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The expression of full length Gp91-Phox protein is associated with reduced amphotropic retroviral production

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ABSTRACT

Background and Objectives. **As a single gene defect in mature bone marrow cells, chronic granulomatous disease (X-CGD) represents a disorder which may be amenable to gene therapy by the transfer of the missing subunit into hemopoietic stem cells. In the majority of cases lack of Gp91-phox causes the disease. So far, studies involving transfer of Gp91-phox cDNA, including a phase I clinical trial, have yielded disappointing results. Most often, low titers of virus have been reported. In the present study we investigated the possible reasons for low titer amphotropic viral production.**

Design and Methods. **To investigate the effect of Gp91 cDNA on the efficiency of retroviral production from the packaging cell line, GP+envAm12, we constructed vectors containing either the native cDNA, truncated versions of the cDNA or a mutated form (LATG) in which the natural translational start codon was changed to a stop codon. Following derivation of clonal packaging cell lines, these were assessed for viral titer by RNA slot blot and analyzed by non-parametrical statistical analysis (Whitney-Mann U-test).**

Results**. An improvement in viral titer of just over two-fold was found in packaging cells containing the start-codon mutant of Gp91 and no evidence of truncated viral RNA was seen in these cells. Further analysis revealed the presence of rearranged forms of the provirus in Gp91-expressing cells, and the production of truncated, unpackaged viral RNA. Protein analysis revealed that LATG-transduced cells did not express full-length Gp91-phox, whereas those containing the wild-type cDNA did. However, a truncated protein was seen in ATG-transduced cells which was also present in wild type cells. No evidence for the presence of a negative transcriptional regulatory element was found from studies with the deletion mutants.**

Interpretation and Conclusions. **A statistically significant effect of protein production on the production of virus from Gp91-expressing cells was found. Our data point to a need to restrict expression of the Gp91-phox protein and its derivatives in order to enhance retroviral production and suggest that improvements in current vectors for CGD gene therapy may need to include controlled, directed expression only in mature neutrophils.**
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Key words: CGD, Gp91-phox, retroviral vector, gene therapy, immunodeficiencies

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Gp91-phox is a transmembrane protein that, with p22-phox, forms cytochrome b558, the effector part of the enzyme NADPH-oxidase. This enzyme is responsible for the formation of superoxide with consequent killing of pathogens in the phagosome.[1,2] The Gp91-phox gene spans approximately 30kb, is divided into 13 exons and is transcribed in terminally differentiated phagocytes and at a much lower level in B-cells. A locus control region (LCR) activity spanning a contiguous DNA fragment of 130kb, covering 40kb of the human Gp91-phox gene, 60kb of 5' flanking sequences and 30kb of 3' flanking sequences[3] directs efficient, tissue-specific and integration site independent expression in terminally differentiated hemopoietic cells of myeloid origin. This presumably reflects the requirement for stringent control of Gp91-phox gene expression.

Patients with a defect in Gp91-phox suffer from a rare disorder, X-linked chronic granulomatous disease (X-CGD), and exhibit the greatest morbidity and mortality of all CGD patients with serious (often antibiotic resistant) fungal and bacterial infections mainly localized in subcutaneous tissues.[4] Bone marrow transplantation (BMT) is the only curative therapy available so far. However few patients have been able to take advantage of this therapy because of lack of suitable donors and severe side effects due to graft-versus-host disease (GVHD) and immunosuppression.

Gene transfer of Gp91-phox into hemopoietic stem cells and subsequent expression of the gene in mature phagocytes may be an attractive therapeutic alternative. Studies on heterozygotic carriers of CGD alleles indicate that as few as 5-10% of phagocytic cells with wild-type levels of NADPH oxidase can confer a phenotype largely indistinguishable from that of genetically normal individuals.[5] In the last five

years, several studies have shown that, in principle, it is possible to partially restore expression of any of the missing subunits and NADPH-oxidase function in hemopoietic cells.[6-9] However so far, long term studies, including a phase I clinical trial, have yielded disappointing results and no real improvement has been achieved in the efficacy of gene therapy for CGD.[10] Most often, low titers of virus have been reported and the consequent low transduction efficiencies of primary hemopoietic cells, together with an inevitable dilution of transduced cells with the uncorrected cells of the marrow following reconstitution of patients, is likely to be a major limitation to therapeutic efficacy. In the present study we investigated the possible reasons for low titer amphotropic viral production.

