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A new cell-based assay for measuring the forward mutation rate of HIV-1

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Abstract

Over 20 years into the ever-worsening AIDS pandemic, genetic variation remains the greatest obstacle for treating and preventing HIV-1 infection. Mutation rate assays for HIV-1 have been reported; however, none measure directly the forward mutation rate during replication of the virus in cell culture while still retaining the ability to propagate and further study mutant proviruses. Therefore, the objective of the current study was to develop such a phenotypic cell-based assay for measuring the forward mutation rate of HIV-1. Conventional recombinant DNA techniques and polymerase chain reaction were used to create a replication defective HIV-1 vector, pNL4-3Δ+cass, which is based on the NL4-3 strain and contains the thymidine kinase gene from human herpes virus type 1 as the mutational target. A series of transfection and infection steps were used to introduce the vector into 143B cells, which are negative for thymidine kinase function, and produce vector virus for a single cycle of replication. Viral titers were measured by counting the number of drug resistant colonies on the assay plates, and forward mutation rates were calculated from the viral titers. Mutant proviruses were sequenced to determine the types of genetic alterations that occurred. The average forward mutation rate for HIV-1 was 2.2×10^{-5} mutations/base/cycle. The majority of mutations were base substitutions, including high frequencies of C → U and G → A transitions. Single adenosine insertions were also observed frequently. The new assay is economical and provides a direct measurement of the mutation rate during a single cycle of viral replication. Target cells containing mutant proviruses survive the drug selection process and may be propagated for further analysis. The new assay is novel and has many advantages over previous mutation rate assays and thus will be very useful in future studies on genetic variation of HIV-1.

Keywords: HIV-1; Retroviridae; Mutation rate; Variation; Reverse transcription; Thymidine kinase

1. Introduction

A high degree of genetic variation is associated with HIV-1 during the course of infection. This variation enables the virus to escape the host immune response, use multiple cell surface proteins for viral entry, become more pathogenic, mount resistance to antiretroviral drugs, and prevent effective vaccination.

Early in the AIDS epidemic, measurements of the HIV-1 mutation rate were performed in cell-free studies using artificial templates and purified enzyme and substrates (hereafter referred to as in vitro studies). These in vitro studies predicted the mutation rate for HIV-1 to be on the order of 10^{-4} mutations/base/replication cycle (Bebenek et al., 1989; Preston et al., 1988; Roberts et al., 1988). Other in vitro studies followed these initial reports using various assays to characterize the types of mutations such as base substitutions, insertions, and deletions that are associated with the high RT mutation rate (Bebenek et al., 1993; Ji and Loeb, 1994; Perrino et al., 1989). In vitro systems, however, may not duplicate accurately the physiologic conditions of

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a replicating virus. For example, Varela-Echavarría et al. (1992) were the first to show that retroviral mutation rates are higher in vitro as compared to rates measured during replication of the virus in cells (hereafter referred to as cell-based studies). Other researchers have reported that in vitro systems can generate reverse transcription products that are not found naturally, such as extended minus-strand strong stop DNA (Swanstrom et al., 1981) and non-templated base additions (Chen and Patton, 2001; DeStefano et al., 2000; Patel and Preston, 1994; Peliska and Benkovic, 1992).

In 1995, the first cell-based assay for measuring the mutation rate of HIV-1 was reported (Mansky and Temin, 1995). This assay measures the mutation rate indirectly because it does not detect mutant proviruses in the target cell. The mutational target sequence, the *lacZα* peptide gene, is excised from the provirus and introduced into a bacterial system, where screening and sequencing of mutations takes place. Colonies of target cells are pooled after a single cycle of viral replication, resulting in a sampling type of measurement as opposed to a direct measurement. Because the cells are pooled, there is no way to know whether identical mutants are independent, and the total numbers of viruses screened and mutants detected cannot be determined. This assay can be used to stain target cells directly with X-gal, but the ability to propagate and further study mutant proviruses is lost after the staining process (Mansky et al., 2003; Weiss et al., 2004). The *lacZα* peptide gene is small (280 bp) and the viral titers produced in this assay are low. These two features decrease the frequency by which mutants are detected and increase the number of infections that must be performed. In order to achieve sufficient viral titers, this assay relies on co-cultivation of target cells with initiator cells that have been treated with the mutagenic antibiotic mitomycin C (Bolzan and Bianchi, 2001).

