

Generation of Retroviral Packaging and Producer Cell Lines for Large-Scale Vector Production and Clinical Application: Improved Safety and High Titer

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For many applications, human clinical therapies using retroviral vectors still require many technological improvements in key areas of vector design and production. These improvements include higher unprocessed manufacturing titers, complement-resistant vectors, and minimized potential to generate replication-competent retrovirus (RCR). To address these issues, we have developed a panel of human packaging cell lines (PCLs) with reduced homology between retroviral vector and packaging components. These reduced-homology PCLs allowed for the use of a novel high multiplicity of transduction ("high m.o.t.") method to introduce multiple copies of provector within vector-producing cell lines (VPCLs), resulting in high-titer vector without the generation of RCR. In a distinct approach to increase vector yields, we integrated manufacturing parameters into screening strategies and clone selection for large-scale vector production. Collectively, these improvements have resulted in the development of diverse VPCLs with unprocessed titers exceeding 2×10^7 CFU/ml. Using this technology, human Factor VIII VPCLs yielding titers as high as 2×10^8 CFU/ml unprocessed supernatant were generated. These cell lines produce complement-resistant vector particles (N. J. DePolo *et al.*, *J. Virol.* 73: 6708–6714, 1999) and provide the basis for an ongoing Factor VIII gene therapy clinical trial.

Key Words: packaging cell line; high titer; reduced homology; retroviral vectors; RCR potential.

INTRODUCTION

Retroviral-based vectors have constituted a significant portion of human gene therapy trials to date (1). We and others have shown that if titers of retroviral vector producer systems can be increased to $>5 \times 10^7$ CFU/ml final titers of the purified material, quite efficient *in vivo* gene transfer can be obtained in animal models of hemophilia, arthritis, cancer, chronic HBV infection, cystic fibrosis, and other diseases (2–8).

For many clinical applications it is necessary to manufacture a large volume of high titer doses in accordance with FDA guidelines and regulations (9). One major safety issue for the use of retroviral vectors is the possibility of generation of replication-competent retrovirus (RCR), and this risk is increased as the total vector produced increases.

Many modifications to packaging cell lines (PCL) and vectors that attempt to reduce the risk of RCR generation have been described (10–15). State of the art PCLs have a split-genome with *gag/pol* and *env* separated on distinct plasmids. Emergence of RCR following introduction of the retroviral vector to generate the vector producing cell line (VPCL) requires three recombination events between the vector, *gag/pol* and *env* genes. Nevertheless, RCR occurrence in these PCLs has been reported (16) and identified as the product of multistep homologous recombinations between the introduced and endoge-

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nous retroviral sequences (17). We have also observed the generation of RCR with our initial split-genome VPCLs that retain homologous overlap between the *gag/pol*, *env* and vector components, as a result of homologous recombination between these components. The mechanism of RCR generation was confirmed by sequence analysis of the three respective PCR-amplified areas of homology between introduced vector components in *Mus dunni* cells transduced with RCR-positive samples (unpublished data). Furthermore, VPCLs derived from early generation PCLs suffer from low titers (1×10^6 CFU/ml) and generate particles that are rapidly inactivated by human complement.

Several approaches have been assessed in attempts to identify scalable methods of reliably producing high potency clinical retroviral vector preparations. One method involved the stable incorporation of polyomavirus early genes in the PCL, whose viral gene products were shown to stimulate the retroviral LTR, producing more genomic RNA and thereby increasing titer (18). Alternatively, substantial improvement of the titer was achieved by increasing the provector copy number in the vector producing cell line, presumably by increasing the level of vector genomic RNA and the ratio of functional to defective vector particles. Approaches to generate high titer VPCLs through increased provector copy number have included "cocultivating" two PCLs that produce viruses with different envelopes, resulting in multiple rounds of infection of PCLs (the "ping-pong" strategy) (19–23). However, the ping-pong method suffers from an increased probability of recombination between the retroviral components, often resulting in RCR (22, 23). Another approach incorporates vector sequences in stable high copy episomes derived from Epstein-Barr (EB) (24). However, episomal vector copy number and vector expression is not consistent over an extended period of time and theoretically increases the inherent probability of RCR. Other high titer strategies involve the production of vector supernatants from transiently transfected cells (25), which may not be feasible for large scale production of clinical-grade vector.

High proviral copy numbers in a producer cell enhances the probability of generating RCR by increasing the number of viral sequences available for homologous recombination. To reduce or eliminate this risk, we developed several canine and human PCLs and VPCLs with reduced sequence overlap or no homology between the three retroviral components. We designated these as "reduced homology" (RH) PCLs to distinguish them from our previously described early generation split-genome PCLs (26) (see Table 1).

We report here the combination of a "high multiplicity of transduction" (m.o.t.) method using vectors having reduced sequence homology, and VPCL clone screening methods that incorporate culture scale-up parameters, to generate stable VPCL clones carrying multiple provector copy numbers that do not generate RCR. Examples include VPCL clones encoding human Factor

VIII and rat IL-4 that reproducibly yield high-titer vector at unprocessed titers greater than 1×10^7 CFU/ml. Southern and Northern blot analyses of low and high titer producing clones demonstrated that an increase in provector copy number correlated with increased genomic vector RNA, increased vector titer, and increased transfer of expression of the therapeutic gene product to target cells. Human Factor VIII (hFVIII) VPCLs have been identified that are suitable for large scale production of recombinant vector which is resistant to human complement inactivation (27), and yield high titers of vector supernatant $>1 \times 10^8$ CFU/ml without RCR occurrence. One human amphotropic FVIII producer cell line with reduced homology is currently being used to produce retroviral vector that, after purification and formulation, is being administered intravenously to individuals within a phase I hemophilia A clinical trial.

MATERIALS AND METHODS

Plasmid Construction

Detailed descriptions of all vectors will be provided upon request.

Retroviral vector constructs. The original N2-derived retroviral vector pKT-1 (Patent Applications WO 91/06852 and WO 92/05266), and all its safety modifications, are summarized in Fig. 1A. Retroviral vector pCB β -gal is derived from pKT-1 and codes for the β -galactosidase and neo^r genes as described in detail (28). The reduced homology vector, pBA-5b, is a result of several safety modifications being incorporated into pKT-1. Vector pKT-1, which already contained the modification ATT in place of the normal ATG start site of *gag*, was modified to contain two stop codons in the extended packaging signal (Ψ); the ATT modified start site was changed to the stop codon TAA, and an additional TGA stop codon was inserted 21 nt downstream. All extraneous MLV-derived retroviral sequences upstream of the 5' LTR, downstream of the 3' LTR, and between the polypurine tract and the stop codon of *env*, were eliminated. The safety-modified retroviral vector pBA-9b/rIL-4 codes for rat interleukin-4 (rIL-4) and pCF8 codes for the B-domain deleted form of human Factor VIII (hFVIII) (29–31).

MoMLV-derived *gag/pol* constructs. Safety-modifications on the original MoMLV-derived *gag/pol* plasmid pSCV10 (patent applications WO 91/06852, WO 92/05266) were carried out to reduce sequence homology to the retroviral vector and *env* expression constructs (Fig. 1B). The expression cassette pCI-WGPM contains degenerate code in approximately the first 400 nt of the coding region for *gag*, as well as deletions of all 5' and 3' untranslated sequences. In addition, the sequence coding for the last 28 amino acids of the *pol* gene is deleted, resulting in a truncated integrase gene. Plasmids pCI-GPM and pSCV10/5',3'tr. contain the same *gag/pol* cDNA as pCI-WGPM except that the 5' area of *gag* contains the native sequence.

Envelope constructs. To reduce sequence overlap in the *gag/pol* and retroviral vector plasmids, the original 4070A-derived amphotropic expression plasmid pCMVenv^{am}Dra (Patent Application WO 91/06852) was used to generate two plasmids (Fig. 1C) with either all 3' untranslated sequences deleted after the *env* stop codon (pCMVenv^{am}DraLBGH), or all 3' and 5' untranslated sequences deleted (pCMV- β /env^{am}). The xenotropic retroviral envelope expression cassette pCMVxeno was derived from NZB9-1 (32) and the amphotropic envelope expression cassette pMLPen^{am} was derived from 4070A (33).