Design and Methods

Vector preparation and generation of producer cells

The Gp91-phox (kindly donated by H.L. Malech, NIH, Bethesda, USA) was blunt-ended and inserted into the ClaI site of the retroviral vector LX by blunt ended ligation to produce L91 (Figure 1A). Mutant L600 was generated from L91 by deletion of a fragment of 663bp from the 5' end Gp91-phox cDNA via an *Nco*I digest (*Nco*I-*Nco*I, Figure 1C). Mutant L1200 was obtained by deletion of a 3' fragment of 1162bp from L91 with a combination of *Dra*III and *Xho*I, filling in the 3'end and re-ligating the vector (*Dra*III-*Xho*I; Figure 1D). LATG was made by mutating (Figure 1E) the ATG translational start codon at position 1635-1637 to a TGA stop codon by site directed mutagenesis. Construction of the Lid vector (which contains the α-L-iduronidase cDNA) is detailed elsewhere.[11]

Polyclonal, ecotropic packaging lines were produced by transfection of GP+E86[12,13] with pL91, pLID, pL600, pL1200 or pLATG as appropriate, and clonal amphotropic lines were obtained following transduction of GP+envAm12 cells[13] with viral supernatant from the ecotropic transfectants.

RNA slot blot analysis

Retroviral RNA was prepared as described elsewhere,[14] following PEG-precipitation of 1 mL of viral supernatant. The viral RNA was blotted onto a nylon membrane (Amersham Life Sciences) and probed with a ^{32}P-labeled LTR sequence The intensity of the radioactive signals obtained were measured using a phosphorimager (Molecular Dynamics) and analyzed by non-parametric analysis (Whitney-Mann U-test).

Southern blot analysis

Genomic DNA was extracted from cell lines by lysis of the cells in 1% SDS, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl plus 40 g/mL proteinase K and phenol extraction. Ten micrograms of DNA were digested with SacI and separated by gel electrophoresis. The DNA was transferred to a nylon membrane, probed with a ^{32}P-labeled Gp91-phox

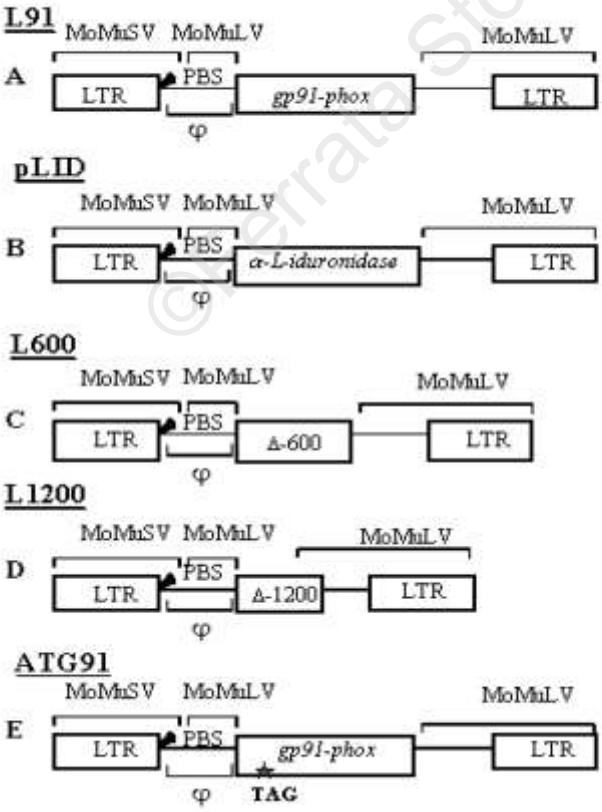

Figure 1. Retroviral construct used in this study. All are based on the Moloney Murine Leukemia Virus, LX. Constructs contain: L91 – wild type Gp91-phox cDNA; LATG – start codon-mutated gp91-phox cDNA; L600 – gP91-phox cDNA with 5' 600bp deletion (D-600); L1200 – Gp91-phox cDNA with 3' 1200bp deletion (D-1200); LID – control, α-L-iduronidase cDNA. *MoMuLV, Moloney Murine Leukemia Virus; MoMuSV, Moloney Murine Sarcoma Virus; LTR, Long Terminal Repeat; PBS, Primer Binding Signal; j, Packaging site.*

