (PGK) promoter was cloned into the EcoRI to BamHI sites of bluescript (KSPGK). The 334-bp PvuII/HindIII fragment of pCH110 (Stratagene) containing the SV40 promoter was blunted and cloned into the EcoRV site of bluescript and oriented towards the SacI site of the polylinker (KSSV). The 280-bp XhoI/PstI fragment of PMCINeo containing the herpes thymidine kinase promoter and mutant polyoma enhancer was cloned into the XhoI to PstI sites of bluescript (KSTK). The myeloproliferative sarcoma virus (MPSV) enhancer was cloned into the 3' Moloney's murine leukemia virus (MoMLV) LTR in G1 by replacing the SacI-ClaI fragment of the MoMLV LTR with the 600-bp SacI-ClaI fragment from the MPSV LTR (provided by Dr D. Bodine, NHLBI, NIH, Bethesda, MD) (GIMP).

Expression cassettes containing the internal promoters in front of the human GC cDNA were constructed in bluescript by digesting the plasmids KSSV, KSTK, and KSPGK with EcoRI, Sma I, and XhoI, respectively, all of which cut at the 3' end of the respective promoters. A second digest was performed with Xmn I, which cuts in the ampicillin-resistance gene of the bluescript plasmid. The 1.35, 1.3, and 1.5-kb fragments containing the respective promoters were isolated. SKGCS was cut with a partial EcoRI digest, Sma I, or Xho I, which all cut at the 5' end of the GC cDNA, and Xmn I in the amp gene. The 4.2-bp fragments containing the human GC cDNA were isolated and ligated to the respective promoter fragments.

The MG vector was constructed by ligating the NotI/XhoI fragment of SKGCS into the NotI/XhoI fragment of G1MP. The SG, TG, and PG vectors were created by ligation of the XhoI fragments of 5SVG, TKGC, and PKGC into the XhoI site of G1. Both orientations of the cassettes were isolated relative to the viral LTR. A polyadenylation site was added to the vectors in which the GC gene was in the backwards orientation by removal of the SalI fragment containing the last 828-bp of the GC cDNA and replacing this with the SalI fragment of SKGCGNPoA that contains the last 828 bp of the GC cDNA plus the inserted polyadenylation signal.

Virus-Producing Cells

The GP + E86 virus-producing cells used in this study were grown in Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal bovine serum, and glutamine. The virus producing clones were created by transfection (LSGN) or cotransfection with pSV2Neo by calcium phosphate precipitation of the recombinant viral constructs into GP + E86 cells. Two days later, the cells were split by serial dilution and replated in media containing 1 g/L geneticin sulfate (G418). Ten days to 2 weeks after plating, individual colonies were picked and expanded.

Titration of LGSN was performed by plating 1 × 10⁶ TK−3T3 cells onto a 10-cm tissue culture dish on day 1. The following day, the 3T3 cells were infected by serial dilutions of an overnight supernatant from the virus producing cells in the presence of 8 µg/mL of polybrene. The following day, the infected 3T3 cells were split 1:10 in media containing 1 g/L G418. Eight to 10 days later, G418-resistant colonies were counted. Viral titers are expressed as colony-forming units (CFU) per milliliter of viral supernatant.

Titration of viruses with no selectable marker was performed by plating 1 × 10⁶ TK−3T3 cells on 10-cm tissue culture dishes and infecting them on day 2 with 5 mL of an overnight supernatant from the virus-producing cells in the presence of 8 µg/mL of polybrene. On day 3, viral supernatant was removed and replaced with normal media. On day 4, the 3T3 cells were harvested and DNA was extracted for Southern blot analysis. The blots were probed with the full-length 2.2-kb human cDNA, and the signal of DNA from infected 3T3 cells was compared with the signal of equal amounts of DNA from uninfected cells containing the equivalent of 1 copy/cell and 0.1 copies/cell of vector-containing plasmid DNA. Viral titers are expressed in copies per cell.