Cells

Human kidney 293 cells (ATCC CRL 1573), human fibrosarcoma HT-1080 cells (ATCC CCL 121), canine sarcoma D-17 cells (ATCC CRL 8468) and retroviral packaging and producer cell lines derived from these parent cells were maintained in DMEM (Irvine Scientific, CA) supplemented

with 10% γ -irradiated defined fetal bovine serum (FBS, Hyclone Laboratories Inc., UT), 20 mM Hepes (Irvine Scientific, CA), 1 \times nonessential amino acids and 1 mM sodium pyruvate. Parent cell lines used to generate clinical vector producing cell lines were banked and tested in accordance with FDA guidelines for origin (i.e., isoenzyme analysis and karyotyping), absence of expressed retroviral sequences and adventitious agents including mycoplasma, bacteria, fungus and viruses.

Production of VSV-G Pseudotyped Supernatant

Large-scale production of concentrated VSV-G (vesicular stomatitis virus glycoprotein) pseudotyped vector supernatant (G-supernatant) was performed as outlined by Yee *et al.* (34) with some minor modifications. Briefly, 2A-LB packaging cells (Table 1, Fig. 2) were plated into T225 flasks at 1×10^7 cells/flask. Twelve to 20 h later the cells were CaPO₄-transfected with the VSV-G coding plasmid pMLP-G and the respective retroviral vector using the Profection kit (Promega Corp., WI). Following incubation with the DNA precipitate for 6–8 h, the DNA suspension was removed and fresh media added. Twelve to 20 h later the supernatant was collected and fresh media applied. Four to five repeat collections were made and the G-supernatant pooled, filtered (0.45 μ m) and concentrated by centrifugation at 9000g and 8°C for 8–18 h. Pellets were resuspended in a small volume of fresh media, aliquoted, frozen under liquid nitrogen, and stored at –70°C. This concentrated viral supernatant was then evaluated for titer by transfer of expression (TOE, see below) and PCR titer analysis before carrying out high m.o.t. generation of producer pools and clones.

Generation and Analysis of MLV-Based Packaging and Producer Cell Lines

Packaging cell lines. Generation of the PCLs DA, 2A, HX, and 2X was described in detail (28, 35, 36) (Patent Nos. WO 91/06852 and WO 92/05266) and the procedure further refined for the generation of the RH PCLs 2A-LB, HA-LB, HAIL, DAII, and DAwob. In general, retroviral *gag/pol* and *env* expression plasmids were sequentially introduced into cells by CaPO₄-mediated cotransfection with a phleomycin or methotrexate marker plasmid followed by the appropriate selection for 2 weeks. Selected *Gag/pol* intermediate pools were analyzed for p30 expression and subsequently dilution cloned into 96-well plates according to standard protocols. *Gag/pol* intermediate clones were analyzed for p30 expression in a Western blot (polyclonal goat anti-p30 antibodies, kindly provided by J. Elder) as well as for titer potential. Clones with the highest titer potential were co-transfected with a retroviral *env* expression plasmid and a marker, transfected cells selected, dilution cloned and PCL clones were analyzed for gp70 expression in a Western blot (polyclonal goat anti-gp70 antibodies; Quality Biotech, MD) and for titer potential. The titer potential was tested by several rounds of transduction of retroviral vectors into PCLs at a high ratio of vector to PCL in order to test the limits of the packaging capacity.

Producer cell lines. Retroviral producer pools and clones were established using a process called the “high m.o.t. approach” with m.o.t.’s >20. The multiplicity of transduction is defined as the number of infectious viral particles used per PCL cell for the production of VPCL pools. Typically, the PCL was seeded at 1×10^5 cells/well in a 6-well plate one day prior to transduction. The appropriate volumes of vector supernatants were then added to PCLs (in the presence of 8 μ g/ml polybrene) corresponding to m.o.t.’s of 0.1, 0.5, 5, 25, and 125, or as indicated in the text. After 20–24 h the vector supernatant was replaced with 2 ml of fresh media. For the HAIL and HA-LB hFVIII derived producer lines, the transduction procedure was repeated for a second day using the same volume of vector supernatant. Producer pools were grown to confluency and supernatants collected daily at 24, 48, and 72 h postconfluency to determine TOE and PCR titers. Selected pools were then dilution cloned and resulting single cell clones analyzed in several rounds of titer determination.

The HX-, 2X-, DA- and 2A/ β -gal producer pools and clones were established similarly, except that the transduction was carried out with concentrated vector derived from the amphotropic DA/ β -gal and xenotropic 2X/ β -gal VPCL clones. HX and 2X PCLs were transduced once

with the DA/ β -gal vector and DA and 2A PCLs were transduced once with the 2X/ β -gal vector each at m.o.t. 0.1, 0.5, 5, 25, and 125. Resulting pools were selected with G-418 and the β -gal titers from filtered pool supernatants determined in triplicates using the Galacto-Light assay described below. The four β -gal producer pools, 2A/ β -gal (m.o.t. 0.1 and 25) and HX/ β -gal (m.o.t. 0.1 and 125), were dilution cloned. Approximately 20 clones from each of the four producer pools were isolated, supernatants harvested, filtered (0.45 μ m) and the Galacto-Light β -gal titers determined.

TOE Assay and Titer Determinations

This general titrating assay utilizes HT-1080 target cells seeded one day prior to transduction at 3×10^5 cells per well in a six-well plate (Corning Costar, NY). Polybrene (8 μ g/ml) was added 2 h before transduction with serial dilutions of vector supernatants. After 20–24 h the supernatant was replaced with 1–2 ml of fresh media. Cells were allowed to grow for an additional 24–48 h before supernatants or genomic DNA were assayed for the expressed gene product (TOE titer) or the number of provector copies present (PCR titer).

β -Galactosidase TOE titer determination. The β -gal TOE titers were determined by two independent methods. The first method was a biochemical staining procedure using X-gal staining following a standard protocol. The second procedure was a chemiluminescent detection method utilizing the Galacto-light Plus Kit (Tropix, Inc., MA).

Human Factor VIII TOE titer determinations. To evaluate FVIII activity, target cells were transduced with an estimated m.o.t. of 0.01–3. Target cell supernatants were assayed for functional hFVIII activity 48–72 h later using the Coamatic *in vitro* diagnostic kit (Chromogenix, Sweden). The samples were directly compared to the previously characterized FVIII vector standard DA/B- (28), which generates a linear FVIII expression standard curve between 3×10^3 to 1×10^6 CFU-eq/ml (m.o.t. 0.01–3).

Rat IL-4 protein determination. Forty-eight hours after transduction of HT-1080 cells with serial dilutions of vector supernatant, aliquots of target cell supernatant were harvested and rat IL-4 levels determined using a capture ELISA (enzyme-linked immunosorbent assay) developed for this purpose (37).

Determination of Titer by PCR

PCR titer analysis of vector samples was carried out as previously described (38), except that MLV-specific primers (5'-GCG-CCT-GCG-TCG-GTA-CTA-G-3', 5'-GAC-TCA-GGT-CGG-GCC-ACA-A-3') and probe (5'-AGT-TCG-GAA-CAC-CCG-GCC-GC-3') were used to amplify a 80-bp product. The amplification reaction was carried out in 50 μ l with 200–400 μ M dNTPs, 900 nM primers, and 100 nM probe oligonucleotide. The resulting fluorescence was detected and titer based on provector copy number expressed as transduction units/ml (TU/ml). Transduction units were defined as the provector copy number per genome equivalent relative to a known copy number standard, and represent a true reflection of vector integration units.