Recently, another cell-based assay was reported to detect unselected mutations across the whole HIV-1 genome (Gao et al., 2004). An *env*-deleted HIV-1 genome, containing a selectable marker, is transfected along with a heterologous *env* construct to produce vector virus for a single cycle of replication. A major problem with this assay is that initiator cells, containing a stably integrated provirus, are not established and characterized. For this reason, mutations and rearrangements resulting from transfection (Butner and Lo, 1986; Heartlein et al., 1988; Murnane et al., 1990) are counted as occurring during the single cycle of replication. Multiple copies of these potentially mutant genomes may be present in the same producer cell, which could result in the co-packaging of different RNA molecules, compounding mutation rates with recombination rates. As noted by the authors, the mutation rate is measured in the absence of viral proteins Vpr and Vpu, which may impact the mutation rate (Mansky, 1996).

For the above reasons, it is clear that a new and improved cell-based assay is needed to measure the mutation rate of HIV-1. Such an assay is described in this report.

2. Materials and methods

2.1. Sources of molecular constructs

The full-length HIV-1 genome that served as the basis for the HIV-1 vector (Fig. 1, top) and the p83-10 plasmid that contains the 3′ half of HIV-1_{NL4-3} were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin (Adachi et al., 1986); p83-10 from Dr. Ronald Desrosiers (Gibbs et al., 1994). The helper plasmids, pCMVΔR8.2 and pMD.G, were generously provided by Dr. Didier Trono, University of Geneva, Switzerland. Plasmid pCMVΔR8.2 encodes structural proteins Gag and Pol, in addition to all of the HIV-1 regulatory and accessory proteins (Naldini et al., 1996). This Gag-Pol helper expresses sequences derived from both the HXB2D (Ratner et al., 1985) and NL4-3 (Adachi et al., 1986) strains of HIV-1 (Naldini et al., 1996). However, the reverse transcriptase portion of pCMVΔR8.2 was derived completely from NL4-3 (accession no. M19921). The pMD.G helper plasmid expresses G glycoprotein of vesicular stomatitis virus (VSV.G) and confers broad tropism via pseudotyping of the HIV-1 vector virus (Naldini et al., 1996). Both of the helper plasmids express genes from heterologous promoters. The pIREShyg2 plasmid was purchased from Clontech, a division of BD Biosciences, Palo Alto, CA. The gene for thymidine kinase (*tk*) was cloned from plasmid pKOS17B2, generously provided by Dr. Donald Coen, Harvard Medical School, Boston, MA.

2.2. Molecular biology

General molecular biology procedures, including restriction enzyme digestion, agarose gel electrophoresis, DNA ligation, and bacterial transformation were performed using standard published procedures (Ausubel et al., 1995; Sambrook et al., 1989). All enzymes were purchased from New England Biolabs Inc., Beverly, MA, unless otherwise noted.

2.3. Construction of the HIV-1 vector, pNL4-3Δ+cass

To create the pNL4-3Δ+cass vector (Fig. 1, top), deletions were made in the *gag*, *pol*, and *env* genes of pNL4-3, and a mutational cassette, encoding the human herpes virus type 1 *tk* and hygromycin phosphotransferase (*hyg*) genes, was inserted at the location of the *env* deletion.

The deletion in *gag-pol* (corresponding to nucleotides 1340–3716 of the original NL4-3 sequence, accession no. M19921) was created by digesting pNL4-3 with *Swa* I and purifying and ligating the 12,503 bp fragment, which contained the plasmid backbone and the *gag-pol*-deleted HIV-1 genome. This intermediate construct was named pNL4-3Δswa.

The deletion in *env* (corresponding to nucleotides 6401–7252 of the original NL4-3 sequence, accession no.