Detection of helper virus by the S′L′ assay was performed by plating D-56 cells (R.H. Bassin, National Institutes of Health, Bethesda, MD) at a density of 2 × 10⁶ cells per 6-cm tissue culture dish. The following day, the cells were infected with serial dilutions of viral supernatant in the presence of 8 µg/mL of polybrene. Two hours later, viral supernatants were aspirated and replaced with normal media. Poci (plaques) were scored on days 5 through 10.

Detection of helper virus by marker rescue assay was performed by plating 3T3 cells transduced with a replication defective retrovirus containing the Neo selectable marker gene at a density of 1 × 10⁶ cells per 10-cm dish. The following day, the cells were infected with serial dilutions of viral supernatant in the presence of 8 µg/mL of polybrene. After the infected cells were passaged for 1 to 2 weeks
to allow time for potential virus to spread, the supernatant was harvested and tested on virgin 3T3 cells. These cells were then selected in 1 g/L G418 and any resistant colonies scored 8 to 10 days later.

BM Infection and Transplantation

Donor C57Bl/6 mice were injected with 150 mg/kg 5-fluorouracil (3-FU; Fluka BioChemika, Ronkonkoma, NY) and BM was harvested from these mice 3 days postinjection. The cells were plated at a density of 1 to 2 × 10^6 cells/10-cm dish and prestimulated for 2 days in DMEM containing 10% heat-inactivated fetal bovine serum, glutamine, Pen/Strep, and 200 U/mL interleukin-3 (IL-3), IL-6, and 100 ng/mL stem cell factor (Immunex, Seattle, WA). On day 3, 1 to 2 × 10^6 cells were plated on a 10-cm dish containing virus producers plated the previous day at 1 to 2 × 10^6 cells/dish and cocultured for 2 days under the same growth factor conditions in the presence of 8 µg/mL of polybrene. Lethally irradiated (850 rad) C57Bl/6 recipient mice were injected with 5 × 10^6 cells/mouse for individual CFU-S foci and 1 to 2 × 10^6 cells/mouse for long-term reconstituted mice. CFU-S foci were examined on 12 to 14 days posttransplantation and BM, spleen, and thymus from long-term reconstituted mice were analyzed 6 to 12 months posttransplantation.

DNA and RNA Analysis

DNA and Southern blot analysis were performed using standard techniques. Proviral copy numbers were determined by scanning the intensity of each band and comparing it with copy number controls, with the endogenous GC bar used as an internal control. All scanning and quantitation was performed with the NIH Image 1.49 software (Dr Wayne Rasband, NIMH, NIH, Bethesda, MD). Total cellular RNA was extracted by guanidine thiocyanate and separated on a formaldehyde/agarose gel. The RNA was transferred to a nitrocellulose filter, prehybridized, hybridized, and washed as described.

Isolation of Macrophages

Macrophages from BM and spleen were isolated by plating 2 × 10^7 cells from a single-cell suspension derived from these two tissues in 10 mL of RPMI medium (Biofluids, Rockville, MD) with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM HEPES, pH 7.3, 5 × 10^{-3} mol/L B-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin. The plates were incubated at 37°C for 3 hours. The nonadherent cells were gently removed from the dish and the remaining adherent cells were grown in the above medium plus 10% L929 (American Type Culture Collection, Rockville, MD) cell-conditioned medium. The macrophages were cultured for 8 days and were refed with fresh medium every other day.

GC Enzymatic Assay

Cell pellets were extracted in a 50 mmol/L potassium citrate/potassium phosphate buffer (pH 5.9) containing Triton X-100 (2 mg/mL) and freeze-thawed for three cycles. Cell extracts were spun for 30 minutes at 12,000 rpm and cleared cellular lysates were assayed for GC activity. GC activity was assayed by cleavage of the synthetic substrate 4-methylumbelliferyl glucopyranoside (Sigma, St Louis, MO) at 4.8 mmol/L in a 0.1 mol/L potassium phosphate buffer (pH 5.9) with 1.5 mg/mL Triton X-100 and 1.25 mg/mL sodium taurocholate at 37°C. The reaction was terminated with 0.4 mol/L NaOH/0.4 mol/L glycine, and the fluorescence of the cleaved 4-methylumbelliferyl was measured using a fluorimeter (excitation: 360 nm, emission 430 nm).

