Cre/loxP-Mediated Excision of a Neomycin Resistance Expression Unit from an Integrated Retroviral Vector Increases Long Terminal Repeat-Driven Transcription in Human Hematopoietic Cells

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Recombinant retroviruses are currently the most attractive vehicles for gene transfer into hematopoietic cells. Retroviral vectors often contain an easily selectable marker gene in addition to the gene of interest. However, the presence and selection for expression of the selectable gene often result in a significant reduction of the expression of the gene of interest in the transduced cells. In order to circumvent this problem, we have developed a Cre/loxP recombination system for specific excision of the selectable expression unit from integrated retroviruses. A retroviral vector, containing both a neomycin resistance expression unit flanked by loxP sites and granulocyte-macrophage colony-stimulating factor cDNA, was used to transduce the human hematopoietic K-562 cell line. Four transduced cell clones were then superinfected with a retrovirus containing a Cre recombinase expression unit. Molecular analyses of 30 doubly transduced subclones showed a strict correlation between cre expression and loxP-flanked selectable cassette excision, thus implying that Cre recombinase activity is very efficient in a retroviral context. Moreover, the excision of the selectable cassette results in a significant increase of granulocyte-macrophage colony-stimulating factor transcription driven by the retroviral promoter.

Introduction of foreign genes into human hematopoietic progenitors is a method of choice for studying hematopoietic differentiation and promises to be an effective strategy for the therapy of hematopoietic disorders. Retroviral vectors are widely used for this purpose, since they are efficient agents for gene transfer into this cell type and lead to stable integration of the transferred genes into the host genome. Moreover, high-titer helper-free stocks of recombinant retroviruses can be easily obtained (reviewed in reference 17). Most retroviral vectors have been derived from Moloney murine leukemia virus (Mo-MuLV) and are designed to express not only the gene of interest but also a selectable marker gene. The selectable gene facilitates titration of virus-producing cell lines and allows isolation of the cell population containing the gene of interest. The most widely used is the bacterial neomycin phosphotransferase gene (neo), which allows easy positive selection of transduced eucaryotic cells in vitro by conferring resistance to the antibiotic analog G418 (34). Selectable genes coding for resistance to others drugs (25, 36) or for human cell surface proteins (15, 28) are also used but to a lesser extent.

The presence of a selectable gene within the vector, although advantageous, raises the difficulty of designing retroviral vectors able to achieve efficient coexpression of two exogenous genes. The main approach used so far consists of expression of the upstream gene from the retroviral promoter located within the 5’ long terminal repeat (LTR) and of the downstream gene from an internal heterologous promoter. These bicistronic vectors allow expression of both genes in the transduced cell population but at a lower level than when only one of the genes is present in the vector (1, 7). Moreover, selection for expression of one gene often results in a significant reduction in the transcription of the unselected gene, regardless of the positions of the genes relative to each other (11, 26). This suppressive effect is cis acting, epigenetic, and stable even when selected cells are maintained without further selection (10, 26). Thus, the biological activity corresponding to the protein of interest may be undetectable in an important fraction of transduced cells. Reversal of this suppressive effect by removal of the selectable gene in selected transduced cells is an attractive possibility which remains to be demonstrated.

The Cre/loxP recombination system from bacteriophage P1 has been widely used in cultured eucaryotic cells and in animals for genetic engineering (reviewed in reference 18), since it is particularly simple and well characterized. A single 38-kDa protein, Cre, is both necessary and sufficient to catalyze site-specific recombination between two 34-bp repeats termed loxP. The result of the recombination event depends on the orientation of the interacting loxP sites. Repeats of loxP in the same orientation dictate an excision of intervening DNA sequences, whereas inverted repeats specify inversion (35).

