Analysis of Partial Recombinants in Lentiviral Vector Preparations

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Abstract

The presence of replication-competent lentivirus (RCL) in lentiviral vector preparations is a major safety concern for clinical applications of such vectors. RCL are believed to emerge from rare recombinant vector genomes that are referred to as partial recombinants or Psi-Gag recombinants. To quantitatively determine the fraction of partial recombinants in lentiviral vector preparations and to analyze them at the DNA sequence level, we established a drug selection assay involving a lentiviral packaging construct containing a drug-resistance gene encoding blasticidin (BSD) resistance. Upon transduction of target cells, the *BSD* resistance gene confers BSD resistance to the transduced cells. The results obtained indicate that there were up to 156 BSD-resistant colonies in a total of 10^6 transducing vector particles. The predicted recombination events were verified by polymerase chain reaction using genomic DNA obtained from BSD-resistant cell clones and by DNA sequence analysis. In an attempt to reduce the emergence of partial recombinants, sequence overlaps between the packaging and the vector constructs were reduced by substituting the Rev response element (RRE) present in the vector construct using a heterologous RRE element derived from simian immunodeficiency virus (SIV-mac₂₃₉). The results obtained showed that a reduction of sequence overlaps resulted in an up to sevenfold reduction of the frequency of BSD-resistant colonies, indicating that the capacity to form partial recombinants was diminished.

Introduction

LENTIVIRAL VECTOR-MEDIATED GENE transfer strategies involving vectors based on human immunodeficiency virus type 1 (HIV-1) or on equine infectious anemia virus are being pursued in a number of clinical protocols (O'Reilly *et al.*, 2012).

Lentiviral vectors have traditionally been produced by transient cotransfection of human embryonic kidney (HEK) 293T cells using a lentiviral vector plasmid carrying a transgene of interest, plasmids encoding packaging (helper) functions, and plasmids encoding envelope (Env) glycoproteins, respectively (Kutner *et al.*, 2009).

For many of the advanced lentiviral vectors, there is considerable overlap between sequences that are shared by the packaging construct and the vector genome, including the Gag-encoding region and the Rev response element (RRE) (Sastry *et al.*, 2003; Cornetta *et al.*, 2011). Such regions of overlap have been shown to facilitate the formation of hybrid vector genomes that are referred to as partial recombinants or Psi-Gag recombinants (Sastry *et al.*, 2003).

Partial recombinants may serve as precursors for the generation of replication-competent lentivirus (RCL), particularly in cells expressing a heterologous Env glycoprotein capable of forming pseudotyped lentiviral vector particles, such as human cells bearing endogenous retroviruses (Mi *et al.*, 2000; Lee and Bieniasz, 2007). Such an outcome would clearly not be desirable since continued RCL replication is expected to result in multiple vector insertion events, thereby enhancing the potential for insertional mutagenesis (Cornetta *et al.*, 2011). Thus, the presence of RCL in clinical-grade vector products is a major safety concern, and current FDA guidance (FDA, 2006) requires that vector products be tested to ensure the absence of RCL.

Partial recombinants are typically detected by polymerase chain reaction (PCR) involving genomic DNA from transduced cells (Sastry *et al.*, 2003; Cornetta *et al.*, 2011), or using cell-based colony assays involving drug selection (Wu *et al.*, 2000; Fuller and Anson, 2001; Koldej *et al.*, 2005). A limitation of PCR-based methods is that only a small fraction of the total genomic DNA can be sampled. Thus, rare vector recombinants can be missed. Also, the analysis of

recombination events in individual cell clones using PCR-based methods can be challenging. In the present study, we employed a drug selection assay based on blasticidin (BSD) selection to detect rare Psi-Gag recombinants in lentivirus vector preparations. We constructed safety-enhanced lentiviral vectors by substituting the RRE present in the vector construct using a heterologous RRE elements derived from simian immunodeficiency virus (SIVmac₂₃₉), and we determined the influence of such sequence modifications on the emergence of partial recombinants using the BSD selection-based recombination assay.

Materials and Methods

Plasmid constructs

The pNL-EGFP(MSCV) and pNL(CMV)EGFP(CMV)/WPΔU3 lentiviral vector plasmids and the pCD/NL-BH*ΔΔΔ packaging plasmid were described before (Zhang *et al.*, 2004; Ou *et al.*, 2012). These plasmids contain a 168 bp SV40 origin of replication (ORI) sequence (Reiser *et al.*, 1996; Mochizuki *et al.*, 1998) and a 150 bp cPPT sequence (Zhang *et al.*, 2004). To construct the pCD/NL-BH*ΔΔΔ/BSD packaging plasmid, the BSD coding region was PCR-amplified using the pcDNA6/TR plasmid (Invitrogen, Carlsbad, CA) as a template, a forward primer (BSD-XbaI: ggccctctagaccacatggccaagccttctctcaag), and a reverse primer (BSD-EcoRI: gtggtggaattcttagccctccacataaccagagg). The PCR product was digested with *XbaI* and *EcoRI* and cloned into *XbaI*- and *EcoRI*-digested pNL-rHSAS plasmid DNA (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH], from Drs. Beth Jamieson and Jerome Zack) (Jamieson and Zack, 1998), to generate pNL-r-BSD. The *AflIII*-*EcoRI* fragment of the pNL-r-BSD plasmid was then subcloned in the pCN/NL-BH*ΔΔΔ plasmid to generate pCD/NL-BH*ΔΔΔ/BSD.