RESULTS

Retroviral Vectors

A series of retroviral vectors containing the human GC cDNA was constructed (Fig 1). In these vectors, expression of the human GC gene is directed by either the MoMLV LTR (LGSN and LG), the MoMLV LTR containing enhancer sequences from the MPSV LTR (MG), or an internal simian virus 40 immediate early promoter (SG), thymidine kinase promoter and mutant polyoma enhancer (TG), or phosphoglycerate kinase promoter (PG) in either orientation relative to the viral LTR sequences. The selectable marker neomycin-resistance gene has been deleted from all constructs but one (LGSN). All of the vectors were packaged in the GP + E86 packaging cell line. The expected transcripts generated by each of the vectors are also shown in Fig 1.

Several individual clones of each vector packaged in GP + E86 cells were used to infect 3T3 cells, and DNA from these infected cells was analyzed by Southern blot analysis. A Southern blot of the highest titer clone found for each vector is shown in Fig 2. The titers of these viruses range from 0.3 to 2.6 copies/cell. Although there is no guarantee that the highest possible titer will be obtained for each vector, for every vector but one, a clone with a titer of at least 1 copy/cell was isolated. However, despite screening more than 50 clones containing the PG vector, the highest titer obtained was only 0.3 copies/cell on 3T3 cells. The virus-producing clones shown in these experiments were used for all subsequent experiments.

To estimate how these titers relate to other published titers, eight LGSN clones were analysed by colony-forming assay and Southern analysis for comparison (Fig 3). Six of the eight clones produced titers of close to 1.0 copy/cell (0.6 to 1.2 copies/cell) on the Southern blot. The colony-forming titers of these clones ranged from 2 × 10^4 to 1 × 10^6 CFU/mL. In most cases, the intensity of the band on the Southern blot corresponded well to the G418 titer. Two of the eight LGSN clones with titers of 3 × 10^3 and 4 × 10^4 CFU/mL did not produce a detectable band on the Southern blot analysis. This indicates that titration by Southern blot analysis will not detect a titer of less than approximately 5 × 10^4 CFU/mL. Furthermore, this indicates that all of the clones used in this study most likely have a titer of at least 1 × 10^6 CFU/mL or greater, with the exception of the PG virus, for which the titer is considerably lower. Clone no. 6 with a titer of 1 × 10^6 CFU/mL is the one shown in Fig 2 and used in the experiments presented here. The difference in copy number between the two experiments reflects the variation sometimes seen from one infection to another.

Viral supernatants from each of the producer clones used in this study were tested for the presence of wild type virus by the S' and/or the marker rescue assay. Helper virus was not detected by these methods in any of the supernatants tested.

The Effect of Vector Design on Transduction of Hematopoietic Cells

Because certain as yet unidentified characteristics of particular viral producer clones may be important for transduc-
Fig 1. A diagram of the vectors used in this study. All vectors are based on the LN series of retroviral vectors. Open boxes denote MoMLV LTR; light hatched boxes, human GC cDNA; dark hatched boxes, polyadenylation signal from SV40; SV, SV40 immediate early promoter; NEO, neomycin-resistance gene; TK, herpes thymidine kinase promoter and mutant polyoma enhancer; PGK, human phosphoglycerate kinase promoter; MPSV, myeloproliferative sarcoma virus; Kb, kilobase. Arrows indicate origin and direction of transcription. The expected RNA transcripts are shown below each vector. The spliced transcripts are shown as dotted lines because they are not always detected.

