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Replicative fidelity of lentiviral vectors produced by transient transfection

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Abstract

Previous investigations have estimated the human immunodeficiency virus type 1 (HIV) base pair substitution rate to be approximately 10^{-4} to 10^{-5} per round of viral replication, and HIV has been hypothesized to be more error-prone than other retroviruses. Using a single cycle reversion assay, we unexpectedly found that the reversion rates of HIV, avian leukosis virus and Moloney murine leukemia virus were the same, within statistical error. Because both the viral enzyme reverse transcriptase (RT) and cellular RNA polymerase II (RNAP) are required for viral replication, we hypothesized that the similar reversion rates actually reflect the intrinsic error rate of RNAP, which is the enzyme common to all three retroviruses in the reversion assay. To address this possibility, HIV vectors with the U3 region replaced by a reporter reversion cassette were constructed and vector supernatant produced by transient transfection. All single integrant revertant cell lines showed the identical mutations at both long terminal repeats. This indicates that either RNAP or another cellular enzyme is responsible for these reversions, or that HIV RT only makes errors during first strand synthesis. Additionally, when HIV particles were rescued from an integrated vector as opposed to being produced by transient transfection, the reversion rate was significantly lower, suggesting that one or more factors in the virus-producing cells plays a role in the fidelity of retroviral replication. These results have implications regarding the fidelity of the transgene after transient transfection production of lentiviral vector supernatants.

Keywords: Lentiviral vector; Fidelity; Transient transfection; RNA polymerase II

Introduction

Human immunodeficiency virus type 1 (HIV) is the causative agent of AIDS, the acquired immunodeficiency syndrome. Because it is able to infect non-dividing or mitotically quiescent cells, HIV has enormous potential as a gene transfer agent for a variety of primary or terminally differentiated cell types. For more than a decade, the HIV genome has been manipulated as a vector with increasing sophistication. Additionally, many ex vivo and in vivo animal studies have demonstrated successful lentivirus-mediated gene transfer of a variety of primary cell types and tissues, suggesting imminent widespread use in man for treatment of both inherited and acquired disorders (Dupre et al., 2004; Nash et al., 2004; Sumimoto et al., 2004). The first HIV-based vector gene transfer approach was recently initiated for ex vivo transduction of peripheral blood T cells from advanced patients who are seropositive for HIV (MacGregor, 2001).

Despite advances in vector design, for the most part, lentiviral vector supernatants are produced by transient transfection of 293T cells, using purified plasmid DNA templates. The FDA-approved Phase I clinical trial involving HIV + patients utilized concentrated vector that was produced after transient transfection of 293 cells (MacGregor, 2001). Although several HIV packaging cell lines have been developed, compared to transient transfection, these typically yield lower vector titers, and particle production may be transient (lasting only 5–10 days) after induction of vesicular stomatitis virus (VSV) G protein expression (Kafri et al., 1999; Strang et al., 2005; Yoshida et al., 1997). There are two reports, however, of HIV packaging cell lines with titers that approach those of transient transfection and more prolonged vector production (Ikeda et al., 2003; Ni et al., 2005). While transient

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transfection (or transient vector production) is suitable for many laboratory-based experimental applications, it may not be very amenable to clinical studies that may require hundreds of liters of vector supernatant.

It is well-established that HIV mutates or evolves during replication, which allows the virus to escape from both the cellular and humoral immune response and to develop drug resistance against all licensed anti-retroviral medications (Barre-Sinoussi, 1996; Domingo et al., 1997; Johnson et al., 2005). The rate at which HIV mutates, or the viral error rate, has implications for gene transfer, as well as for anti-HIV drug and vaccine development. Under immune or drug-selective pressure, a virus that has rare sequence changes may pass through a genetic bottleneck and become the dominant circulating species.

This critical lack of fidelity of HIV has long been attributed to reverse transcriptase (RT) (Goff, 1990). All tested RTs demonstrate poor fidelity in vitro, especially when compared against cellular DNA polymerases, partly due to an absence of proof-reading function (O'Neil et al., 2002; Roberts et al., 1988). The mutation rate can be influenced by multiple variables including the exact assay conditions (e.g., dNTP and salt concentrations), the mutation being introduced, the RT being studied, and the sequence context (Golinelli and Hughes, 2002; Mansky et al., 2003). This makes it virtually impossible to assign a single numerical value to the error rate, but in most cases, it is on the order of 10^{-4} to 10^{-5} per nucleotide or base pair per round of replication (Huang and Wooley, 2005; Mansky, 1996a).

Cell-based assays have been established to measure the fidelity of retroviruses, typically involving a single cycle of replication using a cell line with an integrated vector encoding the reporter gene and a defective provirus to provide missing trans functions. Either forward or reverse genetic screens are used to identify mutations in the reporter and also calculate the fidelity. For HIV, forward genetic screens using LacZα peptide or thymidine kinase resulted in error rates of 3.4×10^{-5} and 2.2×10^{-5} mutations/base pair/cycle, respectively (Huang and Wooley, 2005; Mansky and Temin, 1995). Reversion assays have also been used to estimate fidelity. For example, for reversion of an amber codon, the mutation rate of spleen necrosis virus (SNV) was 2×10^{-5} (Dougherty and Temin, 1988), whereas use of the forward LacZα peptide screen showed that for the same virus the error rate per target bp was 1×10^{-5} (Mansky and Temin, 1994). Similarly the error rate for bovine leukosis virus (BLV) was determined to be 5×10^{-6} per bp, just two-fold lower than that of SNV (Mansky and Temin, 1994). Interestingly, there was a similar spectrum of bp substitutions, frameshifts, deletions, and additions for SNV, BLV, and HIV, suggesting that a common property of retroviral replication was responsible for the observed mutations.

An RNA fingerprinting assay for murine leukemia virus (MLV) AKV strain demonstrated a similar base substitution rate of 2×10^{-5} (Monk et al., 1992). This was similar to the mutation rate of 1.4×10^{-5} for the eukaryotic gene hG6PD, determined by direct sequencing of integrated MLV vectors (De Angioletti et al., 2002). Using an amber reversion assay, Varela-Echavarría et al. determined the MLV error rate to be 4.0×10^{-6}, which was three- to five-fold lower than other estimates of MLV fidelity (Varela-Echavarria et al., 1992).

In all of the above studies, the reversion or forward mutation rate was measured using virus produced from an integrated provirus in a stable cell line. Although superficially similar in most cases, the results may not be directly comparable since variables such as exact method of vector production, producer and target cell lines utilized, and reporter construct were often not identical. An additional confounding factor was low viral titer in many cases, which resulted in relatively few mutational events that could be scored.