To construct the pNL-BSD(MSCV) lentiviral vector plasmid, the BSD coding region was amplified using the pcDNA6/TR plasmid as a template, a forward primer (BSD-Agels: tagcgctaccggctccaccatggccaagccttctctcaagaag), and a reverse primer (BSD-KpnI: tctaaaggtacctgcttagccctccacacataaccagaggc) and subcloned using *AgeI*/*KpnI*-digested pNL-EGFP(MSCV) plasmid DNA.

To construct the pNL-SRRE-EGFP(MSCV) lentiviral vector plasmid containing a SIVmac₂₃₉ virus-derived RRE sequence, a *NotI* site was introduced upstream of the HIV-1 RRE sequence present in pNL-EGFP(MSCV) and an *AgeI* restriction site downstream of the RRE, resulting in plasmid pNL-RTEEmCTE-EGFP(MSCV)/*NotI*. A 1045 bp fragment containing the SIVmac₂₃₉ RRE sequence (Srinivasakumar, 2008) was PCR-amplified from plasmid p239SpE3' (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Ronald Desrosiers) (Regier and Desrosiers, 1990) using a forward primer (SIV*NotI*s: gatcttgcggccgcgcatcgattggaattgggagatta) and a reverse primer (SRRE1045a2: cgtatcgaccggtctagtaactcagacggcctggaccgcctcatg). The PCR product was digested with *NotI* and *AgeI*, and subcloned into *NotI*-*AgeI*-digested pNL-RTEEmCTE-EGFP(MSCV)/*NotI* plasmid DNA, to generate pNL-SRRE-EGFP(MSCV). An overlap PCR was used to construct the pNL-SRRE/ΔORI-EGFP(MSCV) vec-

tor plasmid. The pCEF-VSV-G plasmid was described before (Ou *et al.*, 2012).

Cell lines

HEK 293T cells (ATCC CRL-11268) and HEK 293 cells (ATCC CRL-1573) were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 2.5 mM L-glutamine, 100 u/ml penicillin, and 100 μ/ml streptomycin (all from Invitrogen).

Lentiviral vector production

Lentiviral vector was produced using 6-well plates or 15 cm dishes as described before (Kuroda *et al.*, 2009, 2011). For transfection using 6-well plates, HEK 293T cells were plated at a density of 5×10^5 cells/well 1 day before transfection. The transfection cocktail consisted of 0.51 μg of the vector plasmid, 0.34 μg of the packaging plasmid (pCD/NL-BH*ΔΔΔ or pCD/NL-BH*ΔΔΔ/BSD), and 0.17 μg of the pCEF-VSV-G plasmid (Ou *et al.*, 2012). For 15 cm dishes, the number of cells and the quantities of the various plasmids were proportionally increased according to the surface area. The cell culture media were changed the day after transfection, and the vector-containing supernatants were harvested on the third day (about 60 hr posttransfection), filtered through a 0.45 μm filter (Millipore, Billerica, MA), and stored at -80°C for subsequent experiments. Vector stocks were titrated on HEK 293 cells by FACS, and vector titers are expressed as EGFP transduction units per milliliter (Kuroda *et al.*, 2009).

BSD selection assay

HEK 293 cells were plated in 6-well plates at a density of 1×10^5 cells/well and transduced 1 day later with 10^6 transduction units of the vector stocks to be tested. One day later, cells were fed with a medium containing 10 μg/ml of BSD. The medium was replaced every 4 days until the appearance of BSD-resistant cell clones was apparent, 2–3 weeks later. Upon selection, BSD-resistant cell clones were counted after staining with crystal violet.

Vector rescue from BSD-resistant HEK 293 cell clones

Cells from pooled BSD-resistant HEK 293 cell clones were transfected using the pCEF-VSV-G plasmid, the pCEF-VSV-G plus pCMV-Rev plasmids, or the pCEF-VSV-G plus pCD/NL-BH*ΔΔΔ plasmids. Three days later, the vector-containing cell supernatants were collected and 200 μl aliquots were used to transduce fresh HEK 293 cells. BSD selection was carried out as described above.

PCR and sequence analysis of PCR products

The primers used to amplify the Psi-Gag region consisted of a forward primer complementary to a vector sequence upstream of the packaging signal (PNL-F1: gcagtggcgcccg aacagggacttg) and a reverse primer (GrecR2: tgtctatgtccag aatgct) (Sastry *et al.*, 2003) complementary to a gag sequence present in the packaging construct but absent in the vector construct. To amplify the Tat-EGFP region, a forward primer (Tat_s: agcggttactcgacagaggagagcaag) located in the

Tat-encoding sequence of the packaging construct and a reverse primer (GFP_a: gccctcgccctcgccggacacgctgaactgtg) located in the *EGFP* gene present in the vector construct were used. The *BSD* was amplified by PCR using the primers *BSD*-XbaI and *BSD*-EcoRIa described above.

Genomic DNA was extracted from *BSD*-resistant cell clones using the QiaAmp DNA Blood mini kit (Qiagen, Germantown, MD) as described by the manufacturer, and 70–200 ng of genomic DNA was used for each PCR. The PCR assay was performed using the GoTaq Green Master Mix (Promega, Madison, WI) as described by the manufacturer. Samples were incubated at 95°C for 5 min. This step was followed by 35 cycles consisting of 30 sec at 95°C, 30 sec at 55°C, and 4 min at 70°C, followed by a 10 min incubation at 72°C. The PCR products were analyzed by agarose gel electrophoresis.