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sequence and results were analyzed via a phosphorimager (Molecular Dynamics). The intensity of the signals obtained was compared with those of the controls for copy number (prepared by spiking DNA from untransduced cells with appropriate amounts of vector DNA) and the number of viral particles was evaluated as follows:

$$\text{no. viral particle/mL supernatant} = \frac{\text{no. cells infected x copy number/cell}}{\text{mL viral supernatant used}}$$

Northern blot analysis of viral particles

Viral particles contained in 1 mL of viral supernatant were PEG-precipitated, resuspended in 50% formamide, 6.6% formamildehyde, 1 x SSC and loaded onto a 1% agarose formaldehyde gel. The gel was blotted onto a nylon membrane and this was probed with a Gp91-phox cDNA probe as previously described.

Western blot analysis of packaging cells

Cells were lysed in NP40 lysis buffer pH7.5 (50 mM Tris acetate buffer, 1 mM EGTA, 1 mM EDTA, 120 mM NaCl, 50 mM NaF, 10 mM 2-mercaptoethanol, 1% nonidet P-40) plus 1 mM Na_3VO_4 and protease inhibitors [aprotinin 6 µg/mL, pepstatin 10 µg/mL, trypsin inhibitor 10 µg/mL, N-tosyl-L-phenylalanine chloromethylketone 10 µg/mL, benzamidine 10 µ/mL, antipapain 10 µg/mL and 4-(2-aminoethyl-benzenesulphonylfluoride hydrochloride)]. Protein extracts were fractionated by SDS-PAGE (12%) and transferred to a Hybond C-extra (Amersham, UK) by semidry blotting. The filter was blocked for 1 h in 5% dried milk powder in PBS plus tween 20 0.01%, incubated 3 hours with the monoclonal antibody MoAb 48 (kindly donated by D. Roos, Red Cross Blood transfusion Center, Netherlands) at a dilution of 1:200 in blocking buffer and developed with horse radish peroxidase-conjugated sheep anti-mouse IgG (1:3000, Dako) using enhanced chemiluminescence detection (Amersham, UK).

Transduction of X-CGD PLB-985 cell line with L91 and LATG virus

X-CGD PLB 985 cells in log phase of growth were resuspended in RPMI medium/10% FCS plus polybrene at 8 µg/mL (10^6 cells/mL) and overlaid onto confluent L91 and LATG packaging cells irradiated at 30 Gy with a caesium source. Cells were incubated for 48h at 37°C and then collected for cytochrome c reduction assay.

Cytochrome c reduction assay

Cells ($4\text{-}5\times10^6$) were resuspended in Hanks balanced salt solution in the presence of 1% gelatin, cytochrome C 0.7 mg/mL and 10 µM PMA, with or without superoxide dismutase (SOD, 0.3 mg/mL) as required. The reaction was incubated at 37°C for 30 minutes after which an absorbance at 550 nm was determined. The amount of superoxide produced was calculated as the mean difference in A550 in the absence and presence of SOD.

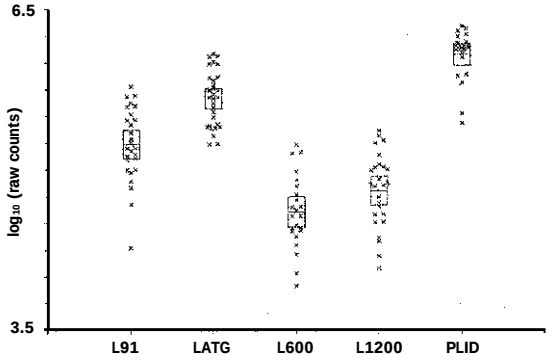

Figure 2. Strength of signal from phosphorimager (expressed as $\log_{10}$ of raw phosphorimager counts), following slot blot analysis of RNA isolated from retroviral supernatant from individual packaging cell lines. Each cross indicates data from one individual packaging line. Boxes denote mean ± SEM.

Results

Generation of constructs and amphotropic producer lines

Different constructs were derived from a MoMuLV based vector in which Gp91-phox cDNA was inserted (L91), driven by the MoMuLV LTR (Figure 1). Two deletion mutants (L600 and L1200) were generated from the L91 vector in an attempt to localize possible inhibitory sequences that regulate RNA accumulation. To test the possible detrimental effect of gp91-phox protein on the packaging cells the ATG starting codon at position 1635-37 of the L91 construct was mutated to a TGA stop codon by site directed mutagenesis. The vector Lid, in which the Gp91-phox cDNA was replaced by one encoding human α-L-iduronidase was used as a control in these studies. Amphotropic producer lines were stably transfected with the ecotropic virus to minimize differences in transfection efficiency. At least 20 clones for each construct were isolated and analyzed for virus production to minimize differences due to position effects resulting from random integration of the gene in the genome of the packaging cells.