Fig 2. Southern blot analysis of DNA from NIH3T3 cells infected by each of the viral supernatants used for this study. The DNA was digested with Nhe I, which cuts in the viral LTRs, and 10 µg was loaded per lane. The blot was probed with the human GC cDNA. 1 and 0.1 copies/cell, genomic DNA from uninfected 3T3 cells + the equivalent of 1 and 0.1 copies/cell of LG plasmid DNA; 3T3, DNA from uninfected 3T3 cells; GP + E86, DNA from 3T3 cells infected with supernatant from untransfected GP + E86 cells. Molecular weight standards are shown on the left in kilobases. The proviral copy number, which is shown below each lane, was determined by scanning with the genomic GC gene used as an internal control for loading.
Fig 3. (A) Southern blot analysis of DNA from 3T3 cells infected by supernatants from eight separate clones of GP + E86 cells transfected with LGSN. The DNA was digested with Nhe I, which cuts in the viral LTRs, and 10 μg was loaded per lane. The blot was probed with the human GC cDNA. 1 and 0.1 copies/cell, genomic DNA from uninfected 3T3 cells + the equivalent of 1 and 0.1 copies/cell of LGSN plasmid DNA; 3T3, DNA from uninfected 3T3 cells. Molecular weight standards are shown on the left in kilobases. Proviral copy numbers, determined by scanning, are shown below each lane. (B) Titers of the same eight LGSN clones shown in (A) as determined by Neomycin-resistance. CFU/mL, colony-forming units per milliliter of viral supernatant.

Table 1. Infection Efficiency of Viral Producer Cells in CFU-S

<table>
<thead>
<tr>
<th>Vector</th>
<th>No. Positive/Total</th>
<th>Infection Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGSN</td>
<td>9/9</td>
<td>100</td>
</tr>
<tr>
<td>LG</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>MG</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>SG Exp. no. 1</td>
<td>3/7</td>
<td>43</td>
</tr>
<tr>
<td>Exp. no. 2</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>TG</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>PG</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>...</td>
<td>11/11</td>
<td>100</td>
</tr>
<tr>
<td>PG</td>
<td>10/10</td>
<td>100</td>
</tr>
</tbody>
</table>

The LGSN vector has the highest infection efficiency of the vectors tested, with an average copy number of 3 copies/cell. This vector has consistently transduced HSCs at a high efficiency.
 efficiency, with 2 to 5 copies/cell detected in every mouse analyzed thus far. Consistent with the viral titer on 3T3 cells, the PG virus had the lowest transduction efficiency in HSCs, with an average copy number of 0.3 copies/cell (approximately 1 log lower than the efficiency of the LG vector) in long-term reconstituted mice. The remainder of the viruses tested had intermediate infection efficiencies, with average copy numbers ranging from 0.4 to 2.4 copies/cell. Vectors containing additional promoters, enhancers, or selectable markers, in general, generated lower transduction efficiencies than the LG virus. The vector containing an internal SV40 promoter (SG) had a greater transduction efficiency than those containing the TK (TG) or PGK (PG) promoters.

It is also interesting to note that when the PGK promoter was used in the opposite orientation relative to the viral LTR, a higher transduction efficiency was obtained, correlating with the higher titer of this virus on 3T3 cells. The virus in which the MPSV enhancer was substituted for the MoMLV enhancer (MG) also had a lower transduction efficiency of both 3T3 cells and HSCs. Finally, the virus containing an internal neomycin-resistance gene (LGSN) had a lower transduction efficiency in HSCs than the LG vector or the same vector without the selectable marker (SG).

DNA from BM, spleen, and thymus of several of the long-term reconstituted mice was also analyzed by Southern blot analysis for the presence of the Y chromosome using a Y-
specific probe. The disappearance of the Y-specific band in male mice transplanted with BM from female donors indicates that, in all animals tested, the recipient mice were fully reconstituted with the donor marrow (data not shown). These results support the idea that the variability in copy number obtained with the different viruses in fact reflects a variability in transduction efficiency rather than differences in the extent of repopulation from one experiment to another.

The Effect of Vector Design on Gene Expression in Hematopoietic Cells

To determine how efficiently the various promoters can direct expression of the GC cDNA in hematopoietic cells in vivo, expression of these vectors was examined in the progeny of the transduced HSCs. Nonadherent cells (Fig 4A) from the BM of several long-term reconstituted mice transplanted with BM transduced by each of the vectors were analyzed for expression of human GC RNA. The RNA in Fig 4A was isolated from a pool of the same nonadherent BM cells analyzed for copy number in the mice indicated by asterisks in Table 2. The number of mice and the average proviral copy number for those cells are indicated below each lane.