In the work presented here, we have developed a Cre/loxP-based recombination system to remove in vitro a neomycin resistance expression unit (neo*) from a retroviral vector in a transduced human hematopoietic cell line. Our goal was to test the possibility that this removal may restore a high level of expression of a gene of interest placed under the control of the 5’ LTR. To verify this hypothesis, we introduced in the vector a second gene coding for granulocyte-macrophage colony-stimulating factor (GM-CSF) as a reporter for the activity of the retroviral promoter. GM-CSF is a cytokine essential for survival, proliferation, and differentiation of hematopoietic progenitors (reviewed in reference 13). In addition, its use in
gene therapy has been proposed as a strategy to enhance host immunity against tumor cells (9). Our study shows that expression of Cre recombinase leads to a very efficient excision of the loxP-flanked neo\(^+\) and to a subsequent increase of the GM-CSF transcription driven by the retroviral promoter.

**MATERIALS AND METHODS**

Vectors are designated as follows: the letter p indicates plasmid vectors (e.g., pN-U-GNix-Nl), and names without a p denote integrated proviruses or viruses produced by the packaging cells (e.g., N-U-GNix-Nl).

**Plasmid construction.** The loxP-neo-\(\text{loxP}\) excision cassette was constructed from plasmid pCBM11 (kindly provided by J. Rossant), which contains two head-to-tail lox\(\text{P}\) sites flanking the combination of a neomycin resistance expression unit (comprising the phosphoglycerate kinase [pgk] promoter, the neo gene, and the pgk polyadenylation signal) and a thymidine kinase (TK) expression unit. The TK expression unit was deleted as an \(\text{XbaI-HindIII}\) fragment, thus resulting in the loxP-neo-\(\text{loxP}\) cassette, composed of two direct repeats of lox\(\text{P}\) flanking the neomycin resistance expression unit. loxP-neo-\(\text{loxP}\) was then isolated as a 1.8-kb EcoRI fragment and inserted into the unique EcoRI site of the retroviral vector pNU (14) in reverse orientation relative to viral transcription. Finally, a 1.4-kb BamHI fragment from the human GM-CSF coding sequence was isolated from pCD Hu GM-CSF (a gift from F. Lee, DNAX, Palo Alto, Calif.) and ligated into the corresponding site of the vector in the same orientation as the retroviral genome, thus producing the pNU-GNix-Nl construct. To create packaging cell lines, pBabePuro-Cre, the cre gene was isolated from pBS185 (32) as a 1.4-kb \(\text{XbaI}\) fragment and cloned, blunt ended, into the unique SnaBI site of the BabePuro retroviral vector (25).

**Cell culture and selection.** Packaging cell lines and \(M. \text{dunii}\) cells were grown in Dulbeccoo’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM glutamine. K-562 cells (provided by American Type Culture Collection) were cultivated in RPMI 1640 medium containing 10% fetal calf serum. All media and sera were supplied by Biowittakter, Walkersville, Md. Nu-GNix-Nl expressing cells were selected in the presence of 1 mg of G418 (Sigma) per ml for 7 to 10 days, and cell lines expressing BabePuro-Cre were selected for 10 days in medium containing 2.5 \(\mu\)g (for GP+envAM12 and \(M. \text{dunii}\) cells) or 1 \(\mu\)g (for K-562 cells) of puromycin (Sigma) per ml.

**Productor cell lines and infections.** To obtain high-titer producer cell lines, plasmids were first transduced into GP+ Env86 packaging cells (24), and then these cells were treated with mitomycin and cocultivated with GP+ envAM12 cells (22, 23) as described previously (14). The Nu-GNix-Nl and BabePuro-Cre-producing GP+ envAM12 cells were isolated by culture in the appropriate selective medium. Viral titers were determined by using \(M. \text{dunii}\) cells as targets for viral transduction. Briefly, \(M. \text{dunii}\) cells were seeded at \(10^7\)/cm\(^2\) dish on day 1, infected with a range of viral supernatant dilutions in the presence of 4 \(\mu\)g of Polybrene per ml on day 2, and split 1:10 in selective medium on day 3, and resistant colonies were counted 10 to 12 days later. Viral titers are expressed as CFU per milliliter of viral supernatant. K-562 cells were transduced by cocultivation with mitomycin-treated GP+ envAM12 producer cells for 3 days in the presence of 4 \(\mu\)g of Polybrene per ml, selected with the appropriate antibiotic, and sorted as single cells into 96-well culture plates with a cell sorter (Vantage; Becton Dickinson, Paramus, N.J.) to generate individual clones.