Detection of Replication-Competent Retrovirus (RCR)

Two procedures were used to determine the presence or absence of RCR in the VPCL or the vector product, respectively. The first procedure tested post-production VPCLs. These cells were seeded into culture with an equal number of the replication permissive cell line *M. dunni*. VPCLs were seeded into flasks at a small scale (1×10^7 cells) or roller bottles at a large scale (1×10^8 cells). Cells were cocultured for several passages and finally harvested. Cell free culture supernatant was tested using a marker rescue or PG45 + L- assay (39). An RCR producing cell line generated by infection of *M. dunni* cells with a hybrid murine leukemia virus served as a positive control for the cocultivation procedure (11). Naïve *M. dunni* cells served as the negative control.

The second procedure tested the vector preparations directly. Unprocessed production harvest or purified bulk product was applied to *M. dunni* cells using 100 ml inoculation volume per roller bottle. After a brief inoculation period, 150 ml of additional media was added to the culture and cells were passaged four to five times before a portion of the

TABLE 1
Summary of Characteristic Features of MLV-Based Packaging Cell Lines

PCL clone	Gag/pol construct	Envelope construct	Parent Line	Configuration (see Fig.2)	Maximum pool titer ^a (CFU/ml)	Maximum clone titer ^a (CFU/ml)	Research or clinical trials
DA ^b	pSCV10	pCMVenv ^{am} Dra	D-17	A	5 × 10 ⁶	2 × 10 ⁷	Clinical ^c
2A ^b	pSCV10	pMLPenv ^{am}	293	A	8 × 10 ⁶	1 × 10 ⁷	Res
HX ^b	pSCV10	pCMVxeno	HT-1080	A	4 × 10 ⁶	4 × 10 ⁶	Res
2X ^b	pSCV10	pCMVxeno	293	A	7 × 10 ⁶	2 × 10 ⁶	Res
2A-LB	pSCV10	pCMVenv ^{am} DraLBGH	293	B	5 × 10 ⁶	5 × 10 ⁷	Res
HA-LB	pSCV10	pCMVenv ^{am} DraLBGH	HT-1080	B	2 × 10 ⁷	2 × 10 ⁸	Res
HAII	pSCV10/5',3'tr.	pCMV-βenv ^{am}	HT-1080	C	1 × 10 ⁶	1 × 10 ⁷	Clinical ^d
DAII	pCI-GPM	pCMV-βenv ^{am}	D-17	C	2 × 10 ⁶	n.d.	Res
DAwob	pCI-WGPM	pCMV-βenv ^{am}	D-17	D	5 × 10 ⁵	n.d.	Res

Note. n.d., not determined.

^a Titer values represent VPCL pool and clone titers described in this publication or from data not shown.

^b PCLs previously described in Refs. 26, 28, and 36.

^c A total of five distinct DA-based retroviral vector products were used for clinical trials (56).

^d HAII-based human Factor VIII VPCL was used in a hemophilia A trial sponsored by Chiron Corp.

culture supernatant was harvested, filtered, and assayed for RCR by a marker rescue or PG4S + L- test. As per recent FDA guidance documents (www.FDA.gov), 300 ml of crude vector from FVIII(V) clinical production lots was assayed for RCR. The *M. dunni* amplification (large scale) and PG4S + L- detection method used for product release was validated for single unit RCR detection (unpublished data).

Hybridization Techniques

Relevant cells were harvested and either genomic DNA or total RNA isolated using Qiagen columns (Qiagen Inc., CA). For Southern blot analysis, 10 µg of genomic DNA was digested overnight using *Bgl*III for the hFVIII sequence containing DNAs and used in a standard Southern blot procedure. For Northern blot analysis, total RNA samples were electrophoresed in a 1% agarose/0.66 M formaldehyde gel. Gels were transferred onto nylon membrane (Zetaprobe; Bio-Rad Laboratories, CA) using 10× SSC. The blots were crosslinked using a Stratalinker (Stratagene, CA), prehybridized in Rapid-hyb buffer (Amersham Life Science Inc., IL) and hybridized overnight at 65°C in fresh Rapid-hyb buffer containing 100 ng of [³²P]-random-primed labeled (Rediprime kit; Amersham Life Science Inc., IL) 855-bp probe which hybridizes to the 3' region of the human FVIII cDNA. As an internal control for the Northern analysis, β-actin RNA levels were determined using [³²P] end-labeled oligonucleotides. The hybridized blots were then visualized using a PhosphorImager (Molecular Dynamics, CA) or following scanning of autoradiograms.

Large-Scale Human Factor VIII Vector Production Using a CellCube Perfusion System

Large-scale retroviral vector production utilized a static culture expansion train. Initially 225-cm² tissue culture flasks were inoculated and the cells cultured using DMEM formulated with 10% γ-irradiated defined fetal bovine serum for 3–4 days until subconfluent, and then progressively passaged while increasing the surface area to 4 × 10-layer cell factories (Nalge Nunc International, IL) prior to bioreactor inoculation. Large scale production utilized the CellCube (Corning Costar Inc., MA) perfusion fed cell culture system. Production media was a custom DMEM (Hyclone, UT) formulated with 10% γ-irradiated FBS (Hyclone, UT). Perfusion was controlled based on glucose consumption with up to a maximum media exchange of eight system volumes per day. Production volume ranged from 200 to 400 liters over a period of up to 13 days.

Representative bioreactor samples were taken for metabolic profile and TOE titer analysis.

RESULTS

Incorporation of Safety Modifications into Retroviral Components to Decrease/Eliminate Sequence Overlap and Reduce RCR Risk

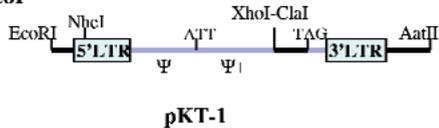
All three retroviral components (*gag/pol*, *env* and vector) of previously described split-genome packaging cell lines (Table 1) were modified to decrease the RCR potential through partial or complete elimination of sequence overlap between the components (Fig. 1). The safety-modified vector, pBA-5b (Fig. 1A), was extensively tested and compared to the unmodified pKT-1 vector for its ability to generate titer from transient and stable VPCL pools in various PCLs, as well as its potential to confer similar gene expression levels on HT-1080 target cells. No reduction of titer potential or expression level of the gene of interest after transfer to a target cell was observed (data not shown).

The two safety-modified *gag/pol* expression plasmids pSCV10/5',3'tr. and pCI-GPM have complete deletions of the 5' and 3' untranslated regions, including a truncation of the 3' end in the *pol* gene to prevent sequence overlap with the *env* plasmid (Fig. 1B). This truncation of integrase was reported previously to not impact integrase function (40). To further reduce homology, plasmid pCI-WGPM carried silent mutations in the first 420 nt of the 5' coding region of *gag/pol*. The use of degenerate code has been described before as a means to reduce overlap (41). However, modifications of this particular area have not been reported previously and were designed according to the most common codon usage of eukaryotic cells.