For Gaucher’s disease, we are ultimately interested in obtaining expression in differentiated macrophages derived from the transduced HSCs. Therefore, macrophages isolated from BM of these same mice were also analyzed for human GC RNA (Fig 4B). Due to a limited number of cells, proviral copy number was not assessed directly in this cell population; however, the results from the macrophages correlates well with the expression pattern seen in the nonadherent cell population for which proviral copy number data is available.

The Northern in Fig 4 were quantitated and the results from this analysis are shown in Table 3. The values obtained for both the nonadherent cells and macrophages are a sum of all transcripts expressing the human GC cDNA. The values for the GAPDH control are also shown as well as the corrected values for human GC transcripts. For the nonadherent cells, the values are also corrected for proviral copy number. The values shown in parentheses are those for which the corresponding copy number data are not available. For these vectors, the overall average copy numbers obtained with these vectors are used. Therefore, there is likely some error in these numbers.

Comparison of promoters/enhancers for expression in hematopoietic cells. The results from these experiments indicate that, overall, the viral LTR is the most efficient of the promoters tested in all of the hematopoietic cells analyzed, although the levels of LTR-driven transcript vary among the vectors tested. Of the internal promoters tested, the PGK promoter in the forward orientation directs the highest levels of human GC message in these experiments. There are much
The numbers represent arbitrary densitometry units.

lower, but detectable, levels of GC RNA generated by the internal TK and SV40 promoters. These results correspond to those obtained when expression of the vectors was tested in three separate CFU-S experiments (data not shown).

Interestingly, levels of RNA from the viral LTR containing the MPSV enhancer (MG) are lower than those from LG in the nonadherent cells; however, in the macrophages, these levels are roughly equivalent. Because these respective samples are from the same mouse BM, it appears as though transcription from the hybrid LTR may be more efficient in the differentiated macrophages than in other nonadherent hematopoietic cells.

### Effect of a second transcription unit on expression from the viral LTR

We were also interested in determining what effect, if any, the inclusion of an internal transcription unit has on the levels of expression from the viral LTR. For these comparisons, it is most informative to focus on expression in the nonadherent fraction of the BM in long-term reconstituted mice for which proviral copy number data has been determined. It is difficult to assess the effect of integration site of the provirus on viral expression; however, due to the fact that the RNA is pooled from several transplanted mice rather than individual mice, some general observations can be made.

The levels of human GC transcripts generated by the LGSN vector are lower than those generated by the LG vector; however, the lower viral copy number obtained with the LGSN virus (0.6 copy/cell) when compared with the LG virus (3.1 copies/cell) almost certainly contributes to the lower levels of expression from the LTR seen with this virus. When corrected for the copy numbers, the levels of these transcripts are roughly equivalent. On the other hand, the levels of LTR-generated transcript in long-term reconstituted mice infected with the SG virus are not reduced when compared with those generated by the LG virus. Because the LG and SG mice in this experiment contained similar proviral copy numbers, it appears as though the presence of the internal SV40 promoter alone or in combination with the NeoR gene in the viral construct has a minimal effect on the expression levels from the viral LTR.

The levels of message from the viral LTR generated by both the TG and PG viruses also remain consistently high in all the hematopoietic cells tested. Interestingly, although the TG virus is present at a lower copy number than the LG virus in the BM of these long-term reconstituted mice (1.8 v. 3.1 copies/cell, respectively), the levels of LTR-derived message generated by this vector are higher than those generated by the LG virus. In addition, levels of LTR-generated transcript from the PG vector are also high in these mice. Although no proviral copy number data is available for the BM of these mice because of experimental error, the overall average copy number obtained in BM with the PG virus is 0.3 copies/cell, and, of the 11 animals tested, the highest copy number in an individual mouse was 0.9 copies/cell. These data indicate that the presence of an internal transcription unit driven by the TK or PGK promoters does not decrease, but may even increase expression from the viral LTR in hematopoietic cells.