**DNA and RNA analysis.** Genomic DNAs and total RNAs were prepared by the cesium chloride gradient method (31). DNA restriction enzyme digests, nucleic acid agarose gel electrophoresis, blotting procedures, DNA probe labeling, and hybridization procedures were done according to standard protocols (31). Membranes (Hybond N \(^\text{+}\); Amersham) were washed for 1 h in \(2\times\) SSC (1 \(\times\) SSC is 0.15 M NaCl plus 0.015 sodium citrate)-0.1% sodium dodecyl sulfate and for 15 min in 0.2\(\times\) SSC-0.1% sodium dodecyl sulfate at 65°C. DNA bands were visualized by autoradiography. DNA and RNA species (and sizes) expected in Northern blots (dashed lines) are indicated. Note that the AU-rich rich run present in the GM-CSF coding sequence contributes a polyadenylation signal. The diagrams are not drawn to scale.

**RESULTS**

**Production of loxP site-containing and Cre-expressing retroviral vectors.** To direct the specific excision of a neomycin resistance expression unit from an integrated retroviral vector in hematopoietic cells, we designed a Cre/loxP excision system based on two retroviral vectors. The first vector, pNU-GNix-Nl, was derived from the Mo-MuLV-based retroviral backbone pNU (14), by inserting both the GM-CSF coding sequence under the control of the 5′ LTR and a loxP-neo\(^+\)-loxP excision cassette composed of neo\(^+\) flanked by two head-to-tail lox\(\text{P}\) sites (Fig. 1A). The second vector, pBabePuro-Cre, is a Mo-MuLV retroviral vector which contains both the cre recombinase gene driven by the retroviral promoter and the puromycin resistance gene, whose expression is directed by the internal simian virus 40 (SV40) immediate early promoter (Fig. 1C). To produce recombinant viruses, pNU-GNix-Nl and pBabePuro-Cre were introduced into the amphotropic packaging cell line GP+envAM12 as described in Materials and Methods. A Nu-GNix-Nl-producing polyclonal population exhibiting a viral titer of 3 \(\times\) \(10^6\) CFU/ml and a BabePuro-Cre-producing clone ex-
hibiting a viral titer of $2 \times 10^6$ CFU/ml were isolated and used to transduce target cells.

**Cre expression mediates efficient excision of the neomycin resistance expression unit in human hematopoietic cells.** The human chronic myelogenous leukemia K-562 cell line (20) was transduced with Nu-GlxNx retroviral vector and selected in G418-containing medium, and then 10 G418-resistant clones were isolated. As assessed by Southern blot analyses, these clones carried one or two intact provirus copies inserted at different chromosomal sites (data not shown). Four independent clones, containing one (clones 1 to 3) or two (clone 4) Nu-GlxNx proviruses, were then superinfected with the BabePuro-Cre retrovirus in order to express the Cre recombinase. Puromycin-resistant (puror) cells were isolated and sorted as single cells to generate subclones which contain both proviruses. In a first step to evaluate the efficiency of the Cre-mediated excision of neo$, 103$ doubly transduced subclones were cultivated in the presence of G418. A total of $74\%$ of these subclones exhibited loss of G418 resistance, thus suggesting removal of the neomycin resistance expression unit.

The structure of the Nu-GlxNx vector carried by 30 doubly transduced subclones was then analyzed at the molecular level. For this study, we chose at random 16 G418-sensitive (G418$^s$) and 14 G418-resistant (G418$^r$) subclones derived from parental clones 1, 2, and 4. Parental clones and native K-562 cells were also used as controls. Representative results are shown in Fig. 2, and the complete analysis is summarized in Table 1. Genomic DNAs were digested with $KpnI$, an endonuclease that cleaves within the LTRs, and assayed for hybridization to the $32P$-labeled neo$^*$ probe. The neo$^*$-containing fragment (4.3 kb) corresponding to the parental proviral structure is indicated. (B) Samples (15 μg) of $KpnI$-digested DNAs were hybridized to the $32P$-labeled GM-CSF probe. Fragments corresponding to the endogenous GM-CSF gene (6 kb), the parental proviral structure (4.3 kb), and the recombined proviral structure (2.5 kb) are indicated. Lane designations are as for panel A.