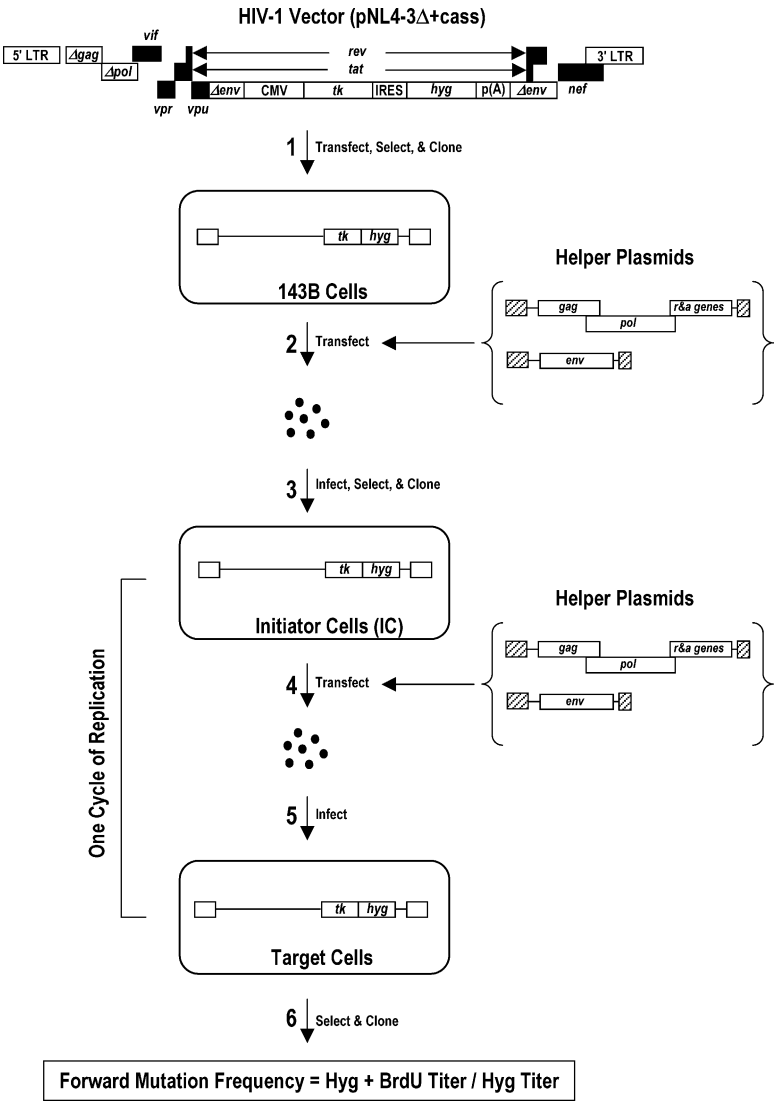

Fig. 1. HIV-1 vector and flow chart for the forward mutation rate assay. The HIV-1 vector, pNL4-3Δ+cass, is shown at the top. *Step* 1: The vector is stably transfected into naïve 143B cells. The cells are placed under selection with hygromycin B (Hyg) and cloned. *Step* 2: The 143B cell clones containing an integrated HIV-1 vector are transiently transfected with HIV-1 helper plasmids to produce vector virus. *Step* 3: Vector virus is used to infect fresh 143B cells. The cells are placed under selection with Hyg and cloned. *Step* 4: The 143B cell clones containing an integrated HIV-1 vector are transiently transfected with HIV-1 helper plasmids to produce vector virus (same as step 2 except cells are infected, not transfected). *Step* 5: Vector virus is used to infect fresh 143B cells. *Step* 6: Parallel infections are placed under selection with Hyg alone and Hyg plus BrdU. Viral titers are calculated by counting the number of drug resistant colonies. The forward mutation frequency is calculated as shown. Cells are cloned for further analysis.

M19921) was created in p83-10 (6253 bp), which contains the 3′ half of the HIV-1 genome. Working with the 3′ half of the HIV-1 genome allowed the use of restriction enzymes that were not unique in the full-length pNL4-3 plasmid. Plasmid p83-10 was digested with *Nde* I and *Nhe* I; the 5402 bp fragment, which contained the plasmid backbone and the *env*-deleted 3′ half of HIV-1, was purified and ligated with complementary oligonucleotides that inserted a multiple cloning site (MCS), creating intermediate construct p83-10MCS. The sequences of the oligonucleotides are as follows: MCS1 5′-TAT GGG CGC GCC ACG CGT CCC GGG G-3′ and MCS2 5′-GCT AGC CCC GGG ACG CGT GGC GCG CCC ATA TG-3′. Restriction enzymes *Sal* I and *Xho* I were used to isolate a fragment from p83-10MCS that contained the *env* deletion. This fragment was then used to replace the corresponding fragment in pNL4-3Δswa that contained the intact *env* sequence. These cloning steps created intermediate construct pNL4-3Δ.