Multiple factors may influence HIV fidelity, including cellular DNA deaminases (notably APOBEC3G (Lecossier et al., 2003; Zhang et al., 2003)) and viral proteins such as Vpr (Chen et al., 2004; Mansky, 1996b; Mansky et al., 2000). Although the error rate of RT has been the subject of much investigation, the contribution of cellular RNA polymerase II (RNAP), which transcribes the full-length viral RNA prior to packaging, to replicative fidelity has not been as well studied. One report suggested that RNAP plays little role in HIV error rate (O'Neil et al., 2002), and at present, there are no reliable methods to measure RNAP fidelity in a cell-based system.

Here, we establish a two-marker genetic reversion assay to examine the fidelity of HIV and two other retroviruses. At least under transient transfection conditions, the fidelity of all three was the same, within statistical error. However, the fidelity of HIV was significantly lower when vector was produced by transient transfection compared to stable producer cell lines. In addition, double copy, self-inactivating vectors showed identical sequence changes in both long terminal repeats (LTRs). The data are consistent with RNAP or another cellular enzyme being responsible for most of the reversions made when vector was produced by transient transfection. These results suggest that precisely how vector supernatant is made can influence the frequency of mutations in a transgene, and this may have implications for lentiviral gene transfer and transgene fidelity.

Results

HIV, MLV, and ALV have similar reversion rates

It has been suggested that HIV is much more error-prone than other retroviruses (Achaz et al., 2004; Bakhanashvili et al., 2004; Berkhout et al., 2001). We decided to test this hypothesis by incorporating precisely the same bicistronic reporter cassette encoding a mutant blasticidin resistance gene (*bsd*), coupled by an internal ribosome entry sequence (IRES) to enhanced yellow fluorescent protein (eYFP) into an HIV, an ALV, and a MLV vector (Fig. 1A). In all three vectors, the reporter cassette was driven off the viral LTR. A genetic reversion assay was used to measure the frequency at which single nucleotide or frameshift mutations were corrected. The *bsd* gene, which contained either a single nucleotide mutation that created an early stop codon or a frameshift mutation, was

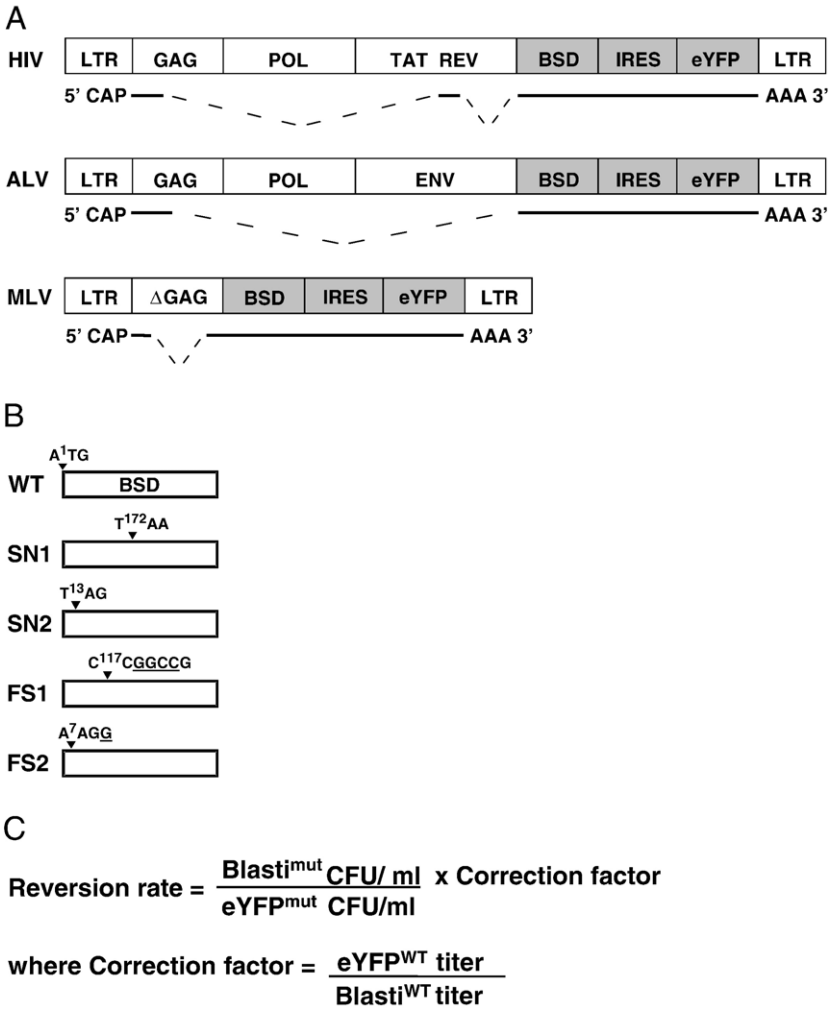

Fig. 1. Two marker genetic reversion assay. (A) Schematic of HIV, ALV, and MLV vectors, each with the identical bicistronic reporter cassette. HIV vector had deletions in *vif*, *vpr*, *vpu*, *nef*, and most of *env*. Left intact were *gag*, *pol*, *tat*, and *rev*. MLV vector encoded only the cassette, whereas ALV vector was replication competent in avian cells. Splicing patterns for the reporter cassette are shown beneath the vectors. Gene products are not precisely to scale. (B) Sequence of frameshift (FS) and single nucleotide (SN) null mutants of *bsd*, each of which resulted in complete loss of blasticidin deaminase function. Numbers are relative to the ATG start codon; underlined nucleotides were those added such that the downstream sequence was out of frame. (C) Calculation of reversion rate. Note reversion rate was normalized to WT *bsd* titers.

used as a marker for reversion (Fig. 1B). Reversion frequency was used to calculate reversion rate, as shown in Fig. 1C. Note that both the eYFP and wt *bsd* titers were used for normalization purposes. The reversion assay measures a subset of all possible sequence changes in the target gene. Some changes are silent and therefore not scored in the assay, which is based on phenotypic selection. The reversion assay is a means of comparison among viruses and methods of vector production; it is not necessarily an accurate measure of the mutation or error rate since it does not score all possible sequence changes. It has been estimated that assays based on phenotype score 40% of all sequence changes in the target gene (O'Neil et al., 2002). Frameshift corrections may behave somewhat similarly to a forward mutation since the correction requires an insertion or deletion.

bsd was chosen because it is relatively short (~400 bp), selection in the presence of antibiotic is quite rapid (complete within 5–7 days), and it is facile to sequence in its entirety. In addition, the single nucleotide or frameshift mutations chosen were null over greater than seven orders-of-magnitude so that any blasticidin-resistant cell colonies would be due to a reversion within *bsd*, as demonstrated by PCR followed by DNA sequencing. In the case of the HIV vector, because the bicistronic expression cassette lies within *nef*, both *bsd* and eYFP are translated from the last exon of a multiply-spliced mRNA so that nonsense-mediated decay is non-operational. To compare the reversion rates of the three retroviruses, pseudotyped vector particles were produced by transient transfection of 293T cells with vector and VSV G envelope expression plasmids, and titered on 293T cells. All reversion rate experiments were performed a minimum of two times, with three measurements per virus per target cell line for each experiment. Thus, the reversion rate measurement for each virus is based on at least 2×10^7 target cells.