A forward primer (Rev-s: gacctcctcaaggcagtcagactcatca) that binds downstream of the Tat_s primer binding site was used together with a reverse primer (GFP_a) to generate PCR products for sequencing. For DNA sequencing, the PCR products were separated on an agarose gel and extracted using the Qiagen gel extraction kit (Qiagen). For DNA sequence analysis, 150 ng of the purified PCR products was used. DNA sequences were analyzed using the Vector NTI Advance 11.5.1 software (Invitrogen, Carlsbad, CA).

Determination of vector copy numbers

Vector copy numbers in transduced HEK 293 cells were determined by quantitative PCR involving a gag sequence-specific primer/probe set as described before (Kutner *et al.*, 2009).

Results

Detection of partial recombinants in lentiviral vector preparations

To detect partial recombinants in lentiviral vector preparations, we employed a recombination assay involving a modified lentiviral packaging construct, pCD/NL-BH* $\Delta\Delta\Delta$ /*BSD*, which is based on a second-generation packaging construct (pCD/NL-BH* $\Delta\Delta\Delta$) (Zhang *et al.*, 2004). This modified packaging construct contains a *BSD* resistance gene inserted within the reading frame of the Vpr coding region (Jamieson and Zack, 1998) (Fig. 1A). The assay also includes an *EGFP*-encoding lentiviral vector (Fig. 1B). *BSD*-encoding partial recombinants (Fig. 1C) emerging as a result of recombination events involving sequences shared by the packaging and vector constructs result in the formation of *BSD*-resistant colonies upon transduction of HEK 293 target cells.

Significant regions of sequence overlap between the vector and packaging components include a 463 bp gag region, a 67 bp sequence upstream of the gag ATG involving a part of the packaging signal, and the 824 bp RRE sequence (Fig. 1A and B). Another overlap involves a 168 bp region involving an SV40 ORI sequence (Fig. 1A and B). A 150 bp cPPT (central polypurine tract)-derived sequence is present in the vector downstream of the ORI sequence (Fig. 1B). The 150 bp cPPT sequence overlaps with the pol sequence present in the packaging construct. All these sequence overlaps are 100% identical.

To verify the presence of vector recombinants in *BSD*-resistant cell clones, genomic DNA derived from such clones was subjected to PCR. To do this, two separate sets

FIG. 1. Assay to detect partial recombinants. (A) Packaging construct. (B) Vector construct. (C) Structure of a putative partial recombinant. The gag and RRE sequences present in the packaging and vector constructs are highlighted in black. A short, 168 bp SV40 origin (ORI) sequence upstream of the RRE is present both in the packaging construct and in the vector construct (Reiser *et al.*, 1996; Mochizuki *et al.*, 1998). The horizontal arrows shown in (C) refer to the PCR primers used. To analyze partial recombinants arising from gag sequence overlaps, the PNL_F1 and GreCR2 primers were used. To analyze recombinants arising from ORI and RRE sequence overlaps, the Tat_s/Rev_s and GFP_a primers were used. To amplify the *BSD* coding region, the *BSD*-XbaI and *BSD*-EcoRI primers were used (broken arrows). $\Delta\Psi$, deleted packaging signal; Ψ , packaging signal; *BSD*, blasticidin resistance gene; CMV, human cytomegalovirus immediate early promoter; cPPT, 150 bp central polypurine tract sequence downstream of the ORI sequence; *EGFP*, *EGFP* transgene sequence; LTR, long terminal repeat; pol, pol coding region; polyA, polyA signal; RRE, Rev response element; tat and rev, tat and rev exon sequences.

of PCR primer pairs corresponding to the Psi-Gag and Tat-EGFP regions, respectively, were designed (Fig. 1C). The Psi-Gag forward primer (PNL_F1) recognizes sequences corresponding to the tRNA binding site present in the lentiviral vector backbone, while the Psi-Gag reverse primer (GrecR2) (Sastry *et al.*, 2003) matches gag sequences that are present in the packaging construct but absent in the vector construct. To amplify the Tat-EGFP region, the Tat_s forward primer, corresponding to a sequence present in the first Tat exon of the packaging construct, and a reverse primer (GFP_a) corresponding to the EGFP coding region present in the lentiviral vector genome were used (Fig. 1C).

The performance of the assay was tested as follows. Lentiviral vectors were generated by transfecting HEK 293T cells using an EGFP-encoding lentiviral vector plasmid, pNL-EGFP(MSCV), containing a hybrid HIV-1 3' LTR bearing an MSCV-derived enhancer sequence (Zhang *et al.*, 2004), the pCD/NL-BH* $\Delta\Delta\Delta$ /BSD lentiviral packaging plasmid described above, and the VSV-G-encoding pCEF-VSV-G plasmid (Ou *et al.*, 2012). HEK 293 cells were then transduced with these vectors, and transduced cells were subjected to BSD selection to enrich for putative partial recombinants. Genomic DNA from BSD-resistant cell clones was then isolated and subjected to PCR. Figure 2A displays the PCR products obtained using genomic DNA from three representative BSD-resistant cell clones, referred to as HRRE1, HRRE2, and HRRE3, respectively. Use of the PNL_F1 and GrecR2 primer pair yielded a PCR product of about 1000 bp, while the Tat_s and GFP_a primer pair

yielded a product of about 1600 bp. Figure 2B shows the DNA sequences obtained for the PCR products corresponding to the Psi-Gag and Tat-EGFP regions of the HRRE1, HRRE2, and HRRE3 cell clones. Consistent with the recombination model presented in Fig. 1, all three clones revealed hybrid vector sequences bearing vector-derived as well as packaging construct-derived sequences. For clone HRRE2, the 39 bp deletion ($\Delta\Psi$) affecting the packaging signal was retained, indicating that the recombination event involved sequences upstream of this deletion. The Psi-Gag-specific DNA sequences obtained for clones HRRE1 and HRRE3 suggest that recombination took place involving sequences downstream of the $\Delta\Psi$ deletion.