![Southern blot analysis of Nu-GlxNlx proviruses in parental clones 1, 2, and 4 and in their doubly transduced derived cells.](http://jvi.asm.org/)

**FIG. 2.** Southern blot analysis of Nu-GlxNlx proviruses in parental clones 1, 2, and 4 and in their doubly transduced derived cells. (A) Samples (15 μg) of genomic DNAs were digested with $KpnI$ and assayed for hybridization to the $32P$-labeled neo$^*$ probe. The neo$^*$-containing fragment (4.3 kb) corresponding to the parental proviral structure is indicated. (B) Samples (15 μg) of $KpnI$-digested DNAs were hybridized to the $32P$-labeled GM-CSF probe. Fragments corresponding to the endogenous GM-CSF gene (6 kb), the parental proviral structure (4.3 kb), and the recombined proviral structure (2.5 kb) are indicated. Lane designations are as for panel A.
is summarized in Table 1. The expected hybridization pattern revealed by both
cre probes, provided that the cre gene is intact. Representative results of Southern blots hybridized to
the integration of a full-length Cre expression unit by hybridization of
between neou deletion (or persistence) and Cre expression in
5.9-kb fragment following digestion with
KpnI fragment; +*, presence of abnormal
probe, –, no hybridization to the indicated probe.

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* Doubly transduced subclones 1.1 to 1.7. 2.1 to 2.11, and 4.1 to 4.12 were derived from Nu-GlxNlx-containing parental clones 1, 2, and 4, respectively.
+*, presence of the proper 3.9-kb transcript; +*, presence of a KpnI fragment of inappropriate size; –, no hybridization to the indicated probe.

In summary, these analyses show that a normal BabePuro-Cre proviral structure leading to a full-length 3.9-kb transcript was present in all the neo-deleted subclones. Conversely, the BabePuro-Cre proviruses were recombined and thus cannot give rise to correct cre-containing transcripts in all the neo-containing subclones. Assuming that only the 3.9-kb transcript generates a functional Cre protein, these results strongly implicate that the neo deletion is the result of an excision event specifically mediated by Cre recombinase activity.

Excision of the neomycin resistance expression unit increases LTR-driven transcription of the GM-CSF cDNA. We then tested the hypothesis that excision of neo from the Nu-GlxNlx vector increases expression of the GM-CSF transgene. First, an enzyme-linked immunosorbent assay was performed to assess GM-CSF protein secretion by cultured cells from clone 1 and from derived subclones 1.1 to 1.7, cultivated under similar conditions (see Materials and Methods). Control K-562 cells secreted 25 ± 5 pg (mean ± standard deviation) of GM-CSF/ml106 cells. Averages (± standard deviations) of 450 ± 50 and 700 ± 100 pg of GM-CSF/ml106 cells were detected in the culture supernatants of transduced cells in exponential growth and at saturating density, respectively, with no clear difference between neo-deleted and neo-containing cell clones. We also compared the amounts of GM-CSF transcript initiated from parental and recombined Nu-GlxNlx proviruses in cell clones collected after the same period of culture. Total RNA extracted from parental cell clones 1, 2, and 4, from the 30 doubly transduced subclones, and from K-562 control cells was tested by Northern blotting for hybridization to the GM-CSF probe. Representative results are shown in Fig. 4A. As expected, the GM-CSF probe failed to detect transcripts in native K-562 cells (Fig. 4A, lane 6). This probe detected a 4.4-kb RNA species in neo-containing cells (Fig. 4A, lanes 2, 5, 8, 10, 11, 13, 14, and 16) and a 2.6-kb RNA species in neo-deleted subclones (lanes 3, 4, 7, 9, 12, and 15), as expected for complete transcription of the parental and the recombined Nu-GlxNlx provirus, respectively. The 2.2-kb fragment corresponding to RNA polyadenylated at the GM-CSF cDNA polyadenylation signal was also revealed in all the transduced cell clones. The level of GM-CSF transcription in each cell clone was quantified by densitometric analysis, using the endogenous GAPDH hybridization level as an internal tran-
DESCRIPTION standard. This analysis showed that GM-CSF transcripts initiated from neo\(^{-}\)-deleted proviruses were two- to eightfold more abundant than those initiated from neo\(^{+}\)-containing proviruses, independently of the Nu-GlxNlx integration site (Fig. 4B). Thus, Cre/loxP-mediated excision of neo\(^{-}\) from Nu-GlxNlx proviruses increases significantly the level of GM-CSF transcription initiated from the retroviral promoter in hematopoietic cells.