The resultant reversion rates were not significantly different from each other (Fig. 2A), suggesting that either the fidelity of

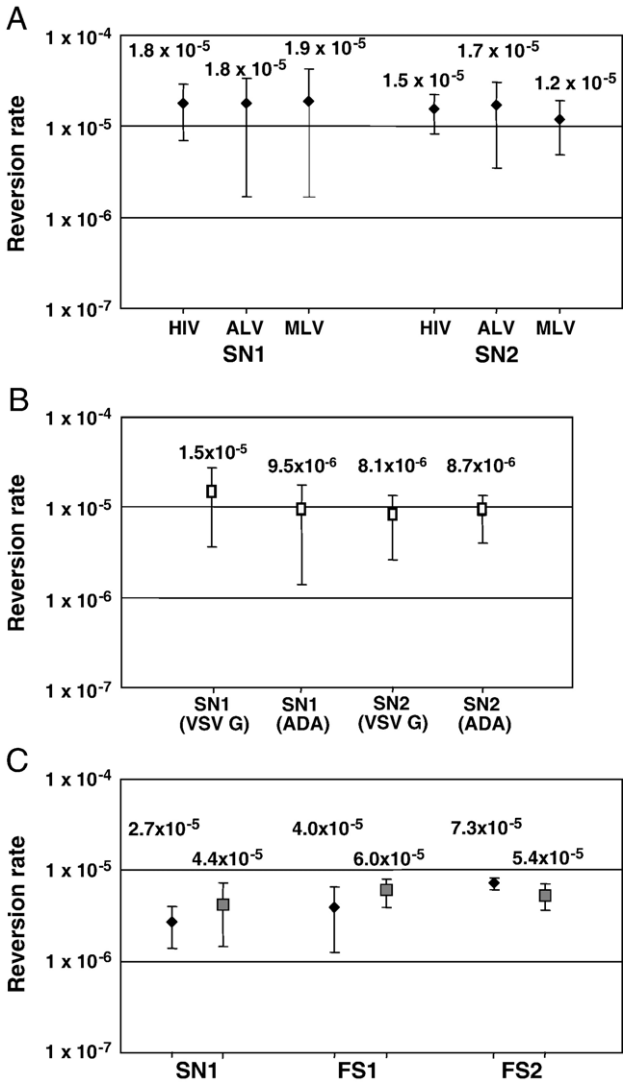

Fig. 2. Measurement of retroviral reversion rates. (A) Reversion rate of VSV G-pseudotyped HIV, ALV, and MLV vectors, as measured on 293T cells; $P > 0.05$ for all intervector comparisons. (B) Comparison of HIV reversion rates using VSV G- or ADA-pseudotyped vector on HOS.CD4.CCR5 targets; $P > 0.05$. (C) HIV reversion rate determined on 293T (closed diamond) and HeLa (closed square) target cells; $P > 0.05$ for all intravector comparisons.

HIV RT is comparable to that of the other two retroviruses or that a shared replication factor is responsible for the errors generated. For the SN1 mutation in HIV, ALV, and MLV vectors, the reversion rates were 1.8×10^{-5}, 1.8×10^{-5}, and 1.9×10^{-5}, respectively. For the SN2 mutation, the reversion rates were 1.5×10^{-5}, 1.7×10^{-5}, and 1.2×10^{-5}, respectively. There were no significant differences when intervector reversion rates for each mutant bsd gene were analyzed by Student's *t* test. This was repeated several times with similar results. It should be noted that the HIV vector utilized does not encode *vif*, but the 293T cells do not express CEM15 (APOBEC3G) or APOBEC3F so that particular hypermutation system is non-functional. In particular, the stop mutation reversions could not be G to A changes, which are the vast majority of mutations observed in the absence of Vif and the presence of APOBEC3G (Goff, 2003; Lecossier et al., 2003). Additionally, the deaminases are not known to correct frameshift mutations.

Reversion rate is independent of viral envelope and transduction temperature

Because we were using VSV G-pseudotyped particles which enter the cell through an endocytic pathway that is pH-dependent, it is possible that the reversion rate is altered compared to when an HIV envelope is used, especially since HIV typically fuses directly with target cell plasma membrane and the precise cellular machinery involved is different. In order to address this, we prepared HIV vector supernatants that had been pseudotyped with ADA, a macrophage-tropic HIV envelope. Both VSV G- and ADA-pseudotyped vector supernatants were tested on GHOST HI 5 cell targets that express both human CD4 and CCR5, and the resulting reversion rates were not significantly different (Fig. 2B). For VSV G- and ADA- pseudotyped vectors, reversion rates for the SN1 mutation were 1.5×10^{-5} and 9.5×10^{-6}, respectively. For the SN2 mutation, reversion rates for VSV G- and ADA-pseudotypes were 8.1×10^{-6} and 8.7×10^{-6}, respectively. This suggests that despite the differences in the way the two pseudotyped particles enter the cell, replicative fidelity is the same and thus independent of immediate post-entry events.

We also wished to determine whether the observed reversion rate was dependent on the target cell type. VSV G-pseudotyped HIV was used to transduce two human cell lines, 293T and HeLa, and the resultant reversion rates determined. The differences in the HIV reversion rate for the single nucleotide and frameshift mutations on these target cell lines were not statistically significant (Fig. 2C). This is consistent with the fact that there are no host proteins in the target cells known to participate in viral cDNA synthesis.

Although like most other laboratories, we grow all mammalian cells at 37 °C, this is not the core temperature of the typical hosts for ALV. Both chickens and quail have a core body temperature of about 41.5 °C, and for mice, it ranges from 36 to 38 °C. We questioned if the reversion rate observed could be temperature-dependent. HIV vector supernatant was produced transiently using 293T producers at 37 °C but tested on target cells at both 37 °C and 41 °C. Reversion rates for HIV-SN1-IY were 1.0×10^{-5} at 37 °C and 1.1×10^{-5} at 41 °C, which were not significantly different ($P > 0.05$, data not shown).