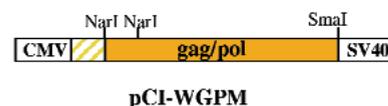
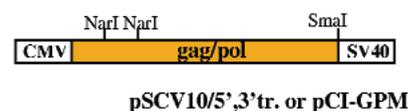
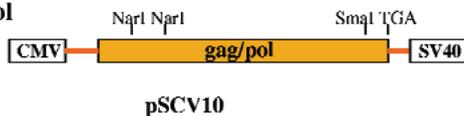
MLV-based retroviral constructs

Safety-modified MLV-based constructs

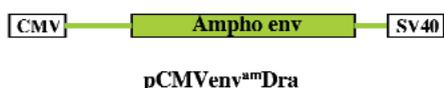
A. Vector



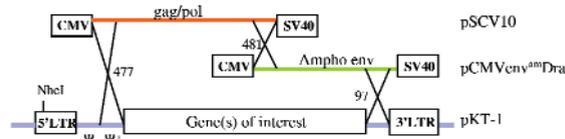
B. Gag/pol



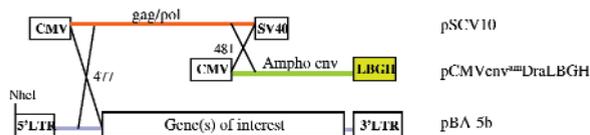
C. Envelope



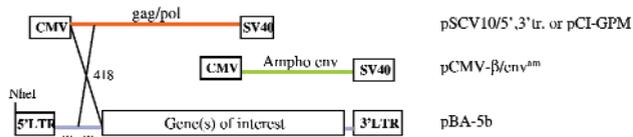
A. Three areas of overlap



B. Two areas of overlap



C. One area of overlap



D. No area of overlap

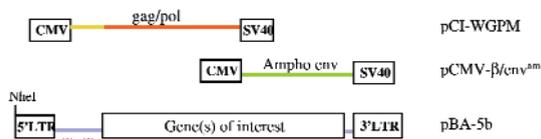


FIG. 1. Schematic presentation of the three safety-modified MLV-based retroviral components amphotropic *env* (4070A-derived), *gag/pol* (MoMLV-derived), and vector (MoMLV-derived). (A) In the pKT-1 retroviral vector, extraneous sequences (denoted in black) upstream of the 5' LTR, downstream of the 3' LTR and between the unique *ClaI* site and the TAG *env* stop codon were deleted. In addition, two stop codons were introduced in the extended packaging signal Ψ to prevent production of Gag/pol proteins. The first TAA stop codon replaces the ATG start codon of *gag/pol*, with the second TGA stop codon introduced 12 nt after the first TAA stop. All these changes were incorporated in the safety-modified vector pBA-5b. (B) In the original *gag/pol* construct pSCV10 all 5' and 3' untranslated sequences were removed and sequence coding for the last 28 amino acids of the integrase gene in Pol truncated (pSCV10/5',3'tr. or pCI-GPM). Additionally, degenerate code was incorporated in the first 420 nt of *gag/pol* to prevent overlap to the extended packaging signal of the vector (pCI-WGPM). (C) The original amphotropic envelope construct pCMVenv^{am}Dra was modified to minimize sequence overlap such that either the 3' untranslated sequences (pCMVenv^{am}DraLBGH) or the 5' and 3' untranslated sequences (pCMV-β/env^{am}) were deleted.

FIG. 2. Areas of sequence overlap and possible homologous recombination between the retroviral components *env*, *gag/pol* and vector. Presented are different configurations of all three retroviral components. Areas of overlap (denoted by X) indicate regions of sequence homology between the retroviral plasmids. The theoretical risk for homologous recombination decreases from configuration A to D due to elimination of the sequence homology between components. Numbers of nucleotides involved in the sequence overlap are indicated.

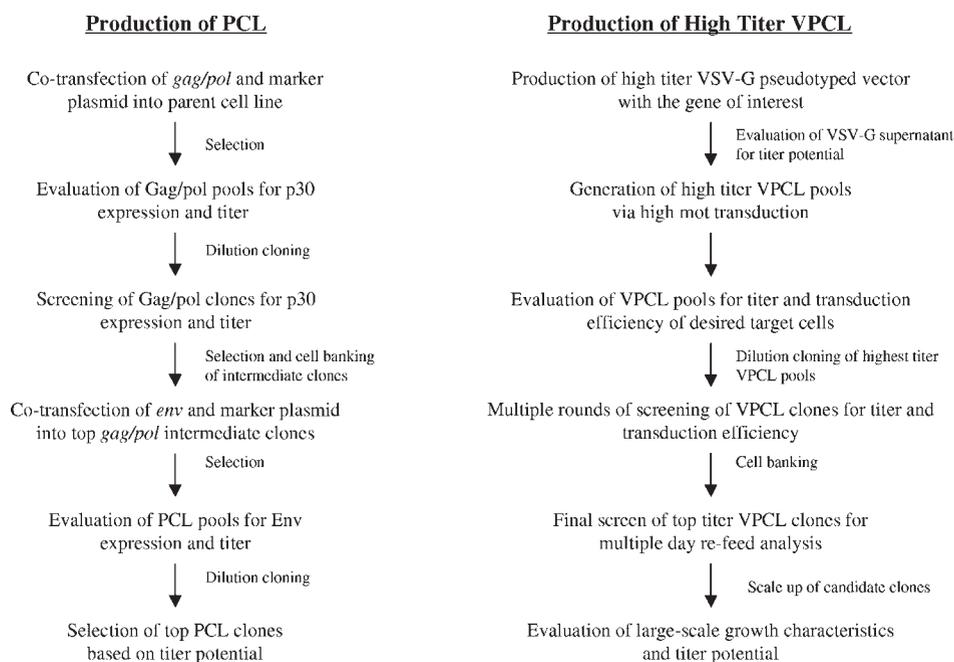


FIG. 3. Flow chart outlining the process for generating a packaging cell line (PCL) and high-titer vector-producing line (VPCL) starting with a parent cell line.

This approach resulted in a decrease in overall sequence homology to approximately 60%, with the longest area of perfect homology of 5 nt. To date, the smallest area of sequence overlap that reportedly leads to homologous recombination is 8 nt long (42). Analysis of relative expression levels of the original and safety-modified *gag/pol* constructs showed that both pSCV10/5',3'tr. and pCI-WGPM, resulted in somewhat reduced p30 levels while the processing of the *gag/pol* multiprotein appeared unchanged (data not shown).

The amphotropic envelope plasmid was modified such that the 5' and 3' untranslated regions were deleted in pCMV- β /*env*^{am}, whereas only the 3' untranslated region was deleted in pCMV*env*^{am}DraLBGH (Fig. 1C). Analysis of relative gp70 expression levels showed slightly increased levels from pCMV*env*^{am}DraLBGH compared to pCMV- β /*env*^{am} and the unmodified pCMV*env*^{am}Dra which we attributed to the bovine growth hormone poly(A) signal (data not shown) (43).

Using these components, we have constructed novel PCLs so that all configurations of sequence homology between the three retroviral components (Fig. 2) are available for testing and use.

Use of Safety-Modified Retroviral Components Results in Packaging Cell Lines with High-Titer Potential

To generate new RH PCLs, retroviral components were introduced sequentially into the parent line to reduce the risk of RCR generation. The approach for introducing and screening for the expression and function of the different retroviral components to generate a

RH PCL is outlined in Fig. 3 and under Materials and Methods. We originally identified the cell line D-17, HT-1080 and 293 as potentially useful parent lines on the basis of the literature (44) and pilot titer testing using rescue of a vector with replication competent virus (26). In general, detectable expression levels of *gag/pol* and *env* are a prerequisite for a PCL with high titer potential but there is no clear correlation between expression levels of *env* and titer. Along the same lines, high p30 expression levels did not guarantee a high titer but most PCLs with high titer potential did generate high p30 levels.