To rule out the possibility that the hybrid vector sequences detected may have emerged as a result of a PCR artifact involving residual pNL-EGFP(MSCV) and pCD/NL-BH* $\Delta\Delta\Delta$ /BSD plasmid DNA present in the crude (unpurified) vector preparation, a control experiment was carried out. In this experiment, 3.33×10^5 copies of the pNL-EGFP(MSCV) and pCD/NL-BH* $\Delta\Delta\Delta$ /BSD plasmids either singly or in combination were tested. Genomic DNA (3.33×10^4 genomic copy equivalents) extracted from the HRRE1 cell clone or from untransduced HEK 293 cells served as positive and negative controls, respectively. Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/hgtb) shows that there was no PCR signal with either one of the plasmids or with the two plasmids mixed together, while the HRRE1 genomic DNA generated the expected fragment. This result indicates that

FIG. 2. Analysis of BSD-resistant cell clones. **(A)** PCR analysis using genomic DNA extracted from BSD-resistant HEK 293 cell clones. HEK 293 cells were transduced using 10^6 transducing units of the NL-EGFP(MSCV) lentiviral vector. Transduced cells were subjected to BSD selection ($10 \mu\text{g/ml}$) for 3 weeks. To detect partial recombinants, genomic DNA extracted from three BSD-resistant cell clones, referred to as HRRE1, HRRE2, and HRRE3, respectively, was analyzed by PCR. The PNL_F1 and GrecR2 primers were used to amplify sequences corresponding to the Psi-Gag region and the Tat_s and GFP_a primers to amplify the Tat-EGFP region. The two horizontal arrows refer to the Psi-Gag (lower arrow)- and Tat-EGFP (upper arrow)-specific PCR products. C, genomic DNA from untransduced HEK 293 cells; lanes 1–3, HRRE clones 1–3; M, marker (1 kb ladder). **(B)** Sequence analysis of PCR products. The broken lines indicate gaps in the overlaps between the HRRE1, HRRE2, and HRRE3 sequences, and sequences present in the pCD/NL-BH* $\Delta\Delta\Delta$ /BSD packaging (Pack) construct or the NL-EGFP(MSCV) vector construct. PCR, polymerase chain reaction.

the PCR fragments obtained with genomic DNA from the HRRE1, HRRE2, and HRRE3 cell clones did not emerge as a result of contaminating plasmid DNA.

Design of lentiviral vector genomes containing modified RRE and ORI sequences

We next wanted to determine whether removal of sequence overlaps that are shared between the packaging and vector constructs would reduce the frequency of recombination events. To do this, a series of modified lentiviral vector constructs were generated (Fig. 3). To test whether replacement of the HIV-1 RRE sequence present in the NL-EGFP(MSCV) lentiviral vector genome (Fig. 3A) by an unrelated RRE sequence would lower the emergence of partial recombinants, the HIV-1 RRE sequence was replaced with a 1045 bp RRE fragment derived from SIVmac₂₃₉. The sequence similarity between the SIVmac₂₃₉-derived RRE sequence and the HIV-1 RRE sequence is 18.6%. The SIVmac₂₃₉-derived RRE sequence was previously shown to be functionally equivalent to that of the HIV-1 RRE in the context of HIV-1-based lentiviral vectors (Srinivasakumar, 2008). The resulting vector is referred to as NL-SRRE-EGFP(MSCV) (Fig. 3B).
An additional vector, NL-SRRE/ Δ ORI-EGFP(MSCV), that lacks the 168 bp ORI sequence upstream of the SIVmac₂₃₉ RRE sequence was also constructed (Fig. 3C). The percentage of sequence overlap between the packaging construct and the various vector genomes is indicated in Fig. 3, middle column. It varied from 13.3% for the NL-EGFP(MSCV) vector to 9.1% for the NL-SRRE/ Δ ORI-

EGFP(MSCV) bearing the SIVmac₂₃₉-derived RRE and lacking the ORI sequence.

Reduced emergence of partial recombinants using vector genomes containing an SIVmac₂₃₉ RRE sequence

To test the vector constructs shown in Fig. 3A–C regarding their capacity to lead to the emergence of BSD-resistant colonies, 10⁶ EGFP-based transduction units for each of the three lentiviral vectors were used to transduce HEK 293 cells and the cells were subjected to BSD selection. Figure 3 right column shows the number of BSD-resistant colonies obtained with the various vectors. For the NL-EGFP(MSCV) vector, the number of BSD-resistant colonies obtained was 63.4 $\pm$ 15.4 (mean $\pm$ standard error of the mean) and for the NL-SRRE-EGFP(MSCV) and NL-SRRE/ Δ ORI-EGFP(MSCV) vectors, the numbers were 25.6 $\pm$ 5.3 and 9.5 $\pm$ 2.4, respectively. There were no BSD-resistant colonies with mock-transduced cells (data not shown). A quantitative PCR analysis carried out using a gag-specific primer/probe set (Kutner *et al.*, 2009) was used to determine vector copy numbers in BSD-resistant HEK 293 cell clones. The vector copy numbers for 5 of the NL-SRRE-EGFP(MSCV) vector-derived clones tested varied from 6 per cell to 25 per cell (data not shown).
To verify the presence of partial recombinants, genomic DNA from BSD-resistant HEK 293 cell clones obtained using the NL-EGFP(MSCV), NL-SRRE-EGFP(MSCV), and NL-SRRE/ Δ ORI-EGFP(MSCV) lentiviral vectors was extracted and subjected to PCR. The results presented in Table 1 show that the reduction of sequence overlaps