Reversions due to plasmid amplification in E. coli or transient transfection do not contribute substantially to HIV reversion rate

It is conceivable that the reversions observed were due to mutations in the plasmid template that arose during amplification in *E. coli*. Purified plasmid HIV vector DNA (encoding both mutant and wild-type *bsd*) was used to stably transfect 293T cells using the cationic lipid DOTAP, and blasticidin-resistant colonies were selected. Although the

Table 1
Reversion rate after stable transfection

Plasmid [a]	Stable transfection rate [b]	Reversion rate [c]
pHIV-WT-IY	9.6×10^{-2}	–
pHIV-FS1-IY	$1.4 \pm 1.1 \times 10^{-8}$	$1.5 \pm 1.1 \times 10^{-7}$
pHIV-SN1-IY	$5.4 \pm 3.5 \times 10^{-8}$	$5.6 \pm 3.6 \times 10^{-7}$

[a] Cells were transfected with 80 μg of plasmid using DOTAP, then selected in blasticidin.
[b] Number of blasticidin-resistant colonies divided by number of input cells.
[c] Calculated as mutant transfection rate divided by WT transfection rate.

reversion rate varied depending upon the mutation being corrected, it was approximately 10^{-7}, 1–2 orders of magnitude lower than that measured after transient production of vector supernatant (Table 1). Thus, it is unlikely that the observed reversion rate was simply due to mutation of DNA sequences during plasmid propagation in the bacterial host *E. coli*.

It is also possible that the reversion rate is much higher after transient transfection because the process of calcium phosphate transfection is itself mutagenic to the DNA template. To address this possibility, a plasmid encoding both a wild-type chloramphenicol acetyl transferase and also a point mutant β-lactamase (BLA E166Q) gene was transiently transfected into 293T cells using the same protocol used to make viral vectors. The BLA E166Q mutant is null over 8 orders of magnitude, and enzymatic function can only be restored by the single nucleotide reversion $C^{496} \rightarrow G^{496}$ (Palzkill and Botstein, 1992). Plasmid was recovered from Hirt supernatants of transfected cells and used to transform electrocompetent *E. coli*. Reversion rate was measured as the number of ampicillin + chloramphenicol-resistant colonies divided by the number of chloramphenicol-resistant colonies. Recovery of plasmid by the modified Hirt method was approximately 1% of transfection input. As a comparison, non-transfected plasmid DNA was directly transformed into *E. coli*. Measured reversion rates comparing post-transfected to non-transfected plasmid were not significantly different, implying that the process of transfection itself did not introduce mutations into the DNA plasmid template (Table 2).

RNAP may be responsible for reversions observed during transient transfection

The above results suggest that either the RTs of HIV, MLV, and ALV have finely tuned error rates that have converged due to selective pressures and unknown evolutionary forces to be approximately the same, or that perhaps, another polymerase involved in the replicative cycle, namely RNAP, is responsible for the reversions generated, at least in the transient transfection system. With regards to the latter, rare errors made during mRNA transcription would then simply be duplicated during reverse transcription. Under the transient transfection conditions employed, the 'readout' may be a measure of RNAP fidelity and not that of RT. In order to test this hypothesis, we constructed a self-inactivating (SIN) HIV-based vector in which mutant *bsd* was driven by the cytomegalovirus (CMV) immediate-early enhancer–promoter and placed within the deleted region of the 3′ U3 (Fig. 3A). The second internal cassette of the SRα promoter driving enhanced green fluorescent protein (eGFP) provided an independent measure of cellular transduction. Note that the *bsd* gene had an early stop codon, making it null and also creating an *AflII* restriction endonuclease site; removal or alteration of that codon in the DNA template would eliminate the *AflII* site, facilitating the screening process for mutations.

Vector was produced by transient transfection, and targets were transduced at low MOI to reduce the number of double integrants. Approximately 70 individual blasticidin-resistant cell colonies were isolated, expanded, and genomic DNA prepared. In order to examine both the 5′ and 3′ *bsd* genes of each, unique PCR DNA primers were designed. Based upon the PCR results, only 16 had single vector integrants, which were further analyzed. For each of the 16, both 5′ and 3′ PCR products were digested with *AflII*, and in each case, the *AflII* site had been destroyed at both the 5′ and 3′ ends (Fig. 3B). This was further confirmed by sequencing the *bsd* gene for each. For all 16 revertants, the observed sequence change(s) at the 5′ end was identical to that at the 3′ end (Table 3). The reversion rate for this SIN vector was 4.1×10^{-5}.

To demonstrate that two corrected *bsd* genes were not required for positive cell selection in blasticidin, a SIN HIV vector was constructed in which a CMV promoter-mutant *bsd*-IRES-eYFP cassette was placed within the *nef* region. There were no significant differences between the reversion rates of this 'single copy' vector and that of the 'double copy' vector (5.1×10^{-5} vs. 4.1×10^{-5}). In addition, single copy revertants survived and proliferated well even when the concentration of blasticidin was increased 5-fold, from 10 to 50 μg/ml (data not shown), suggesting that double copy revertants would not have a survival advantage at the low concentrations of blasticidin that were used.

Statistical analysis, coupled with the known HIV replicative cycle, suggested two possible interpretations of the data: either RNAP was much more (>ten-fold) error-prone than RT or RT 1st strand was much more error prone than either RNAP or RT 2nd strand. Since no second strand errors were observed in this experiment, and in vitro data have not established that RT 1st strand is much more error prone than 2nd strand (Bebenek et al., 1999; Boyer et al., 1992), these data are consistent with RNAP being responsible for most of the reversions generated when vector is made by transient transfection.

Table 2
Reversion rate after transient transfection

Source of DNA [a]	Reversion rate [b]
Purified, untransfected plasmid	$3.5 \pm 1.0 \times 10^{-7}$
Hirt lysate (7.5 μg plasmid transfected)	$2.6 \pm 1.4 \times 10^{-7}$
Hirt lysate (75 μg plasmid transfected)	$3.5 \pm 3.0 \times 10^{-7}$

$P > 0.05$ for all comparisons.
[a] Original plasmid was pTP123-TEM-E166Q.
[b] Determined by transforming electrocompetent *E. coli* with 10 ng plasmid and plating on chloramphenicol or ampicillin + chloramphenicol plates.