### Effect of gene orientation on gene expression

Those vectors (TG and PG) that contain internal transcripts in the opposite orientation relative to the viral LTRs consistently show very low levels of both the LTR message and the internally generated message in all hematopoietic cells tested in this study. This is most likely caused by the production of antisense RNA from the internal promoter.

### Protein Production in Macrophages of Long-Term Reconstituted Mice

Macrophages isolated from BM of the same set of mice for which the RNA is shown were analyzed for expression of GC activity. The activity for each mouse is shown in Fig. 5. In this experiment, the PG virus produced the highest overall levels of GC activity in BM macrophages, with an average activity 1.77-fold higher than the uninfected controls. This activity was generated by a combination of transcripts from the LTR and PGK promoters because both transcripts can be translated to produce GC protein. This was followed by the TG, LG, and SG viruses, which increased the enzyme activity in BM macrophages by an average of 1.46- to 1.44-fold, respectively. The majority of the enzyme activity generated by the TG and SG viruses is most likely caused by transcription from the viral LTR, because levels of transcript from the SV40 and TK promoters in these cells are low. The LGSN virus produced lower levels of activity on average than the LG virus, and the TG and PG viruses in which the transcriptional unit was placed in

### Table 3. Expression Levels of Human GC RNA in Long-Term Reconstituted Mice

<table>
<thead>
<tr>
<th></th>
<th>LGSN</th>
<th>LG</th>
<th>MG</th>
<th>SG</th>
<th>TG</th>
<th>PG</th>
<th>TG</th>
<th>PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC RNA nonadherent</td>
<td>562</td>
<td>1,961</td>
<td>1,384</td>
<td>2,014</td>
<td>1,893</td>
<td>2,056</td>
<td>2,674</td>
<td>0</td>
</tr>
<tr>
<td>GAPDH nonadherent</td>
<td>2,998</td>
<td>1,995</td>
<td>3,061</td>
<td>2,014</td>
<td>1,893</td>
<td>2,056</td>
<td>1,710</td>
<td>618</td>
</tr>
<tr>
<td>GC RNA/GAPDH nonadherent</td>
<td>0.16</td>
<td>0.90</td>
<td>0.16</td>
<td>1.16</td>
<td>1.16</td>
<td>1.16</td>
<td>1.16</td>
<td>1.16</td>
</tr>
<tr>
<td>GC RNA macrophages</td>
<td>0.6</td>
<td>1,398</td>
<td>1,545</td>
<td>2,039</td>
<td>4,373</td>
<td>4,156</td>
<td>456</td>
<td>0</td>
</tr>
<tr>
<td>GAPDH macrophages</td>
<td>2,469</td>
<td>2,314</td>
<td>2,612</td>
<td>1,842</td>
<td>2,101</td>
<td>2,388</td>
<td>1,953</td>
<td>1,027</td>
</tr>
<tr>
<td>GC RNA/GAPDH macrophages</td>
<td>0.6</td>
<td>0.60</td>
<td>0.67</td>
<td>1.11</td>
<td>2.08</td>
<td>1.74</td>
<td>0.23</td>
<td>0</td>
</tr>
<tr>
<td>Proviral copy number nonadherent</td>
<td>0.6</td>
<td>3.1</td>
<td>(1.1)</td>
<td>3.1</td>
<td>1.8</td>
<td>(0.3)</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>GC RNA/copy 3, no. of nonadherent</td>
<td>0.3</td>
<td>0.3</td>
<td>(0.15)</td>
<td>0.5</td>
<td>1.5</td>
<td>(4.3)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
very closely the levels of RNA produced by the various vectors in these cells.

The MG virus did not show any detectable increase in GC protein in these cells. This is most likely caused by a mutation in the GC cDNA that may have occurred during subcloning, because the packaging cells also do not express any human GC cDNA (data not shown). When a different clone of this plasmid was transfected into amphotropic packaging cells, it was capable of expressing human GC protein.