FIG. 3. Design and testing of RRE-substituted and SIN lentiviral vectors. (A) NL-EGFP(MSCV) vector. (B) NL-SRRE-EGFP(MSCV) vector bearing the SIVmac₂₃₉ RRE. (C) NL-SRRE/ Δ ORI-EGFP(MSCV) vector bearing the SIVmac₂₃₉ RRE and lacking the ORI sequence. (D) NL(CMV)EGFP/CMV/WPRE Δ U3-based SIN vector. The percentages of sequence overlap between the vector and the packaging constructs are referred to in the middle column. BSD-resistant colonies (mean $\pm$ standard error of the mean) obtained using 10⁶ EGFP transducing units of the various vectors are presented in the column at the right [n = 7 for the NL-EGFP(MSCV) vector; n = 11 for the NL-SRRE-EGFP(MSCV) vector; n = 10 for the NL-SRRE/ Δ ORI-EGFP(MSCV) vector; n = 10 for the NL(CMV)EGFP/CMV/WPRE Δ U3 vector]. SIN, self-inactivating.

TABLE 1. POLYMERASE CHAIN REACTION ANALYSIS OF GENOMIC DNA FROM BLASTICIDIN-RESISTANT COLONIES OBTAINED USING UNMODIFIED VECTORS, REV RESPONSE ELEMENT-SUBSTITUTED VECTORS, OR SELF-INACTIVATING VECTORS

Vector	No. of PCR-positive clones/ No. of clones analyzed	
	Psi-Gag primers ^a	Tat-EGFP primers ^a
NL-EGFP(MSCV) ^b	14/15 (93%)	14/15 (93%)
NL-SRRE-EGFP(MSCV) ^c	18/35 (51%)	29/35 (83%)
NL-SRRE/ Δ ORI-EGFP (MSCV) ^d	2/16 (13%)	1/17 (6%)
NL(CMV)EGFP/CMV/WPRE Δ U3 ^e	2/19 (10%)	1/12 (8%)

^aPrimers are referred to in Fig. 1C.

^bHIV-1 RRE plus 168 bp ORI sequence.

^cSIVmac₂₃₉ RRE plus 168 bp ORI sequence.

^dSIVmac₂₃₉ RRE without ORI sequence.

^eSelf-inactivating vector.

ORI, origin of replication; PCR, polymerase chain reaction; RRE, Rev response element.

resulted in a drop in the percentage of cell clones that yielded an identifiable PCR product. It was evident, however, that removal of sequence overlaps did not completely eliminate the emergence of BSD-resistant cell clones (Fig. 3), or the formation of an identifiable PCR product (Table 1).

The PCR results using genomic DNA extracted from 7 of the BSD-resistant HEK 293 cell clones (SRRE clones 1–7) obtained with the NL-SRRE-EGFP(MSCV) vector are

shown in Fig. 4A. Genomic DNA was amplified using the Tat_s and GFP_a primers (Fig. 4A, top panel) and the PNL-F1 and GrecR2 primers (Fig. 4A, bottom panel). All 7 clones revealed a Psi-Gag-specific PCR product of the expected size (around 1 kb), but only 5 of the clones produced a Tat-EGFP-specific PCR product of the predicted size (around 1.8 kb), while two of the clones (clones SRRE3 and SRRE6) revealed a shorter PCR product.

The DNA sequences of the Tat-EGFP-specific PCR products for SRRE clones 1, 2, 4, 5, and 7 are presented in Supplementary Fig. S2. The results obtained are consistent with the view that recombination took place within the 168 bp ORI sequence present both in the vector and in the packaging constructs (Fig. 4B). The sequences of the Psi-Gag-specific PCR products for SRRE clones 1, 2, 4, 5, and 7 were identical to those obtained for clones HRRE1, HRRE2, and HRRE3 presented in Fig. 2B (data not shown). The DNA sequence analysis for clone SRRE3 revealed that it contains packaging construct-derived sequences as well as vector construct-derived sequences and a partially deleted SRRE sequence (data not shown).