DISCUSSION

This study demonstrates that the Cre/loxP system is able to direct a specific excision of the neomycin resistance expression unit from Nu-GlxNlx proviruses in human hematopoietic cells. This excision has the significant advantage of improving the retroviral promoter activity and thus the transcription of the GM-CSF reporter gene.

Recently, the Cre/loxP system has been used in other laboratories to develop self-deleting Mo-MuLV vectors (8, 30) or to delete an excision cassette from an MESV vector (4) in murine fibroblasts. Those investigators have shown that transfection of the cre gene in cells carrying loxP-containing retroviral vectors or introduction of the cre gene and loxP sites within the same retroviral backbone leads to recombination between the loxP sites of the proviruses with a variable efficiency (from 50 to 100\%) from case to case. The present study extends these observations by establishing the relationship between expression of the Cre recombinase and excision of the loxP-flanked DNA cassette at the molecular level (Table 1).

These molecular analyses demonstrate that (i) Cre recombinase activity is very efficient in a retroviral context, since it leads to complete excision of neo\(^{-}\) in all the subclones expressing the Cre recombinase, regardless of the copy number and of the integration site of Nu-GlxNlx proviruses; and (ii) neo\(^{-}\) excision is specific to Cre activity, since it was never observed in subclones which do not express the full-length Cre expres-
sion unit. This excision is also specific to loxP sequences, since no recombination of the neo gene (not flanked by loxP) from NuNeo-LacZ proviruses occurred in a polyclonal K-562 population following BabePuro-Cre superinfection (data not shown). Therefore, the Cre recombinase can function in a highly efficient manner in directing loxP-specific recombination events in a retroviral context. However, the efficiency of the Cre/loxP system is lowered (26% of the doubly transduced subclones remain G418-sensitive) because of deletions within the BabePuro-Cre vector. The reason for this phenomenon remains unclear, but it is striking that other groups have also observed a high rate of rearrangements within integrative cre-containing vectors (8, 30). We hypothesize that cre might undergo fewer recombinations when introduced by a transient-delivery method. This hypothesis is supported by recent studies which show that injection of cre-containing adenoviruses into...
transgenic mice induces a virtually complete excision of loxP-flanked transgenes in the tissues known to be highly infected by adenoviral vectors (29, 37). Thus, since only a transient expression of the Cre recombinase is necessary, the integrative approach used in this study as a model to express Cre might be replaced by a transient-delivery strategy, when an efficient one is available for the recipient cell type.