Titration by RNA slot blot analysis of virus production

Following collection of viral supernatant from each clone, viral particles were precipitated, blotted and hybridized with an LTR sequence, common to all the constructs, as a probe. All the clones were hybridized on the same day and one of the samples was present on all the membranes as an internal control, to account for any variability in manipulation. The intensity of the radioactive slot was quantified using a Molecular Dynamics Phosphorimager. The amount of quantified radioactivity was proportional to the viral titer of each clone, with a linear range between 1 and 100,000 units.

Data were analyzed by non-parametric analysis (Mann-Whitney U-Test) and are represented in Figure 2. This analysis revealed that the titer of the L91

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producers, relative to that of the LID ones, was significantly different ($p<0.001$): L91 production generates on average approximately 16% of the viral particles obtained from the LID producers. The L600 and L1200 deletion constructs produced titers equivalent to 4% and 6% respectively of LID and 24% and 38% of L91: in both cases differences were statistically significant ($p<0.001$). No difference was found between L600 and L1200 ($p=0.034$). More interesting was the statistically significant difference between L91 and LATG ($p<0.001$). LATG produced virus with a mean titer of 41% of LID and 242% of L91, equivalent to a more than two-fold increase in titer over L91.

Southern blot analysis of transduced 3T3 cells

Three clones each of the L91 and LATG producers, with low, average and high titer, were chosen for further analysis. Serial dilutions of viral supernatant were used to infect 3T3 cells in order to obtain a quantitative viral titer. Cellular DNA was then isolated, digested with SacI and subjected to Southern blot analysis using the Gp91-phox cDNA as a probe (Figure 3A). The same blot was then stripped and rehybridized with a mix of 3 different probes specific for mouse cells (GATA-1, INT-2 and SCL) in order to allow correction for the amount of DNA loaded (Figure 3B). The titer of the best LATG producer was estimated to be around 2×10^5 viral particles/mL, whereas the best L91 producer had a titer of 10^5 viral particle/mL. This confirmed the results obtained by RNA slot blot analysis. The signal seen in all 3T3/NIH cell populations transduced with the L91 vector comprised two bands, one at 3.3Kb consistent with a full length provirus and one smaller in size (2.7Kb) than the parental L91 vector but able to hybridize with the Gp91-phox probe. In LATG infected cells, only the parental-sized proviral signal was detected.

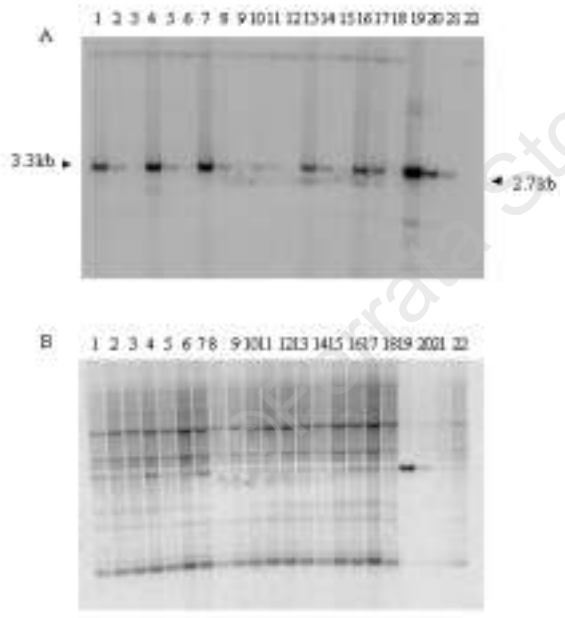

Figure 3. A) Southern blot analysis of titre from LATG (lane 1-9) and L91 (lanes 10-18) packaging cell lines. Data show signal derived from exposing 10^5 3T3 cells to 10-fold serial dilutions of viral supernatant. Lane 1-LATG clone 11, neat supernatant; Lane 2-LATG clone 11, 1:10 diluted supernatant. Lane 3-LATG clone 11, 1:100 diluted supernatant. Lane 4-6, LATG clone 40: lanes 7-9 clone 23; lanes 10-12 L91 clone 29; lanes 13-15 L91 clone 17, lanes 16-18 L91 clone 13, dilution series as described for LATG clone 11 (above). Lanes 19-21 copy number controls, lane 19, 10 copies per genome; lane 20, 1 copy per genome; lane 21, 0.1 copies per genome. Lane 22 negative control. B) Reprobing of blot from A (above), using cDNA of SCL, gata-1 and int-2 as control for amount of genomic DNA loaded.