The titer potential of the RH PCLs HAI1, HA-LB and 2A-LB has been determined repeatedly by using high titer VSV-G pseudotyped vector to introduce various retroviral vector cassettes and determine the vector production capacity of each individual PCL. Although titers varied depending on the combination of PCL and the gene of interest, general trends were observed that indicate VPCL clone titers of 1×10^7 CFU/ml in the human-derived RH PCLs HAI1, HA-LB and 2A-LB (see Table 1) were achievable. Additional comparisons of pool titer data between the human RH PCLs and the DA cell line established that the RH human PCLs consistently produced 10- to 100-fold higher crude vector titers (unpublished data, Fig. 9). We have shown previously that in head to head testing the DA line gives a 4- to 9-fold higher titer than the GP + *env*Am12 line (45). It should be noted that differences in the titer assay can result in big differences in the observed endpoint titer (22). The titer values reported here were consistently determined using HT-1080 target cells which reproducibly yield 3-fold lower titers than observed with NIH 3T3 targets (unpub-

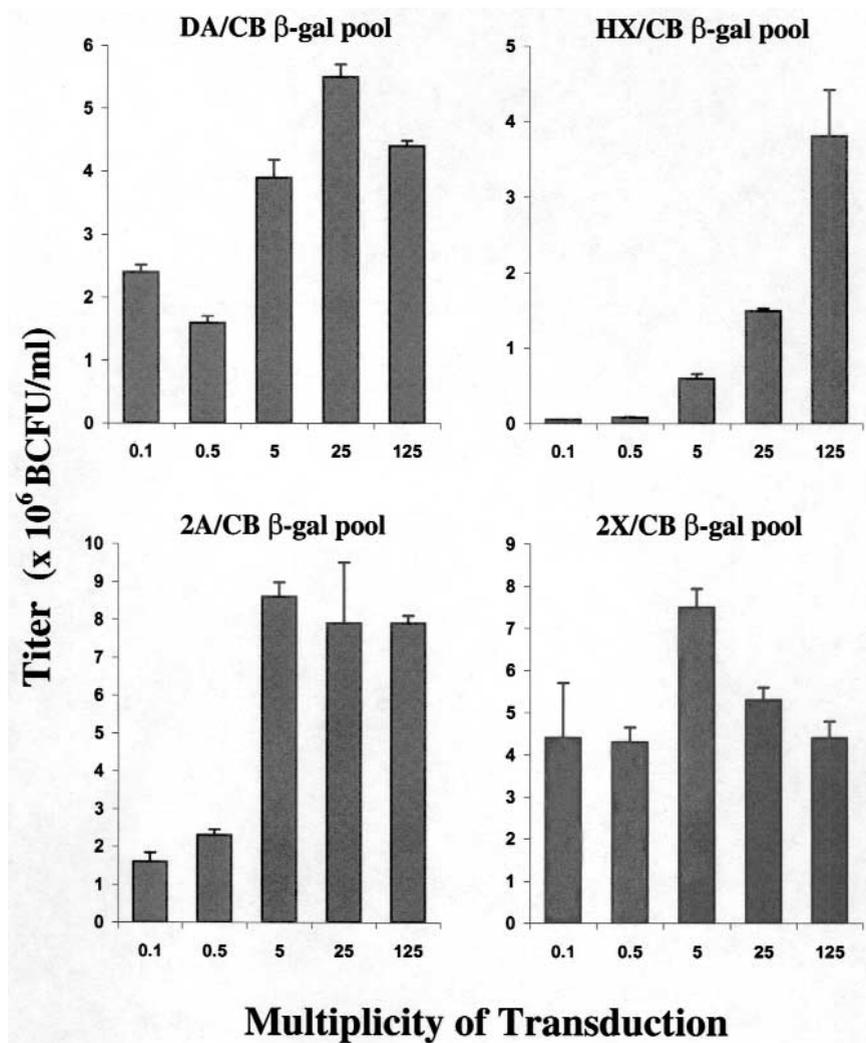


FIG. 4. Correlation between titer and multiplicity of transduction (m.o.t.) in vector producing pools. m.o.t.'s ranging from 0.1 to 125 were used to generate CB β -gal vector producer pools in the packaging lines DA, HX, 2A, and 2X. Titers are given in blue colony forming units (BCFU) and an average of three independent titer determinations were performed.

lished data), and therefore represent conservative VPCL titer values.

Generally, at least 100 clones were tested each time after introduction of a retroviral component at the Gag/pol intermediate, PCL and VPCL stages (Fig. 3). Once candidate PCL and VPCL clones for production of clinical material were identified, manufacturer's master and working cell banks were generated and tested for a variety of parameters including (i) growth characteristics, (ii) stability of retroviral components, (iii) titer over extended culture periods (up to 6 months), and (iv) the absence of RCR, mycoplasma, bacterial contamination and other adventitious agents including bovine virus, HBV, EBV, Parvovirus B-19, AAV, CMV, HIV I and II, and HTLV I and II. Testing for eco- and amphotropic RCR was also performed.

The Use of a High m.o.t. Scheme Generates Retroviral Vector Producers with Improved Titers: Proof of Principle

We examined the correlation between number of provectors per genome and titer using a wide range of m.o.t.'s (0.1 to 125) for VPCL production. Histograms summarizing the experimental results, which represent the trend observed in repeated experiments, are shown in Figs. 4 and 5. Titers from DA-, HX-, 2A-, and 2X-derived VPCL pools indicate that, in general, high m.o.t.'s correlate with high titer producer pools resulting in titer increases ranging from 3- to 100-fold (Fig. 4). Titers of producer pools generated with a m.o.t. of 0.1 and 0.5 within a given PCL were similar since most of the cells are expected to contain a single integrated provec-

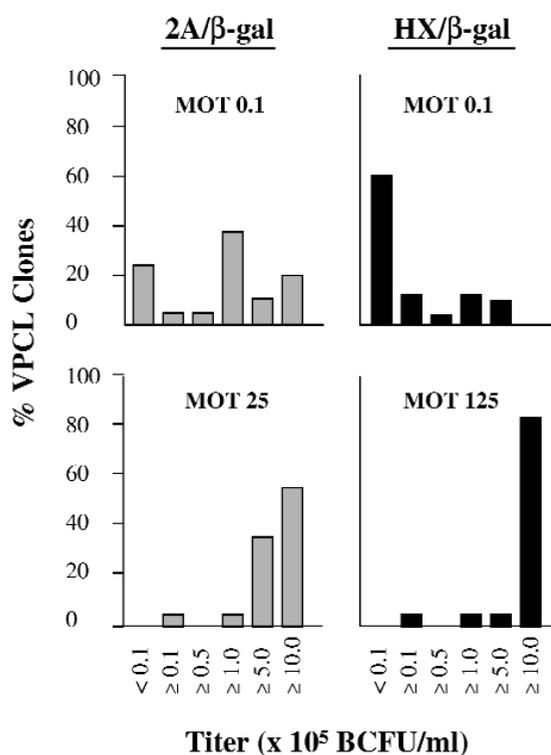


FIG. 5. Titer distribution of individual VPCL clones derived from either low or high m.o.t. 2A/ β -gal (gray bars) or HX/ β -gal (black bars) VPCL pools. A total of 21 clones for 2A/ β -gal m.o.t. 0.1, 20 clones for 2A/ β -gal m.o.t. 25, 22 clones for HX/ β -gal m.o.t. 0.1, and 20 clones for HX/ β -gal m.o.t. 125 were analyzed. Titer results are derived from three replicate titer determinations and shown in a histogram with titer values expressed as percentages of total number of clones yielding a titer in a specific range.

tor after selection. Therefore, the effect of high m.o.t. on titer is expected to be more evident at an m.o.t. > 1 , which is reflected in data shown in Fig. 4. Overall, the maximum titers of the producer pools generated with high m.o.t. were remarkably high and ranged from 3.8 to 8.6×10^6 CFU/ml. However, the correlation between pool titers and m.o.t. is only observed up to a certain m.o.t. The titers between the pools derived from the four PCLs at low m.o.t.'s vary greatly (40-fold) but the maximum pool titers only vary approximately 2-fold.

To analyze the titer distribution in the producer pools, approximately 20 random VPCL clones were isolated from the lowest and highest titer 2A/CB β -gal and HX/CB β -gal pools (Fig. 5). The wide range of up to two orders of magnitude between individual clone titers within the four pools implies that the high m.o.t. approach favors the production of higher titer producers; this does not guarantee the outcome for individual clones. Therefore it is still desirable to screen 100 clones to identify a truly high titer clone.

The data from Figs. 4 and 5 suggest that additional PCL-specific parameters contribute to titer as well. For example the introduction of the CB β -gal vector at low m.o.t. into the 2A and HX PCLs gave titers of $> 1 \times 10^6$

CFU/ml and around 1×10^4 CFU/ml, respectively. As all four PCLs (DA, HX, 2A, 2X) express comparable amounts of p30 (data not shown) which is often recognized as an indication of titer potential, the differences in titer may be due to components other than amount of vector and gag/pol, in this situation.