Emergence of partial recombinants using vector genomes containing the SIVmac₂₃₉ RRE sequence and lacking the ORI sequence

The PCR results for 17 of the BSD-resistant HEK 293 cell clones (SRRE Δ ORI clones 1–17) obtained using the NL-SRRE/ Δ ORI-EGFP(MSCV) vector are shown in Fig. 5. A primer set specific for the BSD coding region generated the predicted BSD fragment for all the clones analyzed, indicating that the BSD sequence was present in all the clones (Fig. 5, bottom panel). The PCR fragments obtained using primers specific for the Tat-EGFP and Psi-Gag regions are shown in Fig. 5 top and middle panels, respectively. For the

FIG. 4. PCR analysis of genomic DNA from BSD-resistant cell clones obtained using the RRE-substituted lentiviral vector. (A) HEK 293 cells were transduced using 10^6 EGFP-based transducing units of the NL-SRRE-EGFP(MSCV) lentiviral vector. Transduced cells were subjected to BSD selection. To detect partial recombinants, genomic DNA extracted from BSD-resistant cell clones was analyzed by PCR using primers specific for the Tat-EGFP and Psi-Gag regions as outlined in the legend to Fig. 2A. C, genomic DNA from untransduced HEK 293 cells; Samples 1–7, BSD-resistant cell clones referred to as SRRE clones 1–7. The two arrows refer to the Psi-Gag (lower arrow)- and Tat-EGFP (upper arrow)-specific PCR products. (B) Representation of predicted recombination events. Top panel: Packaging construct. Middle panel: NL-SRRE-EGFP(MSCV) lentiviral vector genome. Bottom panel: Outline of predicted partial recombinant. SIVmac₂₃₉-derived sequences are highlighted in gray.

FIG. 5. PCR analysis of genomic DNA from BSD-resistant cell clones obtained using the RRE-substituted lentiviral vector lacking the ORI sequence. HEK 293 cells were transduced and BSD-resistant cell clones were selected as described for Fig. 2. Seventeen clones were analyzed by PCR using primers specific for the Tat-EGFP (top panel) and Psi-Gag (middle panel) regions and the BSD coding region (bottom panel). The arrows refer to the BSD (bottom arrow)-, Psi-Gag (middle arrow)-, and Tat-EGFP (top arrow)-specific PCR products.

Tat-EGFP-specific PCRs, 4 of the 17 SRREΔORI clones yielded a PCR product of the expected size (around 900 bp). While SRREΔORI clone No. 9 revealed a strong PCR product, the PCR products for clones No. 1, 2, 3, 5, and 6 were weaker. The reason for the difference in intensity of the PCR products is not known. In addition, there were two other weak bands between 2 and 3 kb (Fig. 5, top panel, SRREΔORI clones 10 and 13), the origin of which is not clear. For the Psi-Gag-specific PCR, two of the clones revealed a PCR fragment of the expected size (around 1 kb). Interestingly, none of the clones that revealed a PCR product using the Tat-EGFP-specific primer pair yielded a PCR product using the Psi-Gag-specific primers, and vice versa. This may indicate that these clones contain truncated/rearranged Psi-Gag or Tat-EGFP sequences. The Tat-EGFP-specific PCR product from SRREΔORI clone No. 9 was sequenced to determine its origin. The sequence analysis revealed that the recombination event took place within the cPPT sequence present in the vector upstream of the SRRE sequence and a residual HIV-1 Env-encoding sequence present in the packaging construct (Supplementary Fig. S3).

Emergence of partial recombinants in the context of a self-inactivating lentiviral vector

Since the BSD resistance assay to detect partial recombinants is dependent on a promoter sequence driving the expression of the BSD resistance gene, it was not clear whether it would be compatible with the self-inactivating (SIN) vector format. The results presented in Fig. 3D were obtained using a third-generation SIN vector. Small numbers of BSD-resistant colonies were consistently obtained under conditions where mock-transduced cells produced no colonies. The results presented in Table 1 show that, of the 19 SIN vector-derived clones analyzed, 2 revealed a PCR fragment of the expected size using primers specific for the Psi-Gag region, and of the 12 clones analyzed using the Tat-EGFP primer set, 1 revealed a fragment of the expected size. The PCR results of the SIN vector-derived clones obtained using a BSD-specific PCR primer set and primer sets specific for the Tat-EGFP and Psi-Gag regions are shown in Fig. 6A and B, respectively. The four clones analyzed yielded the predicted PCR fragment corresponding to the BSD region, while one of the clones (SIN clone No. 3) analyzed revealed the predicted PCR fragment corresponding to the Tat-EGFP region and two

of the clones (SIN clones No. 3 and 4) the predicted PCR product corresponding to the Psi-Gag region.

These findings are in line with the PCR results reported by Sastry *et al.* (2003) involving SIN vector-transduced cell DNA that showed the presence of Psi-Gag recombinants. They are also compatible with the results presented earlier by Hanawa *et al.* (2005) and Logan *et al.* (2004) that showed that integrated SIN vector genomes can be transcribed, possibly mediated by cryptic promoters present in the vector.

Use of an unmodified packaging construct in conjunction with BSD-encoding lentiviral vectors to detect partial recombinants

To extend the use of the BSD selection assay to detect partial recombinants in the context of packaging constructs lacking a *BSD* resistance gene, a BSD-encoding lentiviral

FIG. 6. PCR analysis of genomic DNA from BSD-resistant cell clones transduced with a SIN lentiviral vector. HEK 293 cells were transduced using the NL(CMV)EGFP/CMV/WPREΔU3 SIN lentiviral vector and subjected to BSD selection. Four clones were analyzed by PCR using primers specific for the BSD coding region (A) and the Tat-EGFP and Psi-Gag regions (B), respectively. M, 1 kb ladder; N, negative control (genomic DNA from untransduced HEK 293 cells); P, positive control (HRRE1 DNA). The arrows refer to the Tat-EGFP (top arrow)- and the Psi-Gag (bottom arrow)-specific PCR products.