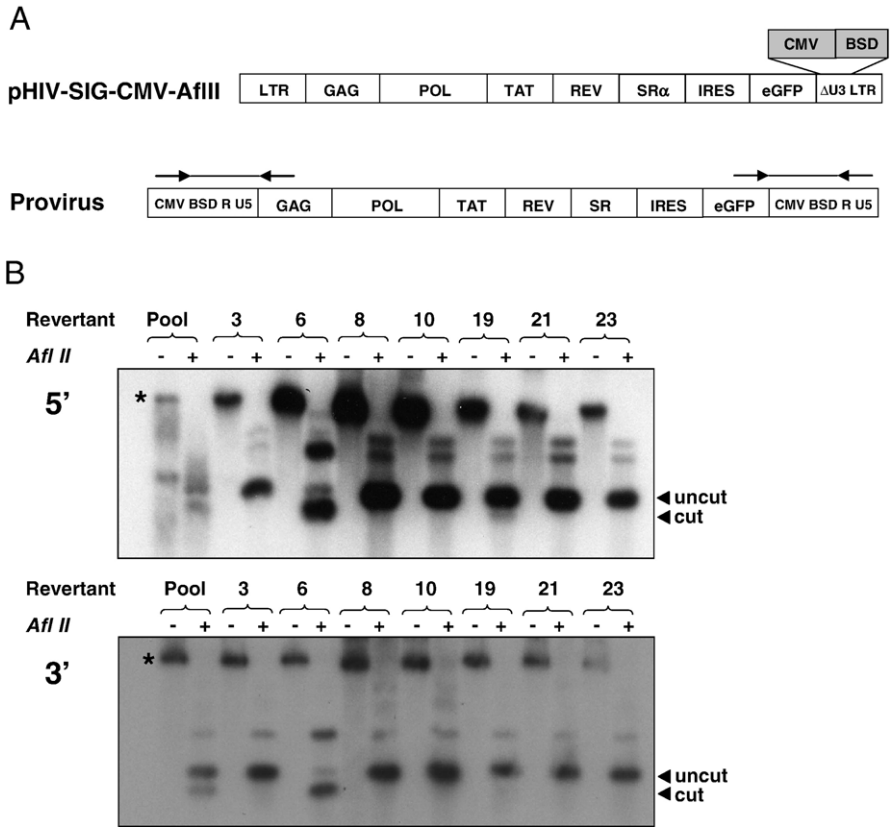

Fig. 3. Double copy *bsd* mutant vectors. (A) Schematic of the SIN HIV-based plasmid vector in which the 1.4 kb reporter cassette (CMV-*bsd*) was placed within the deleted region of the 3′ U3. The proviral form is shown below. Arrows indicate PCR primers for amplification of both the 5′ and 3′ CMV-*bsd* cassettes. (B) Southern blot (using *bsd* coding sequence as ^{32}P-labeled probe) of both the 5′ and 3′ LTR PCR products, cleaved with *AflII* as indicated above each lane. Arrow indicates expected product for presence of *AflII* site; note clone 6 has two bands consistent with >1 integrant (and thus excluded from further analysis). 'Pool' indicates blasticidin-resistant pooled transductants, each of which has one or more *bsd* genes. Arrows indicate positions of both uncut DNA (revertant) and Afl II-cut DNA (mutant) and asterisks mark full-length PCR products.

Differences in reversion rate due to method of vector supernatant production

Although widely used for gene transfer purposes, production of vector supernatant by transient transfection of 293T cells is unquestionably artificial. Because of this, we decided to determine whether reversion rate was similar when virus was produced by an alternative method—recovery of a stably integrated vector. Briefly, 293T cells were transduced at low multiplicity of infection (MOI) with the replication-defective vectors HIV-SN1-IY, HIV-FS1-IY, and HIV-WT-IY. Three YFP + cell clones were picked and expanded for each construct (Fig. 4A). PCR analysis of the cell clones using HIV-specific DNA primers (normalized using β-actin-specific DNA primers) suggested that each of these cell clones had 1 or 2 vector integrants. Vector was then recovered by transiently transfecting the clones with VSV G expression plasmid and titered on adherent human cells. Surprisingly, the measured reversion rate was 4- to 18-fold lower than that of vector produced by transient transfection. There were no significant differences in reversion rates between independently derived cell clones, and the results using one cell clone of each construct are shown in Fig. 4B. The reversion rates for the SN1 mutation on 293T and HOS TK$^-$ cells were 3.6×10^{-6} and 4.3×10^{-6}, respectively (for vector transcribed from integrated proviral template). Vector produced from a plasmid template by transient transfection had a significantly higher reversion rate, 1.9×10^{-5} for 293T cells and 2.1×10^{-5} for HOS TK$^-$ cells. The reversion rates for the FS1 mutation on 293T and HOS TK$^-$ cells were 2.9×10^{-6} and 2.7×10^{-6}, respectively (for vector transcribed from integrated proviral template). Vector produced from a plasmid

Table 3
Sequence changes in *bsd* in SIN vector after transduction

DNA sequence[a]	Frequency[b] (%)	5′ and 3′ Reversions?[c]
CAA GAA GAA[d]	6 (37)	Yes
Δ6 T**T**A GAA GAA	2 (13)	Yes
T**GG** GAA GAA	2 (13)	Yes
AAA GAA GAA	1 (6)	Yes
GAA GAA GAA	1 (6)	Yes
T**T**A GAA GAA	1 (6)	Yes
T**GG** GAA GA**G**	2 (13)	Yes
T**GG** G**G**A GAA	1 (6)	Yes

[a] Mutant sequence is T^{19}AA GAA GAA, relative to *bsd* start codon. Changes in the revertants are shown in bold; Δ6 refers to a deletion of 6 bp.
[b] Total of 16 revertants with single integrants.
[c] Determined by PCR and direct sequencing of *bsd* in both LTRs.
[d] Wild-type sequence.

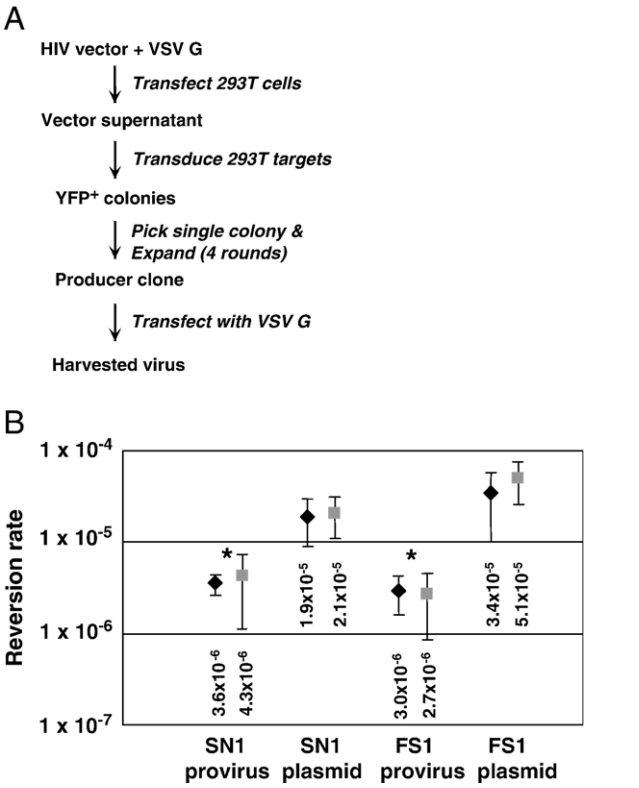

Fig. 4. Reversion rate of vector rescued from stable 293T producers. (A) Schematic of method for generating stable producer cell lines. Vector made by transient transfection was used to transduce 293T cells at low MOI. eYFP$^+$ cell clones (three for each vector) were selected and expanded. Vector was recovered by transfecting stable cell clones with VSV G expression plasmid and harvesting culture supernatant. (B) Measured reversion rates on 293T (closed diamond) and HOS TK$^-$ (closed square) target cells for vector recovered from the stable cell lines (provirus) or produced by transient transfection (plasmid). *$P < 0.001$ compared to the transiently produced vector.