Combination of the High m.o.t. Method with RH PCLs Results in High-Titer Producers

The high m.o.t. method was applied to the new human RH PCLs to demonstrate its general applicability for generating high titer VPCLs coding for different therapeutic genes. Retroviral vectors containing either the B-domain deleted form of human Factor VIII (hFVIII) gene or the rat IL-4 (rIL-4) gene were introduced at various m.o.t.'s into the three RH human PCLs HAI, HA-LB and 2A-LB, and the individual pool data analyzed (Fig. 6).

The hFVIII retroviral vector was introduced into HAI and HA-LB at high m.o.t.'s ranging from 10 to 200 for the clinical producer line (Fig. 6), or at a broader range of m.o.t.'s between 0.1 and 125 for research purposes (pool titer data not shown, clone data in Table 2). These HAI and HA-LB derived hFVIII pools produce the highest reported titers of 2.2×10^6 and 2.2×10^7 CFU/ml of unprocessed supernatant from pools generating hFVIII vector, respectively (Figs. 6A and 6B). The 293-based 2A-LB PCL was examined for titer potential by introducing the hFVIII or rIL-4 retroviral vectors (hFVIII pool data not shown). Supernatants from rIL-4 producer pools were internally compared using the TOE assay, and overall pool titers expressed in terms of rIL-4 protein/ml (ng/ml, Fig. 6C). As seen previously, the highest overall transfer of rIL-4 expression was observed with pools generated with the high m.o.t.'s. Dilution cloning of the m.o.t. 25 and 50 pools resulted in five stable clones with titers between 8×10^6 and 5×10^7 CFU/ml.

The hFVIII and rIL-4 VPCL results (Fig. 6) are in direct correlation with that observed for the β -gal producer pools (Fig. 4), indicating that the high m.o.t. approach to generating high-titer producers is generally applicable. This approach also allows simple VPCL generation with vectors lacking a selectable marker such as the Factor VIII vector.

High-Titer VPCL Clones Have a High Provector Copy Number

Clones from different m.o.t. pools were isolated to demonstrate that overall increases in pool titers did indeed result in high titer VPCL clones. Individual HA-LB derived hFVIII clones from low (0.1–0.5), moderate (5), or high (25–125) m.o.t. pools were evaluated for provector copy number, retroviral RNA, and titer (Figs. 7 and 8, Table 2). In the absence of a selectable marker, only 25% of the low m.o.t. clones contained a provector and produced titer. Southern blot analysis of representative titer-producing clones from 0.5, 5, or 125 m.o.t. demonstrated a clear increase in the number of detectable provector

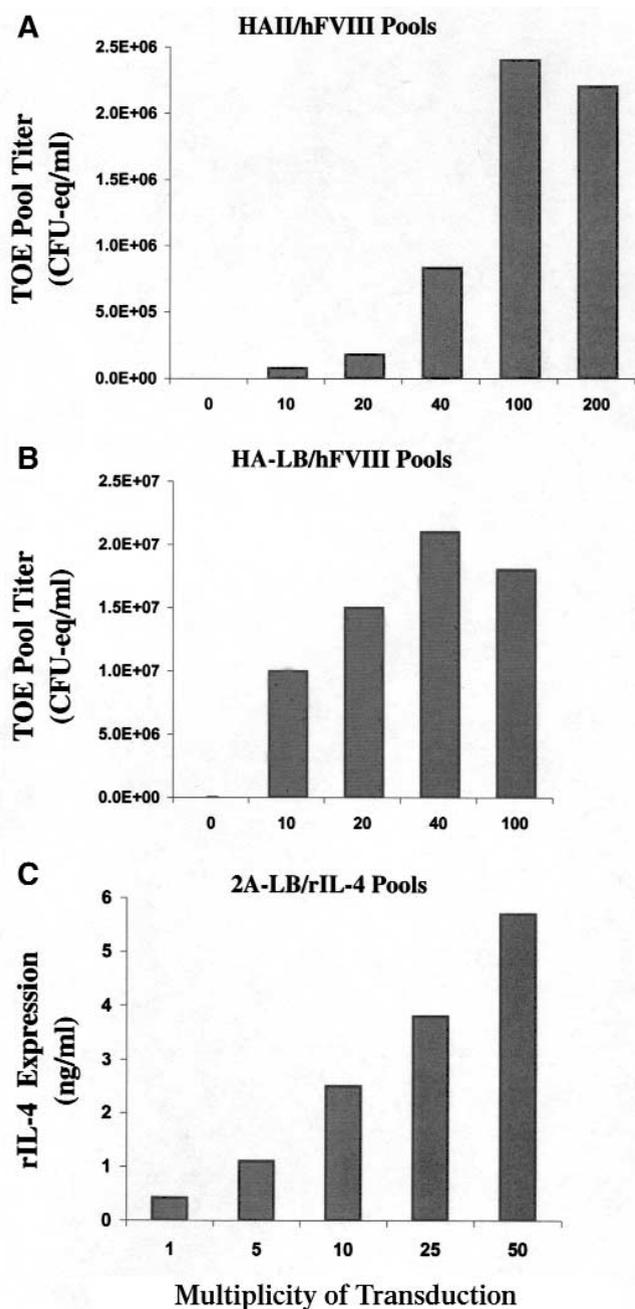


FIG. 6. Correlation between titer and high multiplicity of transduction (m.o.t.) in vector producing pools derived from safety-modified RH PCLs using therapeutic genes of interest such as hFVIII (A, B) and rIL-4 (C). Titer values for the hFVIII and rIL-4 producer pools represent the average of three and two independent titer determinations, respectively.

copies for clones expanded from the m.o.t. 125 pool compared to clones from the 0.5 and 5 m.o.t. pools (Fig. 7). Several exposure times of the Southern blots were used to evaluate the number of provector copies since varying intensities of the provector-representing bands made it difficult to determine provector copy numbers from a single exposure. FVIII TOE titers for these clones

with corresponding estimated provector copy number are shown in Table 2. The average FVIII TOE titer is 34-fold higher for the m.o.t. 125 clones (2.8×10^7 CFU/ml) compared to the 0.5 m.o.t. clones (8.2×10^5 CFU/ml). These titers corresponded to an average provector copy number of 7 for the m.o.t. 125 clones and 1–2 copies for the m.o.t. 0.5 clones. The moderate m.o.t. 5 clones demonstrated intermediate titers and an average of two to three provector copies per genome.

Southern blot analyses of the 2A/ β -gal and HX/ β -gal VPCL clones shown in Fig. 5 generated similar results (data not shown) as that observed for the HA-LB derived human FVIII VPCL clones. Taken together, these findings demonstrate a correlation between multiple provector copies and high titer in VPCL clones.

High-Titer, High-Provector-Copy VPCL Clones Show Increased Expression Levels of Retroviral Genomic RNA

To investigate whether a high provector copy number results in overall higher levels of retroviral genomic RNA in high titer producing VPCLs, we examined the levels of retroviral RNA from HA-LB hFVIII producer clones in a Northern blot (Fig. 8). The data suggest that a correlation exists between RNA levels and m.o.t. Although a quantitative RNA assay (46) was not attempted, these results are consistent with the hypothesis that introduction of multiple provector copies into a PCL is associated with increases in retroviral genomic RNA, resulting in VPCL pools which give rise to high titer individual clones.

Vector Producer Lines Derived from RH PCLs Do Not Generate RCR

Extensive testing of small scale VPCL pools and/or clones derived from the current panel of PCLs has not revealed any RCR. Large-scale RCR testing according to the FDA Guidance for Human Somatic Cell Therapy and Gene Therapy (March 1998) was carried out on post-production cells, which typically had produced 300-liter lots of viral supernatant, and on the culture fluids itself. A total of either 1×10^8 VPCL cells or 5% (or, more recently 300 ml) of the culture fluids was tested for RCR in the co-cultivation or amplification marker rescue assay, respectively. Large scale RCR testing included VPCL clones derived from the DA-, HAI1- and HA-LB cell lines. Using the DA cell line as a packaging cell, at least 5 distinct VPCLs producing different vectors have been made, banked and used to generate large scale vector lots (>200 liters). A total of 77 lots at this scale have been tested, of which 9 have been positive for RCR (12%). The majority of the production and all the RCR positives came from one specific producer line. Large scale testing was performed in multiple flasks or roller bottles. Typically only one or a few flasks out of 20 scored positive. This suggests that the RCR detected represented only a single event.