The mutational cassette was created by using pIREShyg2 from Clontech. The *tk* sequence was amplified by polymerase chain reaction (PCR) from plasmid pKOS17B2 using a set of primers containing *Bsr*G I and *Bsi*W I sites at the 5′ and 3′ ends, respectively; *tk* was inserted into the MCS of pIREShyg2 using these sites after intermediary cloning in a TOPO TA vector (Invitrogen, Carlsbad, CA), creating intermediate construct pTKIREShyg. The sequence

encompassing the CMV promoter, *tk*, IRES, and *hyg* was then amplified from pTKIREShyg using a set of primers containing *Mlu* I and *Nhe* I sites at the 5′ and 3′ ends, respectively. Following intermediary cloning in a TOPO TA vector, these restriction sites were used to insert the CMV-*tk*-IRES-*hyg* mutational cassette into pNL4-3Δ to create the final vector, pNL4-3Δ+cass.

2.4. Polymerase chain reaction

For cloning into the mutational cassette, the *tk* gene was amplified using *Taq* DNA polymerase in a 100 μl reaction, according to the manufacturer's protocol (Invitrogen). Twenty-five nanograms of plasmid pKOS17B2 was used in the reaction, along with 20 pmol of each of the following primers: TK1 5′-GCT ATG TAC AGC CAC CAT GCC CAC GCT ACT GCG GGT-3′ and TK2 5′-GTA CCG TAC GTC AGT TAG CCT CCC CCA TCT-3′. The TK1 primer encodes a *Bsr*G I site near the 5′ end followed by a Kozak sequence. The TK2 primer encodes the 3′ end of *tk*, followed by a *Bsi*W I site. Twenty-five cycles of the following program were performed: 15 s at 94 °C, 15 s at 60 °C, and 1 min at 72 °C. *Taq* DNA polymerase was added to the reaction after an initial heating step at 94 °C for 5 min, and a final extension step was performed at 72 °C for 10 min.

For cloning into the HIV-1 vector, the CMV-*tk*-IRES-*hyg* mutational cassette was amplified using *PfuTurbo*® polymerase (Stratagene, La Jolla, CA) in a 50 μl reaction, according to the manufacturer's protocol. Twenty-five nanograms of plasmid pTKIREShyg were used in the reaction, along with 10 pmol of each of the following primers: CMVCAS 1 5′-GAT CAC GCG TCG CGT TAC ATA ACT TAC GGT A-3′ and CMVCAS 2 5′-GTC AGC TAG CTT CCT TTG CCC TCG GAC GAG-3′. CMVCAS 1 encodes a *Mlu* I restriction enzyme site prior to the CMV promoter sequence, and CMVCAS 2 encodes the 3′ end of *hyg*, followed by a *Nhe* I restriction enzyme site. Twenty-five cycles of the following program were performed: 1 min at 95 °C, 1 min at 58 °C, and 4 min at 72 °C. An initial heating step at 95 °C for 3 min and a final extension step at 72 °C for 10 min were performed.

For verification of the initiator cells, the CMV-*tk* region (1600 bp) was amplified from genomic DNA (from step 3, Fig. 1) using *Taq* DNA polymerase (Invitrogen) in a 100 μl reaction, according to the manufacturer's protocol. Two hundred nanograms of genomic DNA were used in the reaction, along with 20 pmol of primers CMVCAS 1 and TK2. Twenty-five cycles of the following program were performed: 30 s at 94 °C, 30 s at 58 °C, and 2 min at 72 °C. *Taq* DNA polymerase was added to the reaction after an initial heating step at 94 °C for 5 min, and a final extension step was performed at 72 °C for 10 min.

For sequencing of the final mutants, the *tk* gene was amplified from genomic DNA isolated from the Target Cells (final step, Fig. 1) using Accuprime™ *Taq* DNA polymerase (Invitrogen) in a 10 μl reaction, according to the manufacturer's protocol. Three hundred nanograms of genomic DNA were used in the reaction, along with 20 pmol of each of the following primers: TKA1 5′-TCA CTA TAG GGA GAC CCA AGC-3′ and TKA2 5′-CCC TCG CAG ACA GCG AAT TAA-3′. Twenty-five cycles of the following program were performed: 45 s at 94 °C, 45 s at 58 °C, and 1 min at 72 °C. An initial heating step at 94 °C for 2 min and a final extension step at 72 °C for 10 min were performed. Primers and excess nucleotides were inactivated by adding 1.9 U Exonuclease I and 0.38 U Shrimp Alkaline Phosphatase (Amersham Biosciences, Piscataway, NJ) to the PCR reaction. The final reaction volume was adjusted to 12.5 μl using ultrapure water. The reaction was incubated at 37 °C for 30 min, and enzymes were heat inactivated at 80 °C for 20 min.