Although the highest average levels of GC activity in this experiment were produced by the PG virus, the highest expressing single mouse in this experiment was an LC-infected mouse. In this experiment were produced by the PG virus, the highest expressing single mouse in this experiment was an LC-infected mouse in which the LG virus produced a 2.1-fold increase in GC activity in BM macrophages. In addition, much higher levels of GC activity in macrophages of long-term reconstituted mice have been produced by the LG virus. In the 20 mice tested thus far, the LG virus has produced average increases in activity in BM and spleen macrophages ranging from zero to fivefold over those from normal uninfected mice despite consistently high proviral copy numbers, with an overall average increase of 2.23-fold (data not shown).

**DISCUSSION**

In the studies presented here, we have compared the utility of several retroviral vectors for use in the gene therapy of hematopoietic disorders. We have found that the elements present in the viral vector can affect the retroviral titer, its efficiency at infecting murine HSCs, and its ability to express human GC in hematopoietic cells of long-term reconstituted mice. With the data presented here, we can begin to address the questions posed at the outset of this study.

In general, the simplest viral construct (LG) is the most efficient at infecting HSCs. This virus results in a higher vector copy number in the hematopoietic tissues and, on average, the highest overall enzyme production level of the vectors tested. However, the level of enzyme generated by the LG virus is quite variable and does not seem to be dependent on viral copy number, because all of the LG-infected mice in these studies had multiple copies of the viral genome in all hematopoietic tissues tested. These results compare with those obtained by Kaleko et al, who reported similar results with a vector containing the hADA gene driven by the MoMLV LTR. They concluded that, although the viral LTR was capable of generating high levels of hADA in long-term reconstituted mice, the presence of multiple copies was not sufficient to guarantee long-term expression.

Replacement of the MoMLV LTR enhancer with the enhancer from the MPSV LTR in the viral construct (MG) resulted in a lower viral titer and a subsequent decrease in proviral copy number in long-term reconstituted mice. Results from Beck-Engeser et al indicate that the MPSV enhancer caused a reduction in the retroviral infection of primitive hematopoietic cells. In further support of these results, the transduction efficiency of the MG virus, packaged in two different amphotropic cell lines, in both 3T3 and murine myeloid (M1) cells was lower than that for the LG virus in these same cells.

Expression of human GC RNA from the MG vector was lower than expression from the LG vector in the nonadherent cells from the BM of long-term reconstituted mice, possibly reflecting a lower copy number in these cells. However, in the BM-derived macrophages, the expression levels of human GC RNA generated by these two viruses were roughly equivalent. Therefore, for diseases in which macrophages are the target cell, such as in Gaucher’s disease, the increased levels of transcription in these cells may compensate for the lower infection efficiency. This conclusion is based on the assumption that the macrophage compartment was reconstituted to an equivalent extent in these mice. Although an increase in GC enzyme could not be detected in these macrophages because of a defect in the viral construct, we believe that, in this experiment, the level of enzyme production would be similar to that obtained with the LG virus because the levels of RNA are comparable.

Addition of an internal promoter to the viral construct to drive expression of the human GC cDNA also seemed to have a negative effect on the viral titer and its ability to infect HSCs. In addition, some internal promoters had a more deleterious effect on the viral titer than others. The presence of an internal PGK promoter (PG) dramatically reduced the viral titer and its infection efficiency in HSCs. Again, when the PG vector was packaged in amphotropic cell lines and used to infect M1 cells in culture, a similar decrease in the infection efficiency when compared with the LG and MG vectors was observed. However, when the PGK promoter was placed in the opposite orientation relative to the viral LTR, the viral titer increased somewhat. Similar

![Fig 5. GC activity in macrophages isolated from BM of the same long-term reconstituted mice as shown in Fig 4 and marked with an asterisk in Table 2. Mouse numbers from Table 2 are increasing from left to right, ie, LGSN mice are 5, 6, 7, and 8 from left to right. Enzyme activities of individual mice are shown. Specific activity is presented as nanomoles of 4MU cleaved per minute per milligram of protein.](image-url)
results have been shown with the c-fos promoter that did not allow transduction of the target cells in the forward orientation, although the reverse orientation did.\(^{21}\) The presence of the SV40 promoter in the viral construct (SG) seemed to have the least overall effect on viral titer. The TK promoter (TG) had an intermediate effect on the ability of the virus to infect HSCs, and placing the transcriptional unit in the reverse orientation relative to the viral LTRs did not enhance the titer of the virus.