template by transient transfection again had a significantly higher reversion rate, 3.4×10^{-5} for 293T cells and 5.1×10^{-5} for HOS TK$^-$ cells. As expected, reversion rate was independent of target cell type. This was reproduced several times, and in each case, the stable transductants gave reversion rates that were $2.0–4.0 \times 10^{-6}$. Weighted linear regression revealed a significant main effect for method of vector production ($P < 0.001$).

Discussion

Here, we describe a two-reporter genetic reversion assay to quantify the fidelity of HIV and other retroviral vectors produced by transient transfection. Reversion rate using this assay ranged from 10^{-4} to 10^{-5}, depending upon the reversion being corrected. Fidelity was independent of retrovirus, pseudotyping envelope, and target cell type. Neither propagation in bacteria nor the actual process of transfection appeared to be responsible for the generation of reversions. A novel SIN vector suggested that reversions during transient transfection were generated by RNAP and then simply duplicated by RT during cellular transduction, and fidelity was significantly greater when vector was rescued from a stable proviral integrant.

Other investigators have suggested that the replicative fidelity of HIV is much lower compared to that of other retroviruses. Review of the published literature shows that in no case were other retroviruses compared to HIV directly, using precisely the same vector production method and reversion assay, so it is difficult to conclude that HIV is in fact more error-prone than other retroviruses. Here, we used the identical reporter cassette for all three retroviruses tested, which were all produced by the same method of transient transfection of the same cell type. Use of a bicistronic cassette with an auto-fluorescent reporter along with a wild-type *bsd* provided both an internal and external normalization control, respectively, allowing for precise quantitation of reversion errors. Thus, the result here that the reversion rates of HIV, MLV, and ALV were the same is not in contradiction with the published literature.

Most of the viral mutations that arise during retroviral replication have long been attributed to RT, which is not thought to have proof-reading capability but simply an intrinsic fidelity. In addition, no known cellular factors participate directly in reverse transcription. Recently, it has become clear that the cytidine deaminases APOBEC3G and 3F (and perhaps others) may mold HIV sequences, if not those of endogenous and other exogenous retroviruses (Esnault et al., 2005; Turelli and Trono, 2005), resulting in G to A changes and leading to A-T richness of the genome (Conticello et al., 2003; Goff, 2003; Zhang et al., 2003). Although the vectors here did not include *vif* to counteract the editing enzymes, 293T cells do not express APOBEC3G or 3F. In addition, G to A changes would not have corrected either the stop codon or frameshift mutations in the mutant *bsd* genes used here. We conclude that particular hypermutation system was non-operational here, and the mutations observed were most likely due to a polymerase, namely either RT or RNAP.

Cell-free RT has a calculated error rate that is orders-of-magnitude higher than that observed in cell-based assays (Mansky and Temin, 1995), suggesting that there are other viral or cellular factors that increase replicative fidelity in the cell. Most of the cell-based assays cannot distinguish between errors made by RT and RNAP and only recently have efforts been directed towards determining the relative contributions of RT and RNAP to replicative errors. For example, Dougherty and colleagues sequenced a total of 215 paired LTRs after HIV vector transduction of cells and concluded that RT was in fact responsible for most of the observed mutations (O'Neil et al., 2002). However, intervector recombination (and not RT error) could not be wholly excluded as a cause of single LTR mutations. Of note is the use of a stable cell line to produce the viral vectors used for their experiments.

Here, we designed a SIN vector in which the *bsd* mutant reporter was located in the 3′ LTR and thus duplicated to the 5′ LTR after cellular transduction. In all cases in which a single vector integrant was found in the blasticidin-resistant targets, the same mutation in *bsd* was present in both LTRs. This suggests that either all the mutations observed were created in the vector-producing cell by a cellular enzyme and then simply duplicated by RT in the target cell or that the mutations all arose during first strand RT (and were then duplicated during second

strand). We disfavor the latter explanation since statistical analysis suggests that if this was the case, then first strand cDNA synthesis must have at least a 5- to 10-fold higher error rate than that of second strand. In vitro, cell-free data suggest that second strand cDNA synthesis has a similar if not lower fidelity compared to first strand (Boyer et al., 1992; Kerr and Anderson, 1997). It should be emphasized, however, that our results were obtained in a transient transfection system, and vector recovered from stable integrants had significantly greater fidelity. Experiments are now in progress to determine whether similar results (i.e., duplication of LTR mutations) are obtained in a stable integrant system in which the overall reversion rate is closer to 10^{-6}.

It is not clear why the reversion rate of vector in the transient transfection system was much higher than that of the stable integrants. Although previous results have suggested that the process of transient transfection may be mutagenic (Lebkowski et al., 1984), we did not observe this after calcium phosphate transfection of 293T cells. Reversion rate of plasmid recovered from Hirt supernatants was essentially identical to that of purified, untransfected plasmid, independent of the amount of DNA transfected. Although it could be argued that mutagenized plasmids are recovered with less efficiency than unmutagenized ones, this was not true for previous work (Calos et al., 1983; Lebkowski et al., 1984). Prior to Hirt supernatant preparation, transfected cells were extensively washed to remove any extracellular DNA, and the plasmid yields of 1% or less were approximately as expected for cell-associated DNA. Whether the recovered DNA was in fact nuclear is unknown.

It is possible that the mutagenicity of transient transfection observed in the past depended upon the method of transfection, the recipient cell type, and the plasmid itself. It is notable that some of the prior work demonstrating increased mutagenicity of transient transfection involved plasmids which contained the SV40 origin of replication, which was absent from all of the plasmids used here. Many of the mutations after transient transfection observed previously were likely due to acid depurination or cytidine deamination, the former perhaps secondary to transit through endosomes and the latter due to inherent instability of the purine base or the action of cellular deaminases. In addition, previous investigators did not use 293T cells (which lack cytidine deaminases) or chloroquine during the transfection process. Based upon these reasons and the data presented in Tables 1 and 2, we do not believe that the transfection process here was in itself mutagenic, although it cannot be completely excluded.