vector, NL-BSD(MSCV), was constructed. To detect partial recombinants, VSV-G-pseudotyped NL-BSD(MSCV) lentiviral vector particles were generated using the unmodified pCD/NL-BH* $\Delta\Delta\Delta$ packaging construct (Zhang *et al.*, 2004), and the resulting vector supernatants were used to transduce HEK 293 cells, which were then subjected to BSD selection. To rescue partial recombinants, pooled BSD-resistant cell clones were subjected to transient transfection using the VSV-G-encoding pCEF-VSV-G plasmid, the VSV-G-encoding pCEF-VSV-G plasmid plus a Rev-encoding plasmid (pCMV-Rev), or the pCEF-VSV-G plasmid plus the second-generation pCD/NL-BH* $\Delta\Delta\Delta$ packaging plasmid, encoding Gag, Pol, Tat, and Rev. Vector supernatants were used to transduce fresh HEK 293 cells. Transduced cells were subjected to BSD selection. The results obtained are summarized in Supplementary Table S1. They show that supernatants obtained using the VSV-G plus Rev-encoding plasmids, or the VSV-G plasmid in concert with the pCD/NL-BH* $\Delta\Delta\Delta$ plasmid resulted in the formation of BSD-resistant colonies upon transduction of HEK 293 cells, while transfection with the VSV-G-encoding plasmid alone did not. These findings are consistent with the view that transfection using the VSV-G plus Rev-encoding plasmids resulted in the rescue of partial recombinants, while cotransfection using the VSV-G plus pCD/NL-BH* $\Delta\Delta\Delta$ plasmids resulted in the mobilization of partial recombinants plus the original NL-BSD(MSCV) lentiviral vector genomes. The fraction of putative partial recombinants obtained using an unmodified packaging construct in concert with the NL-BSD(MSCV) vector was in the order of 1 such recombinant per 2×10^4 NL-BSD(MSCV) vectors (Supplementary Table S1).

To demonstrate the presence of partial recombinant genomes in BSD-resistant cell clones that emerged after vector mobilization after transfection using the VSV-G plus Rev-encoding plasmids, genomic DNA was isolated and subjected to PCR using BSD and Psi-Gag-specific primers. The results presented in Fig. 7A show that, of the 6 clones analyzed (clones SK 1–6), all yielded a PCR fragment using the BSD-specific primers, while 5 of the clones (clones SK 2–6) produced a Psi-Gag-specific PCR fragment of the expected size. This result is consistent with the view that these clones represent partial recombinants. The lack of a Psi-Gag-specific PCR product for clone SK 1 may indicate that this clone arose because of rescue of the NL-BSD(MSCV) vector genome from transduced cells harboring a recombinant vector genome as well as the original NL-BSD(MSCV) vector. Figure 7B shows an overview of the predicted steps leading to the formation of partial recombinants involving a BSD-encoding lentiviral vector and the unmodified packaging construct.

Discussion

To enhance the safety of lentiviral vectors for clinical use, advanced vector platforms were designed in which the packaging functions were split into separate plasmids (Dull *et al.*, 1998; Wu *et al.*, 2000; Westerman *et al.*, 2007). Codon optimization of the helper construct was also performed in an attempt to improve vector safety by reducing the degree of sequence homology between the vector sequences and sequences present in the packaging construct (Wagner *et al.*, 2000; Fuller and Anson, 2001; Koldej *et al.*, 2005). For these advanced lentiviral vector platforms,

FIG. 7. PCR analysis of vector sequences rescued from HEK 293 cells transduced using a BSD-encoding lentiviral vector. **(A)** Genomic DNA from HEK 293 cell clones bearing rescued vector sequences was PCR-amplified using primers specific for the BSD coding region or the Psi-Gag region. C, negative control (genomic DNA from untransduced HEK 293 cells); m, phage lambda and ϕ X174 marker fragments; M, 1 kb ladder; P, positive control (genomic DNA from the HRRE1 clone); samples 1–6, SK clones 1–6. The arrows refer to the BSD (lower arrow)- and Psi-Gag (upper arrow)-specific PCR products. **(B)** Representation of predicted recombination events. Top panel: Unmodified packaging construct. Middle panel: NL-BSD(MSCV) lentiviral vector genome. Bottom panel: Predicted partial recombinant.

multiple recombination events involving the packaging construct, the vector genome, and an Env-encoding sequence would be required in order to form a fully replication-competent lentivirus. Thus, these advanced lentiviral vectors are assumed to be safe. Indeed, there have been no reports so far documenting the presence of RCL in clinical-grade lentiviral vectors (Sastry and Cornetta, 2009; Cornetta *et al.*, 2011). However, the potential of such vectors to form RCLs remains a significant safety concern. Consequently, current FDA recommendations require testing for RCL, beginning with the vector product and followed by testing the final transduced cell product (FDA, 2006).

While the genomic structure of an RCL remains theoretical (Cornetta *et al.*, 2011), fully replication-competent lentiviral vector genomes encoding VSV-G or an amphotropic Env in *cis* configuration that were artificially generated *in vitro* (Segall *et al.*, 2003) were found to be functional. Viral spread, beginning with a very low inoculum, took several weeks in HEK 293T cells in culture and was characterized by “autoinfection,” resulting in multiple proviral copies per cell, higher levels of viral gene

expression, and eventual cell death (Segall *et al.*, 2003). This shows that lentiviral genomes bearing alternative Env glycoproteins are capable of replicating *in vitro*. It remains to be determined, though, how such replication-competent vectors will perform in other cell types, and how the lack of accessory proteins may affect the replication of such artificial RCLs in other cell types, including primary cells (Jeeninga *et al.*, 2005, 2006).