Southern blot analysis of producer cells

Southern blot analysis of genomic DNA extracted from the same packaging cells used for viral collection and transduction was carried out to determine whether the smaller size signals seen in transduced NIH3T3 cells were present also in the packaging lines. A band of 3.3 kb was found in all packaging cell lines and in the plasmid control corresponding to the full length virus (Figure 4). However, in two out of three L91 packaging cells analyzed a smaller sized band of about 2.7 kb was found which bound the Gp91-phox cDNA probe. This was not present in any of the packaging cells in which the ATG start codon was mutated.

Northern blot analysis of viral particles

In order to establish whether both of the proviruses seen in the L91 producer lines were packaged, viral supernatant was collected from these, virus particles precipitated and the viral RNA obtained was used for northern blot analysis using the full length Gp91-phox cDNA as a probe. Only one band of about 3.3kb, corresponding to the full-length viral genome, was found (Figure 5).

Western blot analysis of packaging cells and cytochrome c assay of X-CGD PLB-985

To check whether or not the LATG construct was producing Gp91-phox protein, western blot analysis was carried out on cellular extracts from the same cell lines used for titration (Figure 6). Two protein

Figure 4. Southern blot analysis of packaging cell lines. Three separate L91 (lanes 1-3) and LATG (lanes 4-6) were analyzed. Lane 7 negative control (empty packaging cells); lane 8, positive control (DNA from empty packaging cells spiked with L91 plasmid and digested as the other samples).

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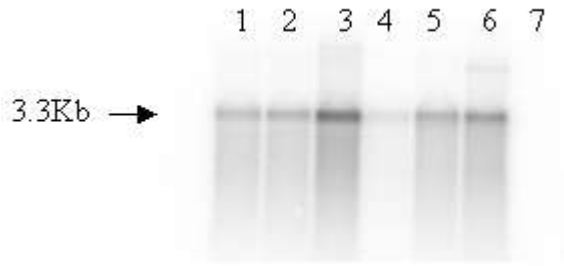

Figure 5. Northern blot analysis of viral particle. Viral supernatant from 3 separate L91 (lanes 1-3) and LATG (lanes 4-6) were analyzed. Lane 7 negative control (supernatant from empty packaging cells).

species of about 61kDa and 69kDa were produced from L91 clones. One protein species of 61kDa was also produced in LATG clones, but was not detected in the negative control (Gp+envAm12).

As expected L91 and LATG virus were able to transduce the human X-CGD cell line, PLB-985. Production of superoxide was seen only in the cells transduced with L91 (65% of wild type PLB-985 line; Figure 7).

Discussion

The ability of packaging cells to produce viral particles containing the L91 vector was compared to the activity of cells containing a similar vector in which the Gp91-phox cDNA was substituted with the α-L-iduronidase cDNA. This cDNA was chosen because it had already been used successfully in our laboratory when inserted into the LX vector.[11] The viral titer of different packaging cell lines derived from each construct was measured using RNA slot blot to quantify the number of viral genomes detectable. Comparing the average titer of L91 versus pLID clones, the insertion of Gp91-phox sequence into a LX retroviral expression vector was associated with a 7-fold reduction in virus production. This analysis indicated that the low titer obtained with the Gp91-phox construct was not due to the vector backbone or the packaging cells used but rather to the nature of the Gp91-phox cDNA itself. Most commonly, problems of low titer are related to low levels of RNA or encapsidation of the viral particle.[15,16] Problems with encapsidation may be related to disruption of the packaging signal or to inappropriate (too small or too large) viral RNA length. The L91 vector, like the LID vector has an extended packaging signal that confers efficient encapsidation. Furthermore the Gp91-phox cDNA is similar in size to (indeed slightly smaller than), the α-L-iduronidase cDNA contained in LID. For these reasons, inefficient encapsidation was not considered to be the likely cause of low titers.