While MLV-based gene therapy vectors are routinely produced from stable producer cell lines, HIV vector supernatants are typically produced by transient transfection of 293T cells, as was performed here, since transient transfection usually results in higher vector titers compared to stable lentiviral producers. Unfortunately, the trade-off for higher titers may be reduced fidelity. Efforts to maximize both titer and fidelity are clearly desirable, and novel vector production methods (i.e., use of helper-dependent adenoviral-HIV chimeric vectors) may have utility in this regard.

Precisely how the method of vector production influences fidelity is uncertain, but we speculate that the amount or state of the DNA template may be critical. For example, the stable 293T producers used here had only one or two integrated proviruses in each cell, whereas after transient transfection hundreds if not thousands of plasmids are likely introduced into each transfected 293T cell. As an example, for Chinese hamster ovary cells, which are transfected at approximately half the efficiency of 293T cells, there was a maximum of approximately 100,000 plasmid molecules per cell 4 h after transfection (Batard et al., 2001). Overall, <5% of transfected cells had fewer than 3000 plasmids per cell. In another study using radiolabeled plasmids, after calcium phosphate transfection, less than 10% of input DNA was found in the nuclear fraction, remaining intact (Orrantia and Chang, 1990).

Based upon this, we speculate that when vector was made by transient transfection, on average approximately 2000–5000 plasmids entered each 293T cell, and of those 10% made it to the nucleus intact. It is therefore possible that cellular transcription factors or subunits of RNAP critical to fidelity may be limiting. Efforts to titrate such factors by co-transfecting plasmids containing solely an intact LTR and demonstrating subsequent reduced fidelity have thus far been unsuccessful. In addition, decreasing or increasing the amount of plasmid DNA used during the transient transfection over a 10-fold dynamic range did not statistically alter resultant reversion rate (not shown).

Alternatively, the exact state of the template may be critical. Transiently transfected plasmid is initially unchromatinized and much remains so, especially when introduced in large amounts, whereas integrated proviruses exist in a nucleosomal structure. Actively transcribed proviruses likely have additional cellular factors bound which maintain the open chromatin structure and participate in transcriptional regulation. Whether those factors have a direct role in transcriptional fidelity is uncertain, but one or more may serve to recruit RNAP subunits that are critical to the intrinsic fidelity or chain cleavage activity of the core polymerase. These may thus be present in the vicinity of stable integrants but absent from transiently transfected DNA templates. We thus speculate that after transient transfection if unchromatinized plasmid is utilized as transcription template fidelity may be lower.

Although the relative fidelity of RNAP has been measured in cell-free systems with or without specific transcription factors (Koyama et al., 2003; Thomas et al., 1998; Wang and Hawley, 1993), it has never been accurately quantified in cell-based assays. The error rate of mammalian genomic DNA replication is thought to be on the order of 10^{-9} to 10^{-10}; there are multiple reasons to believe that RNAP fidelity may be orders-of-magnitude lower. For example, depending upon the particular mRNA, in each cell at steady state, there are tens to thousands of copies of that species. Although some mRNAs are long lived, most degrade relatively rapidly, on the order of minutes to hours. Mechanisms such as nonsense-mediated decay exist to rapidly degrade errant mRNAs that have early stop codons (Hentze and Kulozik, 1999; Mashima et al., 1992; Zhang and Maquat, 1996). Furthermore, ribosomal translational error rate,

estimated to be 10^{-3} to 10^{-4} per residue, is relatively high (Ellis and Gallant, 1982; Weickert and Apostol, 1998). Taken together, these arguments suggest that there might be minimal selective pressure for RNAP to be of extremely high fidelity. It is conceivable that RT, which has its own intrinsic fidelity, may simply rely on RNAP to generate replicative errors.

Unexpectedly, in the SIN vector experiment, we did not observe any templates consistent with genomic recombination, suggesting that the recombination rate in the transient transfection system is <7%. Recombination is a driving force in HIV evolution and is estimated to occur more frequently than point mutations (An and Telesnitsky, 2002; Shriner et al., 2004; Zhuang et al., 2002). HIV infection of T lymphocytes and macrophages generated nine or more recombination events per round of replication, and comparisons with SNV and MLV suggest HIV may be up to ten times more recombinogenic than simple retroviruses (Levy et al., 2004; Onafuwa et al., 2003; Rhodes et al., 2005). Of note, vector recombination here would not per se result in blasticidin resistance. In our experiments, cell revertants which had multiple integrants or proviruses with gross rearrangements were excluded, due to the difficulty of interpreting results, and omission of those transduced cells from further analyses may have underestimated the recombination rate, especially since dual infection is thought to be required for recombination (Fernandez-Medina et al., 1999). Recombination events in cells with single integrants without gross rearrangements would have been scored, however.

In conclusion, we have demonstrated that three different retroviruses, including HIV, have the same replicative fidelity. Results from transient transfection suggest that the errors likely occurred in the producer cell, and those changes were then simply duplicated by RT. The fact that stable producer cell lines generate vector with significantly greater fidelity may have important implications for lentiviral gene transfer.

Materials and methods

Retroviral vectors and expression plasmids

The HIV-1, MLV, and avian leukosis virus (ALV) vectors used in these studies are shown in Fig. 1A. The parent construct for all HIV vectors was pHIV-IRES-eYFP, which has deletions in *vif*, *vpr*, *vpu*, *env*, and *nef* and was derived from the NL4-3 isolate (Adachi et al., 1986). Wild-type *bsd* was inserted just upstream of the IRES element to create HIV-WT-IY. Null mutants of the *bsd* gene were created by PCR-based mutagenesis (SN1, 5′-TGTGCATAACTC-3′, where the underlined nucleotide is mutated; SN2, 5′-GAATTCGCCCTTGGTCTAGACTAAACCATGGCCAAGCCTTAG-3′; FS2, 5′-GGTCTAGAAACCATGGCCAAGGCCTTTGTCTC-3′; antisense for all mutants 5′-GGTTAGCCCTCCCACACATAACTGCA-3′) or restriction digest with EagI followed by fill-in reaction with Klenow fragment of DNA polymerase (FS1). All versions of *bsd* were sequenced by the dideoxy method to confirm the introduced mutation. The ALV-based vector, based on the pRCAS series, is replication-competent in avian cells since it contains *gag*, *pol*, and *env* genes and the cassette replaces *src*, but it is replication-defective in mammalian cells that lack the ALV receptor *tva*. The replication-defective MLV vector was based upon the pBABE series, but it has an extended packaging sequence to increase titer.