TABLE 2
Summary Table Showing the Correlation between Titer and Integrated Provectors per Genome of HA-LB-Derived Human Factor VIII VPCL Clones Produced at Various m.o.t.'s

m.o.t. 0.5			m.o.t. 5			m.o.t. 125		
Clone No.	Titer (CFU-eq/ml)	Provector copy No.	Clone No.	Titer (CFU-eq/ml)	Provector copy No.	Clone No.	Titer (CFU-eq/ml)	Provector copy No.
18	1.5×10^6	1	2	5.3×10^6	3-4	7	1.4×10^7	6
20	1.2×10^6	1-2	3	1.1×10^6	n.d.	8	3.6×10^7	6-7
26	6.3×10^5	1	4	7.0×10^5	2	9	4.1×10^7	7-9
46	2.4×10^5	1-2	10	2.7×10^6	1-2	13	3.5×10^7	6-9
49	5.2×10^5	2	15	n. d.	2-3	19	2.5×10^7	7-9
			17	1.6×10^6	3	22	2.3×10^7	8-9
			22	n. d.	2	23	2.4×10^7	6
Average	8.2×10^5	1-2		2.3×10^6	2-3		2.8×10^7	7

Note. n.d., not determined.

The RCR did not spread throughout the culture as the VPCL is blocked from further amphotropic viral infection. Sequence analysis of the resulting RCR revealed that in all cases investigated the RCR emerged after homologous recombination between the three introduced retroviral components. Large-scale testing of vector material from the safety-modified RH PCLs HAI1 and HA-LB did not result in any detectable RCR.

Large-Scale Production of Safe Retroviral Vector for a Clinical Trial: Generation and Selection of Human Factor VIII Producer Lines

The high m.o.t. approach was used in combination with the human RH PCLs, and large scale cell culture

considerations were incorporated into the screening and selection procedure to identify human FVIII VPCLs that would meet the projected manufacturing demands for the hemophilia A clinical trials.

In brief, concentrated high titer VSV-G pseudotyped hFVIII vector was generated and human 2A-LB, HAI1 and HA-LB PCLs transduced with m.o.t.'s ranging from 10 to 200. Respective producer pools were evaluated for TOE and PCR titer, and individual clones established from the highest titer pools. Approximately 100 producer clones from each PCL were subjected to three rounds of screening with the selection criteria being a functional TOE titer $> 2 \times 10^7$ CFU/ml. All of the selected high titer VPCL clones originated from the high titer 40 and 100 m.o.t. pools and produced titers between 5×10^7 and 2×10^8 CFU/ml in small scale (six-well plates). Candidate clones

HA-LB hFVIII VPCL Clones

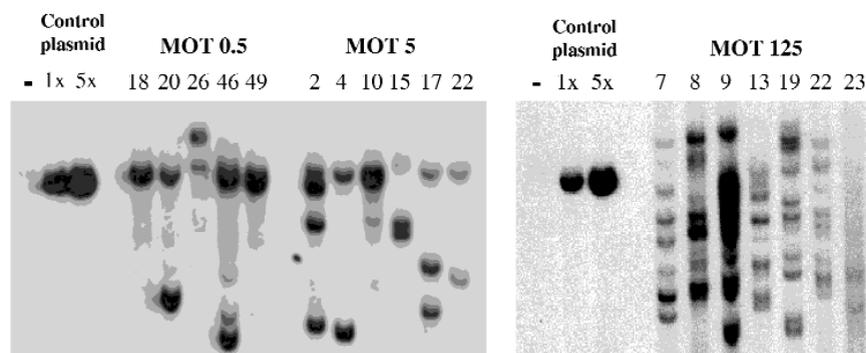


FIG. 7. Southern blot demonstrating the correlation between number of integrated provectors and m.o.t. used to generate the VPCL clones. Shown are VPCL clones derived from producer pools generated at m.o.t. 0.5, 5, or 125. Provectors were visualized after hybridization of the Southern blot with a radioactively labeled fragment of the human Factor VIII coding region. The plasmid control consisted of 0 (-), 1, and 5 copy equivalents of the hFVIII retroviral vector plasmid spiked into digested HA-LB genomic DNA.

HA-LB hFVIII VPCL Clones

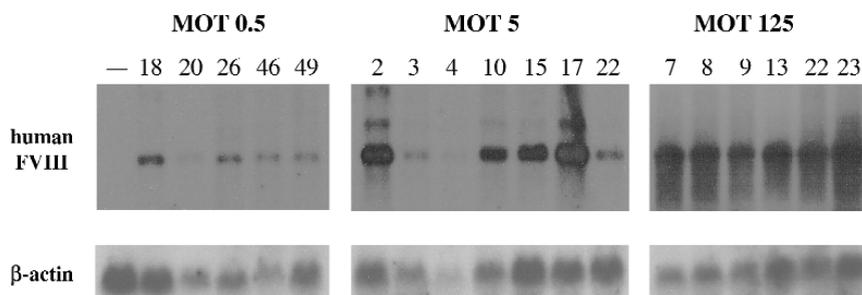


FIG. 8. Northern blot demonstrating the correlation between retroviral genomic RNA levels and m.o.t. used to generate the HA-LB hFVIII VPCL clones. Shown are VPCL clones derived from producer pools generated at m.o.t.'s of 0.5, 5, or 125 (some clones may differ from those represented in Fig. 7). The dash (–) represents control total RNA isolated from the HA-LB PCL. The retroviral genomic RNA levels are visualized after hybridization of the Northern blot with a radioactively labeled fragment of the human Factor VIII coding region. Control β -actin RNA levels for each corresponding VPCL clone are shown below.

were plated at equivalent cell numbers, grown to confluency, and supernatants collected daily for seven days. Both TOE and PCR titer values were determined in this seven-day re-feed experiment, and the two highest titer clones from each hFVIII producer line expanded to establish master and working cell banks. Cell banks were used for large scale characterization, vector processing and stability studies (data not shown).

The highest titer hFVIII producer clones derived from the HA-LB and HAIL PCLs were used to generate 200 liters of unprocessed vector. Their performance in a large scale culture experiment was compared to a DA-derived producer line, whose average titer was 1×10^6 CFU-eq/ml (Fig. 9). The entire process was monitored for

VPCL growth characteristics and extended vector production over 13 days. As shown for both the HA-LB and HAIL hFVIII producer clones, there was an increase in vector titer during the log phase of culture growth out to day 5. After day 5, the HT-1080-based hFVIII clones generated titers in the range of 2×10^7 to 2×10^8 CFU/ml, which translates into a total yield of $1-5 \times 10^{12}$ CFU per day (data not shown). Incorporating the system's increasing perfusion rate into the daily yield calculations, the HA-LB VPCL consistently produced 5×10^{12} vector particles per day out to 13 days. For chronological reasons, the HAIL/hFVIII clone was used for clinical vector production. Fifteen clinical lots of FVIII vector have been assayed for RCR by co-cultivation and supernatant analy-