Northern blot analysis of Gp91-phox and LID packaging cell lines showed reduced levels of RNA in the former with a similar trend to that seen in the RNA slot blot (data not shown). A similar correlation between low levels of retroviral genomic RNA accumulation and low vector titer has been previously described with a vector carrying the human factor VIII gene.[16] That study identified a 1.2 kb fragment of the factor VIII cDNA that retained the ability to act as a dominant inhibitor of RNA accumulation. In

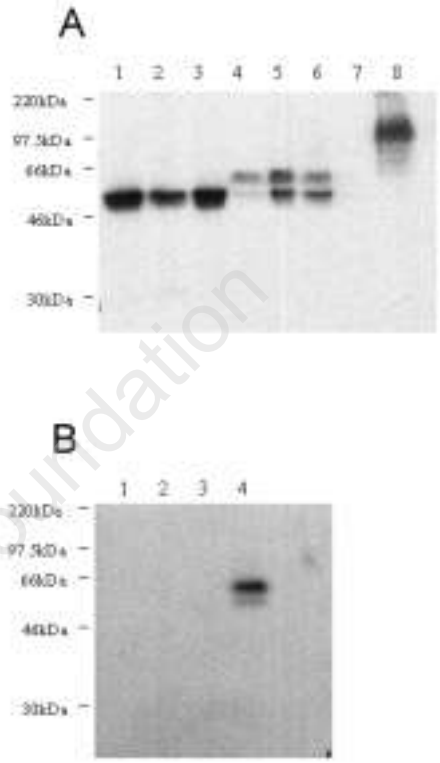

Figure 6. Western blot analysis of packaging cell lines. A) Three separate LATG (lanes 1-3) and L91 (lanes 4-6) were analyzed. Lane 7 negative control (empty packaging cells); lane 8, positive control (normal neutrophils). B) lane 1 L600 producer; lane 2 L1200 producer; lane 3, negative control (empty packaging cells); lane 4 L91 producer.

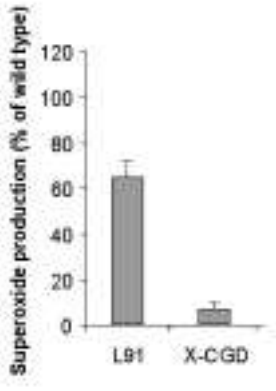

Figure 7. Superoxide production by L91 transduced and untransduced (X-CGD cells). Data expressed as percentage of superoxide produced by wild type PLB-985 cells.

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order to test whether a similar effect was present in the Gp91-phox cDNA, two deletion mutants, spanning the first 600bp and the last 1200bp of the sequence, were generated from the L91 vector. No increase in titer as a result of these deletions was seen in comparison with L91 producer clones. Instead, the L600 and L1200 clones showed a decrease in titer of 3 and 4-fold ($p<0.001$) respectively in comparison with L91. One interpretation of this might be that there are no cryptic sequences in the Gp91-phox gene that may have interfered with the transcription of the viral genome. However, we cannot exclude the possibility that the large deletions examined here might have caused some destabilization of the RNA genome which could mask any effect of removal of a negative regulatory element.

In addition to examining the effect of large deletions, a second approach was to look at the effect of the Gp91-phox on the producer line. To test whether Gp91-phox has a detrimental effect on the fibroblast packaging cells, the ATG starting codon was mutated to a TAG stop codon in the LATG construct. The LATG producer lines consistently established better titers than L91 producer lines. The overall effect was statistically highly significant, resulting in a 2-fold increase in titer, indicating a possible interference of the protein on viral production. Southern blot analysis of transduced 3T3 cells revealed in some L91 transduced cells not only the expected full length provirus but also the presence of a shorter proviral form. This may be due to either aberrant mRNA splicing or to other vector rearrangements leading to deletion of Gp91-phox sequences and either possibility might partially explain the reduction in accumulation of the full-length vector RNA when compared to LATG. No aberrant splicing or rearrangements were observed in LATG transduced cells. Similar results were obtained when cellular DNA from producer lines were subjected to Southern blot analysis. Retroviral vectors can be prone to aberrant splicing, as evidenced from studies with vectors containing CFTR or MDR1 cDNAs, due to the presence of cryptic acceptor and donor sites.[17,18] In the latter case the truncated proviral forms may be transmitted into target cells, leading not only to a reduced viral titer but also to expression of a non-functional, truncated protein and attenuation of P-gp expression. To test whether the shorter Gp91-phox proviral form was packaged and passed to the target cells, viral supernatant was precipitated and used for northern blot analysis. Only one viral form, of a length consistent with the full-length virus, was found in all the viral supernatants examined, indicating that the shorter form was not packaged.