The self-inactivating (SIN) vector pHIV-SRα-IRES-eGFP-CMV-AflII was constructed in three steps. The *bsd* gene with an early stop codon/*AflII* site was amplified by PCR using wild-type *bsd* as template and oligonucleotide primers 5′-GCCGCTCGAGAAACCATGGCCAAGCCTTTGTCTTAAGAAGAATCC-3′ and 5′-GGAATTCTTAGCCCTCCCACA-3′ and cloned into pCR2.1 (Invitrogen Life Technologies). This was digested with *Xho*I and *Eco*RI to release a ~450 bp fragment, which was subcloned into pCI (Promega), which had been modified with an oligonucleotide linker to add an *Mlu*I site at position 4007. *Mlu*I digest released the 1.4 kb CMV promoter and mutant *bsd* gene, which was then subcloned into *Mlu*I-cleaved pHIV-SRα-IRES-eGFP-X-M, which has a 1.9 kb SRα-IRES-eGFP expression cassette in *nef* and a convenient *Mlu*I site just 5′ of the 400 bp deletion in the 3′ U3.

Cell lines

All cells were maintained in water-jacketed, 5% CO_2 incubators at 37 °C, except as indicated. Adherent human cell lines used in this study were cultured in Dulbecco's modified Eagle's medium with high glucose, 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), penicillin, and streptomycin (complete DMEM). Where indicated, complete DMEM was supplemented with 10 μg/ml blasticidin S (Invitrogen Life Technologies). Stable cell lines with single integrated proviruses were constructed by transducing 293T cells at low MOI with VSV G-pseudotyped HIV vector encoding a mutant *bsd* gene. Three independent clonal cells lines for each HIV vector were isolated by repeated rounds of single colony selection, and vector integrant copy number was determined by Southern blotting of genomic DNA cleaved with *Eco*RI and hybridized using an HIV-specific probe and also by semi-quantitative PCR, using β-actin as a normalization control.

Preparation and titration of vector supernatant

Two methods were used to generate replication-defective pseudotyped particles. Vector supernatants were prepared either by transient transfection of 293T cells by calcium phosphate co-precipitation with equimass amounts of pME-VSV G (encoding VSV G) and the HIV or ALV vector (van Maanen et al., 2003), or clonal cell lines bearing one or two HIV vector integrants were transiently transfected with the envelope plasmid alone. MLV vector supernatant was produced by co-transfection of the 293T cells with the MLV vector, pHIT60 (CMV IE promoter driving MLV *gag-pol*) (Soneoka et al., 1995), and VSV G envelope. Supernatants were harvested 72 h later and inoculated directly onto adherent target cells. eYFP vector titers were determined on 293T cells by end-point dilution and enumerating $eYFP^+$ cells 3 days later by epifluorescence microscopy. The titers for VSV G-pseudotyped HIV were typically 2×10^7 IU/ml for virus produced by transient transfection and 5–

10×10^6 IU/ml for virus produced from stable cell lines. In select experiments, HIV vector was co-transfected with ADA envelope expression plasmid and titered on HOS cells expressing CD4 and CCR5 (GHOST HI 5 cells, obtained from the AIDS Reference and Reagent Repository (Morner et al., 1999)). For both ALV and MLV, target cells were typically at low (10–20%) confluence and actively proliferating. For HIV, target cells were at 50% confluence.

Reversion assay for determination of retrovirus reversion rate

Viral supernatant was used to transduce human 293T, HOS TK^- or HeLa cell lines at an MOI of 1–3. In this range of MOI, reversion rate was linear in that increased MOI resulted in a corresponding increase in cells with reversion phenotype. After 3 days, cells were selected by passage into complete DMEM supplemented with 10 μg/ml blasticidin. Cells were refed after 3–5 days, and colonies were stained using 1% crystal violet in 30% methanol and 10% acetic acid after 9 days. Reversion rate measurements were based on the number of blasticidin-resistant colonies, and reversion rate was calculated as shown in Fig. 1C. eYFP titer was determined separately by end-point titration for each revertant vector. All reversion rate experiments were performed a minimum of two times, with three measurements per vector per target cell line for each experiment. Thus, each reversion rate measurement is based on at least 2×10^7 target cells for each cell line tested, typically resulting in tens or hundreds of clonal revertants, respectively, for the stable or transient producers.

Blasticidin-resistant cell revertants after transduction with the HIV SIN vector were clonally isolated by limiting dilution and expanded. Genomic DNA was extracted using DNAzol (Boehringer Mannheim) and used for PCR amplification of *bsd* in both the 5′ LTR (5′-GTGTACGGTGGGAGGTCTATAT-3′ and 5′-TTAATACCGACGCTCTCGCACCCAT-3′) and 3′ LTR (5′-GTGCCACGTTGTGAGTTGGATAGTTGTG-3′ and 5′-TCCCACACATAACCAGAGGGCAGCAATT-3′). For amplification of the 5′ LTR, genomic DNA (300 ng) was used in a 50 μl reaction with Expand Long Template PCR system (Roche Diagnostics), conditions as follows: 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 51 °C for 30 s, and 68 °C for 90 s, then a final elongation step at 72 °C for 7 min. For amplification of the 3′ LTR, annealing temperature was 59 °C. PCR products were directly sequenced by the automated dideoxy method.

Determination of reversion rate after transfection

Plasmids were liquid amplified in *E. coli* and purified by alkaline lysis followed by two rounds of CsCl equilibrium ultracentrifugation. Plasmid pTP123-TEM-E166Q (Palzkill and Botstein, 1992) encoding the point mutant β-lactamase gene BLA E166Q was used to measure reversions due to calcium phosphate transient transfection. Plasmid (≤1% of input DNA) was recovered from transfected cells after 39 h using a modified Hirt lysate procedure after washing cells extensively with phosphate-buffered saline (Arad, 1998). Ten nanograms of this plasmid or non-transfected plasmid (representing approximately 10^8 copies of pTP123-TEM-E166Q) was transformed into electrocompetent TOP10F *E. coli*, and bacteria were selected on LB plates supplemented with chloramphenicol plus ampicillin or chloramphenicol alone.

With regards to reversions made during stable transfection, purified plasmids were directly transfected into 293T cells using the cationic lipid reagent DOTAP (Goldman et al., 1997). After 1.5 h, cells were refed with complete DMEM, and after an additional 30 h, cells were selected in complete DMEM supplemented with 10 μg/ml blasticidin. Cells were refed every 3–5 days, and colonies were stained and enumerated after 9 days.

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