The main advantage of the specific deletion of the selectable expression unit when it is no longer necessary (i.e., after the selection step) is that it improves the LTR-driven transcription at least in some cases, as demonstrated in this study. Indeed, excision of neo<sup>a</sup> from Nu-GlxNx vector leads to a significant increase in GM-CSF transcription (Fig. 4). This result implies that the presence of neo<sup>a</sup> exerts, specifically or not, a suppressive effect on the activity of the retroviral promoter. Several explanations might account for this suppressive effect. (i) Methylation of the cytosine residues in CpG dinucleotides has often been correlated with suppression of gene expression in mammalian cells (reviewed in reference 6) and with silencing of viral control elements (reviewed in reference 21). Since the neo gene contains a ninefold-higher density of CpG (78 of 874 bases) than the average 1/100 ratio characteristic of the vertebrate genome (6), it should be a good substrate for the DNA methyltransferase responsible for methylation of the vertebrate genome (5). Thus, one might expect that neo<sup>a</sup> inhibits GM-CSF transcription via a spreading of a neo<sup>a</sup>-induced methylation into the flanking sequences (including retroviral promoter and/or GM-CSF cDNA). This is unlikely, however, since analysis of the methylation status of four CG dinucleotides (one located in the 5′ LTR and three located in the GM-CSF cDNA) with methylation-sensitive restriction endonucleases Smal and HpaII showed that all these sites are demethylated in neo<sup>a</sup>-containing as well as in neo<sup>a</sup>-deleted proviruses (12). (ii) There is evidence that the neo gene exerts a specific cis-acting negative effect on expression from some retroviral and eucaryotic promoters in both transient- and stable-expression vectors (2). Such a suppressive effect may explain, at least in part, the weak retroviral promoter activity of the parental Nu-GlxNx proviruses. (iii) Addition of a second expression unit in a retroviral vector in either orientation relative to the viral transcription often has a nonspecific detrimental effect on the level of expression of one gene or both genes, as demonstrated by several groups. For example, Bowtell et al. (7) have investigated the ability of several retroviral vectors to simultaneously express the neo gene driven by an internal promoter and a second gene under the control of the LTR in hematopoietic cells. They showed that inclusion of the second gene (such as myb, myc, or the GM-CSF gene) invariably resulted in reduced activity of both retroviral and internal promoters in vitro. Similarly, in vivo experiments showed that the level of expression of a transferred human adenosine deaminase gene in mice transplanted with transduced bone marrow cells is higher when directed by a retroviral vector containing the human adenosine deaminase gene alone than when directed by a vector containing an additional neomycin resistance expression unit (1). In separate studies, Emerman and Temin (10, 11) examined the expression of adjacent cistrons in retroviral vectors that contain two promoters and two selectable genes. They reported that selection for expression of one cistron, driven either by the retroviral promoter or by the internal one (such as SV40, TK, and metallothionein I promoters), has a cis-acting suppressive effect, variable from clone to clone, on the transcription of the second cistron. They proposed a model in which transcription from one promoter causes a change in the chromatin structure in the surrounding DNA, which in turn inhibits transcription from the nearby promoter. Such competitive interferences between promoters may account for the reduced level of GM-CSF transcription initiated from the parental Nu-GlxNx proviruses. In conclusion, we propose that the suppression of GM-CSF transcription exerted by neo<sup>a</sup> is due mainly to a nonspecific effect of the presence and selection of this second cistron and might be reinforced to some extent by a specific silencing action of neo. A similar analysis with another excision cassette containing the puromycin resistance gene, which has little silencer activity (2), will allow us to further define the contribution of each mechanism to the suppression of the LTR-driven transcription.

It is noteworthy that the increase in GM-CSF transcription resulting from the excision of neo<sup>a</sup> does not give rise to a subsequent increase in GM-CSF protein secretion. Moreover, the amount of GM-CSF protein detected in the supernatant of infected cells is rather small (in the range of picograms for 10<sup>6</sup> cells). This small amount is not due to the reentry of the protein in K-562 cells, since this cell line does not express GM-CSF receptors (27). One explanation might be that the K-562 cell line is not able to perform high-level production and/or exocytosis of GM-CSF protein. However, the X63Ag8 myeloma cell line, known to be efficient for the production of transferred cytokine cDNAs (16), similarly secretes a small amount of GM-CSF when transduced with Nu-GlxNx (12). This result strongly suggests that this low protein level is instead a consequence of an intrinsic property of the vector. In this regard, it has been reported that an adenosine-uridine-rich sequence present in most cytokine mRNAs targets them for rapid degradation (33) and inhibits translation (19). Since this sequence is also present in the GM-CSF mRNA, we favor the hypothesis that such a posttranscriptional regulatory mechanism is responsible for the low level of GM-CSF production observed in both neo<sup>a</sup>-containing and neo<sup>a</sup>-deleted subclones.

In conclusion, the use of the Cre/loxP system in retrovirus-mediated gene transfer technology presents an obvious advantage, since it is able to mediate precise and predetermined excision of a retrovirus-contained gene which is only necessary for a short time, as is the case for selection genes. In the future, the strategy described here might be adapted for further applications. For example, the development of inducible promoters or of transient-expression vectors to drive Cre expression could be useful for the study of genes which function at various times of cell differentiation and in gene therapy for the transplantation of transduced cells expressing the gene of interest without undesirable additional foreign gene products.

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