In some studies attempts to derive stable retroviral producer lines expressing *abl-rel* or green fluorescence protein (GFP) showed that cloned cells produced low titers of virus for short periods of time and then were no longer able to produce detectable vector RNA or biologically active viral particles.[19] Moreover in the latter case, Southern blot analysis revealed rearrangements/deletions which abrogated GFP expression, conferring a potential selective advantage to the cells containing altered provirus. Indeed, similarly to that which we have observed with L91 producer cells, cells transduced with GFP showed signs of slow growth and death after one week in culture. This lends support to the hypothesis that Gp91-phox protein had a detrimental effect on the producer cells, favoring cells that express little or no full-length Gp91-phox protein, with a progressive loss of cells able to produce infectious viral particles. This would also explain the lack of a truncated RNA in LATG producer cells, where the expression of the full-length protein was abrogated.

In conclusion, we have shown that low titer of L91 construct is due to a failure in production of full-length viral RNA and this is, at least in part, influenced by an effect of the cDNA itself. Whilst production of the full length protein appeared to be associated with a reduced titer, other phenomena such as increased incidence of rearrangements and/or presence of a lower molecular weight non-packaged mRNA species due to cryptic splicing may also be involved. Rearrangements or aberrant splicing in some cells, which prevented expression of full-length Gp91-phox may have conferred a proliferative advantage over those cells expressing high levels of Gp91-phox and producing infectious viral particles, leading to progressive selection for low titers. In previous studies it proved difficult to derive amphotropic lines capable of producing more than a modest titer (2×10^5 pfu/mL),[20] despite the use of procedures such as *ping-ponging* which have been shown to increase the yield of retrovirus in studies using other genes.[21] Using a derivative SFFV vector and different producer cells, some small improvement in titer has been achieved with an estimated production of 3-5×10^5 pfu/mL.[22] However, this titer is still not optimal for hemopoietic stem cell transduction. Moreover, given that Gp91-phox protein is normally expressed at very low levels or not at all in hemopoietic stem and progenitor cells, it may be the case that uncontrolled Gp91-phox expression will also confer a selective disadvantage in transduced hemopoietic cells. Thus, cells producing very low levels of protein or which undergo deletion, rearrangement and mutations of the gene during the population expansion phase would overtake cells expressing high levels of Gp91-phox with progressive loss of transduced/expressing cells with time. If this is true expression of Gp91-phox in hemopoietic stem cells and their progeny may prove even more problematic than with other genes. In one study, following successful transduction of $CD34^+$ cells with a Gp91-expressing retrovirus, a 20-50% loss of transgene-positive cells was seen over a very short period (12 days) in culture.[22] Were this rate of reduction in gene-modified cells to occur in patients, any genetically corrected graft would soon be lost. This may point to a need to direct expression of Gp91-phox carefully so as to prevent production of protein, not only in retroviral producer cells, but in all hemopoietic cells other than mature neutrophils, and perhaps even then to control the level of expression in those cells. The transcriptional promiscuity of vectors that utilize the viral LTR as promoters makes them a less than ideal choice for such a task. Construct of a tissue stage specific vector able to express Gp91-phox protein at therapeutic levels only in neutrophils and macrophages

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together with a packaging cell line of the appropriate lineage may be required for a definitive therapy of CGD using genetic manipulation.

Contributions and Acknowledgments

The contributions of the authors to this work are as follows. IB was responsible for the design and execution of experimental work, for the analysis of the data and for producing the first draft of the manuscript. This qualifies her as first author. LJF originated the concept of mutational analysis of Gp91-phox and was the principal author responsible for the interpretation of the experimental work. This qualifies him as senior author. LSL and JAR both made important and equal contributions to the design and interpretation of the experiments. Their names are given in alphabetical order. All authors were involved in revising the manuscript for important intellectual content and approved the final version of the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential Implications for clinical practice

- Development of retroviral vectors with tight control of Gp91-phox protein may allow gene therapy of this severe form of X-linked chronic granulomatous disease.

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