The likelihood for the inadvertent incorporation of a heterologous Env coding region into a lentiviral vector genome during vector production is assumed to be low because of a lack of sequence homology between vector and Env-encoding sequences. However, there might be alternative mechanisms to generate an RCL in the absence of sequence overlaps, including illegitimate recombination events. A precedent for such an event has recently been described for murine cells where recombination between an exogenous RNA virus, lymphocytic choriomeningitis virus, and the endogenous intracisternal A-type particle retrotransposon was found to occur (Geuking *et al.*, 2009).

An alternative and potentially more likely avenue to arrive at conditionally replicating lentiviruses would include partial recombinants and their propagation in cells expressing a heterologous Env glycoprotein capable of forming pseudotyped vectors. These could include cells bearing infectious human endogenous retroviruses (Lee and Bieniasz, 2007; Dewannieux *et al.*, 2010), such as placental cells expressing syncytin, a fusogenic protein encoded by the human endogenous retrovirus HERV-W (Mi *et al.*, 2000). Thus, the emergence of partial recombinants during lentiviral vector production is a concern, and attempts to detect them and to lower their incidence are warranted.

Two different kinds of assays have been used to detect partial recombinants. These involve PCR-based assays (Sastry *et al.*, 2003; Cornetta *et al.*, 2011) and assays involving a drug selection step (Wu *et al.*, 2000; Fuller and Anson, 2001; Koldej *et al.*, 2005). PCR-based assays were shown to detect partial recombinants with a sensitivity of about 10 copies/0.1 μ g of genomic DNA (Sastry *et al.*, 2003; Cornetta *et al.*, 2011). A limitation of PCR-based methods to detect partial recombinants is that only a small fraction of the total genomic DNA can be sampled. Assays involving drug selection are compatible with large vector volumes and can easily be scaled up, a property that is not shared by the standard PCR assay for detecting Psi-Gag recombinants (Sastry *et al.*, 2003).

Wu *et al.* (2000) previously described a sensitive biological assay to specifically select and enrich for lentiviral genomes that arose through genetic recombination between the packaging construct and the gene transfer vector. This mobilization assay involved HeLa cells into which a puromycin resistance gene under control of the HIV-1 LTR was inserted. Pseudotyped vector particles expressing functional Tat were shown to confer resistance to puromycin. The formation of recombinant viruses was confirmed by DNA sequencing. The analysis of the 5' and 3' sequences revealed a physical linkage between the packaging construct and the vector genome. Interestingly, a point of vector recombination was found within the polyA tract of the packaging construct, indicating that it involved nonhomologous recombination events (Onafuwa-Nuga and Telesnitsky, 2009).

The results presented in this report are based on a second-generation lentiviral vector system that we described before (Zhang *et al.*, 2004). While second-generation lentiviral vector systems are not representative of the current state-of-the-art third-generation systems that are used clinically, partial recombinants were also detected in the context of these more advanced systems (Sastry *et al.*, 2003). Thus, our finding that a reduction of sequence overlaps between the vector and packaging components reduces the frequency of partial recombinants will also be relevant for improving the safety of the more advanced lentiviral vector systems.

The conclusions from the sequencing results of the PCR products obtained using genomic DNA from BSD-resistant HEK 293 cell clones and a primer pair specific for the Tat sequence present in the packaging construct and the EGFP sequence present in the vector genome are as follows: (i) For the NL-EGFP(MSCV) vector, the data are consistent with the view that recombination took place within the RRE and/or the ORI sequences present in the vector and packaging constructs (Fig. 2B). (ii) For the NL-SRRE-EGFP(MSCV) vector, the results indicate that recombination occurred within the 168 bp ORI sequence (Fig. 4). (iii) For the NL-SRRE/ Δ ORI-EGFP(MSCV) vector, all of the cell clones tested revealed a BSD-specific PCR fragment of the expected size (Fig. 5, bottom panel). However, the Tat-EGFP-specific PCR products (Fig. 5, top panel) and the Psi-Gag-specific PCR products (Fig. 5, middle panel) were not consistent among the various clones tested, heterogeneous with respect to size, or entirely absent for some of the clones. This may indicate that the Psi-Gag and/or Tat-EGFP regions were deleted/rearranged during reverse transcription. The sequence of the PCR fragment obtained using Tat/EGFP-specific primers shown in Fig. 5 (top panel, lane 9) is consistent with the view that a nonhomologous recombination event took place involving the 150 bp cPPT sequence upstream of the SRRE present in the vector and an HIV-1 Env-encoding sequence present in the packaging construct (Supplementary Fig. S3). It is worth noting that the 150 bp cPPT sequence present in the vector is 100% identical to a 150 bp sequence present in the packaging construct close to the end of the pol sequence. This sequence overlap may provide another site for recombination. However, the resulting partial recombinants would lack the BSD resistance gene. Thus, they would be lost during the BSD selection step.

In conclusion, our results show that the frequency of partial recombinants was dependent on the degree of sequence overlap. Also, similar to the findings presented before by Wu *et al.* (2000), there was evidence for the occurrence of nonhomologous recombination events for vectors where sequence overlaps (RRE and ORI sequences) had been eliminated. This shows that partial recombinants appear to emerge at a low frequency, independent of the sequence context. Whether such rare partial recombinants can be mobilized to generate a replication-competent virus needs to be determined.

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