Expression of the human GC cDNA from an internal promoter in hematopoietic cells is most efficient when the PGK promoter is employed. It has been suggested that the viral LTR can sometimes cause transcriptional interference with a downstream internal promoter. To decrease this interference, some groups have chosen to delete the 3' LTR enhancer.\(^{22,23}\) However, when the PGK promoter was used to drive expression of the ADA gene, interference from the LTR was absent or minimal and a deletion of the 3' LTR was not necessary to improve expression in CFU-S colonies.\(^{24}\) When the SV40 (SG) and TK (TG) promoters were used to drive expression of the human GC cDNA, they generated low, but detectable levels of human GC RNA in the hematopoietic cells.

The presence of an internal promoter did not appear to have a detrimental effect on expression levels from the viral LTR in the hematopoietic cells. Levels of LTR-generated transcript in nonadherent BM cells from long-term reconstituted mice transduced with the SG, TG, and PG vectors were equal to or greater than the levels generated by the LG vector. This increase was observed despite the lower proviral copy number present in the mice transplanted with the TG- and PG-infected BM.

Our double gene vector, LGSN, resulted in a lower infection efficiency in HSCs than the LG and SG viruses, indicating that the presence of the Neo\(^6\) gene may have a deleterious effect on retroviral titer. It has been generally agreed that the size of the retrovirus is inversely proportional to the viral titer that it can produce. The LGSN vector also resulted in decreased expression of human GC in long-term reconstituted mice when compared with the LG and SG vectors. However, this decrease in expression seems to be due solely to the lower proviral copy number present in these cells.

Others have reported detrimental effects on expression levels when a second gene is added.\(^{25,26}\) In support of this, all reports of expression of ADA and GC at levels comparable to endogenous mouse levels in hematopoietic cells have used vectors that contain only the ADA or GC gene and no selectable markers. We conclude that the reduction in expression seen with double gene vectors may be due more to a lower transduction efficiency of HSCs than to the presence of a second transcriptional unit.

In this study, the LG virus was capable of transducing HSCs at the highest efficiency and generated the mouse expressing the highest levels of human GC. However, expression from this vector in long-term reconstituted mice is not consistent. Overall, the TG and PG vectors in this study generated the highest levels of human GC expression per copy of the provirus. However, particularly in the case of the PGK promoter, this high level of expression comes at the expense of viral titer. Unfortunately, at the present time, transduction of human hematopoietic progenitors is not as efficient as the efficiencies in the mouse. Therefore, when designing retroviral vectors for human gene therapy, having a high viral titer is important. The TG vector generates higher titers than the PG vector and may be useful, although very little of the expression from this virus is actually coming from the TK promoter itself. The SV40 promoter is expressed at low levels in hematopoietic cells in vivo and its presence in the viral construct seems to be quite neutral in terms of viral titer and expression from the viral LTR, therefore no advantage is gained by its use. Addition of a selectable marker gene causes a decrease in long-term expression from the viral LTR, most likely due to the lower proviral copy numbers it generates, and should be avoided if possible. Given these results, it would be beneficial to continue focusing efforts to design an "ideal" vector that can transduce HSCs with high efficiency and consistently generate high expression levels of the transferred gene in the progeny of those cells. Of the vectors tested here, the LG vector remains the most promising for transduction of primitive human hematopoietic cells due to its high titer.\(^{27}\)

ACKNOWLEDGMENT

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REFERENCES


Retroviral vector design for long-term expression in murine hematopoietic cells in vivo

PH Correll, S Colilla and S Karlsson