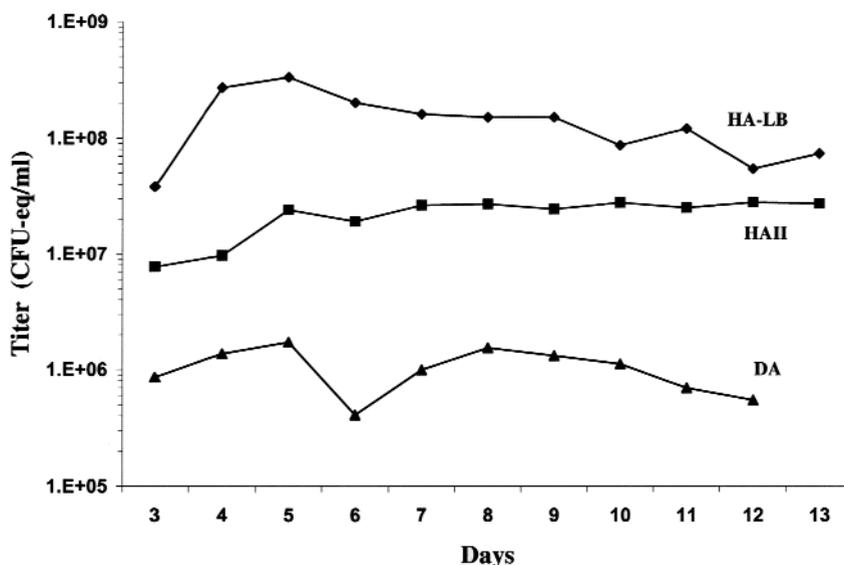


FIG. 9. Titer output of human Factor VIII producing VPCL clones is superior to the clone derived from the canine PCL DA. Relative hFVIII vector titer output for the safety-modified HAIL/pCF8 (■) and HA-LB/pCF8 (◆) VPCLs is compared to the DA/B-canine-derived (▲) VPCL. The titer of the three VPCL clones was followed over 12 days in culture.

sis in the large scale assay; all lots scored negative for RCR. The HA-LB/hFVIII clone has also been scaled up in 200-liter preclinical lots and no RCR has been detected. These findings are consistent with the idea that the RH modifications have reduced the potential for RCR generation in these producer and packaging cell lines.

DISCUSSION

Sufficiently high doses of purified, RCR-free retroviral vectors are critical to the success of clinical trials where the vectors are directly administered. In this study, we describe the development of novel retroviral packaging and producer cell lines that meet these requirements for safety and high titer production.

A major safety concern with retroviral vectors is generation of RCR. A survey of the accumulated data of reported RCR events clearly identifies the genetic background of the parent cell line, the design of the introduced retroviral components, and the strategy used for PCL, VPCL, and recombinant vector production as key factors that determine the potential for generation of RCR. Although other mechanisms are possible, RCR is believed to result primarily from homologous recombination between introduced and/or introduced and endogenous retroviral sequences (13, 16–18, 23) with sequence homology as short as 8 consecutive nucleotides (42). Diminished potential for generation of RCR can be achieved by reducing the sequence overlap between the three retroviral components *env*, *gag/pol* and vector, and several approaches have been described previously (11, 12, 47). We describe canine and human safety-modified PCLs with one or both of the two most common remaining sequence overlaps in split-genome vector systems eliminated. It is important to note, however, that the elimination of sequence homology may be accompanied by a reduction in recombinant retroviral vector titer in cases where functional areas are modified (48). To maximize titer, we introduced multiple provectors per genome. However, this strategy is only recommended when utilizing RH retroviral constructs engineered to minimize overlap.

Truncation of the integrase gene eliminated the overlap between *gag/pol* and *env* in the canine DAII and human HAII RH packaging lines. Additionally, degenerate code was used to eliminate the last overlap between *gag/pol* and vector in the DAwob packaging cell line. To our knowledge, this is the first retroviral MLV-based PCL/VPCL system with no sequence identity stretches of more than five nucleotides between its three retroviral components. The HAII and DAII PCLs produce high titer, however, producer pools derived from the DAwob PCL did not satisfy our criterion of $\geq 1 \times 10^7$ CFU/ml unconcentrated supernatant for producer clones, and therefore the generation of VPCL clones derived from DAwob was not further pursued. It remains to be seen whether this approach may prove useful in future PCL development.

The apparent two- to fivefold titer loss in the HAII and DAII PCLs may be due to slightly impaired function of the truncated integrase, although previous reports of this specific truncation found no compromise of integrase activity (40). Alternatively, the truncation may affect the processing of the Gag/pol multiprotein and impact titer.

During our effort to minimize the retroviral components, retroviral sequences downstream of the 3' LTR were deleted as well. It was important to eliminate these extra murine MLV viral sequences since we have observed that even an incomplete core packaging signal could confer an inefficient packaging function (data not shown), and unwanted copackaging of *gag/pol* components could possibly contribute to the generation of RCR events, as postulated for an HIV-1-based vector system (49).

To further decrease the RCR potential, the 293 and HT-1080-based human parent cell lines were selected as parent cell lines because they lack endogenous murine retroviruses (U.S. Patent No. 5,591,624) (14, 19, 50, 51) and viral supernatant or producer cells derived from the new human RH PCLs have never had a validated positive test result for RCR in the small or large scale assays.

VPCL pool titers as high as 2×10^7 CFU/ml were achieved with the human RH PCLs using the "high m.o.t." approach to introduce multiple provector copies. The optimal m.o.t. was empirical and varied depending on the particular combination of vector and packaging cell line. However, as illustrated by seven different PCL/retroviral vector combinations, a general trend shows that an m.o.t. between 10 and 150 resulted in the highest titer human PCLs. Southern analysis of these high titer producers revealed generally no more than 15 provectors per genome of any long-term viable VPCL. Similar provector numbers were reported in previous investigations (21, 52–55).

This study demonstrates that the correlation between the m.o.t. and resulting VPCL titer is not absolute. In fact, VPCL pools generated with extremely high m.o.t.'s of >250 often show impaired viability in addition to possible decreases in titer (data not shown). This is most likely due to toxic effects of the high titer VSV-G pseudotyped vector preparation and/or limited by how many integrated provector copies can be tolerated in a genome. We have also observed that cellular toxicity, cell viability and propagation can be negatively influenced by the nature of the introduced gene of interest.

Furthermore, the data indicates that there may be additional factors other than level of Gag/pol expression and vector message that contribute to viral particle output of a producer line. Several steps during the complex process of vector production may contribute to the final titer value such as (i) the level and ratios of Env, Gag/pol and retroviral message, (ii) the ability to correctly process the Gag/pol protein, (iii) the efficiency of the insertion of the Env protein into the host membrane and subsequent association with the condensed nucleoprotein complex,

(iv) the availability of other components such as the tRNA primer, (v) the budding efficiency of viral particles from the host membrane, (vi) the stability of viral particles in media, and (vii) the ability to infect target cells.

In selecting the ideal candidate for clinical grade vector production, the final clone should maintain high titer during demanding dynamic cell growth and maintenance conditions (high cell densities, continual glucose consumption, increasing LDH levels, etc.) and produce vector particles which exhibit extended stability throughout extended periods of cell culture confluence. We have found that harvesting a series of multiple supernatant samples over several days after the culture has reached confluency is critical to identifying the highest titer-producing clones. Testing for titer potential during the screening of the Gag/pol intermediates and final PCLs is a critical step that should not be overlooked when selecting a candidate PCL. Once clones are identified, performance needs to be verified under large-scale culturing conditions (100–400 liters), where issues related to culture chemistries, unprocessed vector stability, and the ability of the clone to sustain high titers over extended periods are evaluated.

As this study demonstrates, human PCLs can tolerate the incorporation of multiple provector copies following a high m.o.t. challenge. Increased provector copy number appears to be the most critical, but not sole, parameter associated with superior titers, previously only achievable by amplification techniques (19–23) prone to generating RCR (22, 23). The best-characterized VPCLs are the HAI- and HA-LB/hFVIII clones, which are stable in terms of integrity of retroviral components, expression level and titer over at least 6 months duration. These lines have been adapted to large-scale culture perfusion systems, producing consistently high vector particle yields for at least 15 days. Additional studies demonstrated that these human VPCLs produce complement-resistant vector, which can be used for direct iv injection (27) (data not shown).

In summary, critical issues which once hindered the clinical use and commercial feasibility of retroviral vectors have been resolved.

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