All PCR reactions were performed on a Peltier Thermal Cycler 200 (MJ Research, Reno, NV) with the exception of the final amplification of *tk* from the Target Cells, which was performed on a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA).

2.5. Genomic DNA isolation

The DNeasy Tissue Kit (Qiagen) was used to isolate the genomic DNA from cultured cells per manufacturer's protocol.

2.6. Cell culture

The 143B cell line was purchased from the American Type Culture Collection, Manassas, VA. This cell line is a derivative of the human osteosarcoma cell line, HOS, and is negative for thymidine kinase (TK) function (Bacchetti and Graham, 1977). The cells were maintained in minimal essential medium (MEM) with Earl's BSS (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen). Cells were passaged twice per week and were grown at 37 °C and 5% CO_2.

For selection of drug resistant colonies, cells were cultured for 2 weeks in medium containing the appropriate agent. The final concentration for each agent was determined empirically using 143B cells. The antibiotic hygromycin B (Invitrogen) was used at 0.27 mg/ml. The nucleoside analog bromodeoxyuridine (BrdU; Sigma–Aldrich Co., St. Louis, MO) was used at 0.12 mg/ml. The final concentrations of hypoxanthine, aminopterin, and thymine (HAT; Sigma–Aldrich) were 5×10^{-5} M, 2×10^{-7} M, and 8×10^{-6} M, respectively.

Cells were cloned by choosing well-isolated colonies from plates receiving the highest dilutions of DNA or virus (steps 1 and 3, respectively, Fig. 1) or plates in the linear range of the titration (step 6, Fig. 1). A sterile 5 mm Bel-Art cloning disk (Fisher Scientific, Pittsburgh, PA) treated with trypsin–EDTA (0.25%, Invitrogen) was placed onto the colony, incubated for 3.5 min, and transferred into the well of a 24-well plate containing medium.

2.7. Transfections

Stable and transient transfections (steps 1 and 2, Fig. 1) were performed by the dimethyl sulfoxide (DMSO)/polybrene method (Kawai and Nishizawa, 1984). For step 1, serial five-fold dilutions of DNA, starting with 5 μg, were transfected onto 143B cells plated at 2×10^5 cells/60 mm dish, 24 h pre-transfection. For step 2, 9 μg of pCMVΔR8.2 and 3 μg of pMD.G were transfected onto cell clones (isolated from step 1) plated at 2×10^5 cells/60 mm dish, 24 h pre-transfection. After 6 h of incubation with DNA, cells were shocked with 25% DMSO for 3.5 min and were placed in appropriate selective media at 24 h post-transfection.

Transient transfection (step 4, Fig. 1) was performed using LipofectAMINE™ 2000 per manufacturer's protocol (Invitrogen). Three micrograms of pCMVΔR8.2 and 1 μg of pMD.G were transfected onto ICs (isolated from step 3) plated at 2×10^5 cells/60 mm dish, 24 h pre-transfection. The medium was replaced 24 h after transfection.

2.8. Infections

Virus stock was harvested at 62 h post-transfection by centrifugation of cell supernatant fluid at $1430 \times g$ for 10 min at room temperature. The 62 h time point was chosen empirically based on transduction efficiencies with the HIV-1-based vector pHR′CMV-GFP, which encodes the gene for green fluorescent protein, and helper plasmids pCMVΔR8.2 and pMD.G. The pHR′CMV-GFP vector was generously provided by Dr. Didier Trono (Blomer et al., 1997).

Infections were carried out with fresh virus stock and polybrene (8 μg/ml) in a final volume of 2 ml with cells plated 24 h pre-infection at a density of 2×10^5 cells/60 mm dish. Cells were incubated with virus for 3 h, at which time the viral supernatant was replaced with fresh medium. At 24 h post-infection, the medium was replaced with fresh medium containing the appropriate selective agent. Selective media was replaced twice a week for 2 weeks. Serial 10-fold dilutions of virus were used to determine viral titers, which were measured by counting the number of drug-resistant colonies in the linear range of the titration. Viral titers are reported as the number of colony forming units per milliliter (cfu/ml).

2.9. Southern blotting

Southern blotting was carried out using standard published procedures (Ausubel et al., 1995; Sambrook et al., 1989). Briefly, 15 μg of genomic DNA was digested with *Eco*R I and separated in a 1% agarose gel. DNA was transferred via capillary action onto a Hybond-N^+ membrane (Amersham). The membrane was hybridized with a radiolabeled fragment complementary to the 3′ half of the HIV-1 genome (a 422 bp *Bam*H I to *Xho* I fragment, specific for the Rev-responsive element and *tat* gene). Results were visualized by autoradiography using Kodak X-OMAT AR2 film (Fisher Scientific).

2.10. Calculation of mutations

Forward mutation frequencies and rates were calculated using simple mathematical formulas; the total number of mutants was divided by the total number of viruses (Hyg + BrdU titer/Hyg titer; Fig. 1 and Table 1). The forward mutation rate was calculated by dividing the forward mutation frequency by the number of bases in *tk* (996 bases).

2.11. Sequencing

Purified PCR products were reduced to a concentration of 50 ng/μl by centrifugation under vacuum, and 200 ng of each sample was sent to Lark Technologies Inc., Houston, TX for sequencing. The following two primers were used: FORWARD-TK 5′-TAC CTT ATG GGC AGC ATG ACC-3′ and REVERSE-TK 5′-CTG CAG ATA CCG CAC CGT ATT-3′. The sequencing results were aligned and analyzed for non-synonymous base substitutions, insertions, and deletions using MacVector™ software version 6.5.3 (Accelrys, San Diego, CA).

3. Results

3.1. Successful construction of the HIV-1 vector

A replication-defective HIV-1 vector was constructed based on the NL4-3 strain of HIV-1 (Fig. 1, top). The vector contains all of the *cis*-acting sequences necessary for replication, plus a complete set of intact regulatory and accessory genes for HIV-1. Deletions were made in the *gag*, *pol*, and *env* genes to render the vector replication defective, as described in Section 2. Structural gene products, Gag, Pol, and Env, were supplied *in trans* by helper plasmids, as described in Section 2. In place of *env*, a mutational cassette was inserted that contained the human CMV promoter driving expression of *tk*, which provided the mutational target sequence for the assay. A functional *tk* gene renders cells resistant to medium containing HAT and susceptible to medium containing BrdU. An IRES sequence following *tk* allowed for expression of *hyg*, which provided a selectable marker via resistance to the antibiotic hygromycin B.

The final HIV-1 vector, named pNL4-3Δ+cass, was verified by restriction enzyme analysis, sequencing, and phenotypic testing in 143B cells. All plasmid constructs leading up to the final vector were digested with restriction enzymes to verify the cloning process. The final vector was digested with six restriction enzymes (*Afl* II, *Hin*d III, *Kpn* I, *Pst* I, *Sac* I, and *Xmn* I) and showed only bands of the predicted sizes, indicating that no major rearrangements, insertions, or deletions had occurred during the cloning process (data not shown). Sequencing revealed that the *tk* gene had no mutational defects. The final vector was transfected into naïve 143B cells, which are negative for TK function. The transfected cells were resistant to medium containing HAT and susceptible to

Table 1
Mutation data for individual initiator clones (IC)

	IC2	IC3	IC4	Total
Number of mutants detected	142	53	154	349
Number of viruses screened	5265	3375	7290	15,930
Forward mutation frequency[a] ($\times 10^{-2}$ mutations/cycle)	2.7	1.6	2.1	2.2
Forward mutation rate[b] ($\times 10^{-5}$ mutations/base/cycle)	2.7	1.6	2.1	2.2

[a] Forward mutation frequency = number of mutants detected/number of viruses screened.
[b] Forward mutation rate = forward mutation frequency/number of bases in *tk* gene (996 bases).

medium containing BrdU, indicating that *tk* was functional. The transfected cells were also resistant to medium containing hygromycin B, indicating that *hyg* was functional. Finally, transfection of naïve 143B cells with pNL4-3Δ+cass plus helper plasmids yielded virus, as indicated by successful transduction of the *tk* and *hyg* genes into fresh 143B cells (data not shown).

3.2. Successful construction of the forward mutation rate assay

The forward mutation rate assay is outlined in Fig. 1. Serial dilutions of HIV-1 vector DNA were transfected into 143B cells, which were placed in medium containing hygromycin B to select for resistant cells. Hygromycin B-resistant cells were cloned by picking well-isolated colonies from plates receiving the highest dilutions of DNA (step 1, Fig. 1). In order to remove mutations that may have occurred during transfection (Butner and Lo, 1986; Heartlein et al., 1988; Murnane et al., 1990) and to improve the probability for isolating clones that contained only one provirus vector per cell, a subsequent infection step was performed. The 143B clones containing the HIV-1 vector were transiently transfected with HIV-1 helper plasmids for transient production of vector virus (step 2, Fig. 1). Serial dilutions of this virus were then used to infect fresh 143B cells, which were placed in medium containing hygromycin B to select for resistant cells. Hygromycin B-resistant cells were cloned by picking well-isolated colonies from plates receiving the highest dilutions of virus (step 3, Fig. 1). The resulting cell clones were called the initiator cells (ICs) and served as the basis for the single cycle of replication assay.

The ICs generated in step 3, Fig. 1, were characterized rigorously by molecular and phenotypic analyses to ensure a successful and accurate forward mutation rate assay. Polymerase chain reaction was performed with the genomic DNA of each IC to detect the presence of the provirus vector using primers specific for the sequence that spans CMV through *tk*. The same PCR was carried out with the genomic DNA of naïve 143B cells as a negative control. The predicted PCR product of 1600 bp in length was detected in all ICs and not in the naïve 143B cells (Fig. 2A). A Southern blot analysis was performed on the genomic DNA of each IC to ensure that each IC contained only one HIV-1 vector sequence. The cellular DNA was digested with *Eco*R I, which generated a 3′ fragment of the viral genome with adjacent cellular DNA. A radiolabeled probe specific for this 3′ region of the virus was used to detect a single band in each IC (Fig. 2B). The bands for each IC were different sizes, confirming that each IC was an independent cell clone (Fig. 2B). Each IC was challenged for a 2-week period of selection with medium containing BrdU. The BrdU selection resulted in the complete death of ICs 2–4, indicating functional *tk* expression in these cell clones. These ICs were chosen for the assay and their *tk* genes were sequenced and found to be identical to the *tk* sequence from the original pNL4-3Δ+cass vector. Transient transfection of helper plasmids into the ICs produced

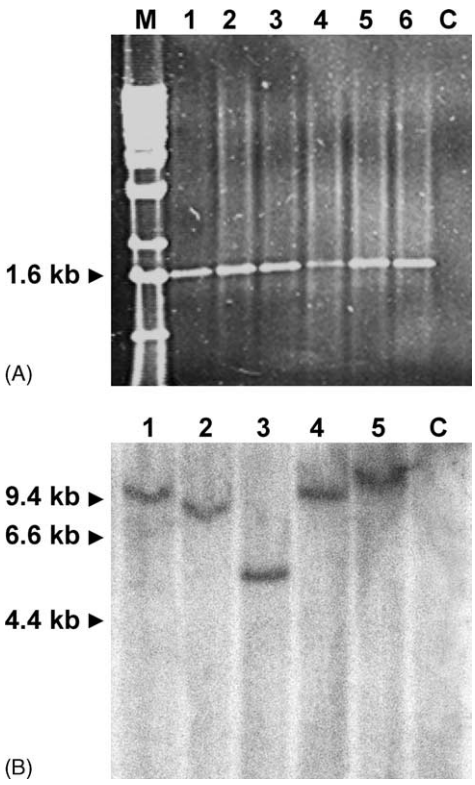

Fig. 2. Analysis of the initiator clones (ICs). (A) Agarose gel showing PCR results performed on genomic DNA of ICs 1–6 and genomic DNA of 143B cells. The 1.6 kb band is specific for the HIV-1 vector sequence spanning CMV through *tk*. Lane M is a 1 kb marker. The predicted 1.6 kb PCR product was detected in all ICs but not in 143B genomic DNA, as expected. (B) Southern blot analysis of ICs 1–5. Lane C represents the 143B genomic DNA negative control. A high molecular weight marker was included on the gel with sizes listed on the left side. Only one band was detected for each IC, indicating one provirus per cell. In addition, the bands for each IC were different sizes, indicating that the ICs were independent cell clones.

viral titers in the range of 10^2–10^4 cfu/ml, which provided a sufficient number of mutants from a practical number of infections.

Once the ICs were established, they were transiently transfected with the HIV-1 helper plasmids to generate virus for the single cycle of replication (step 4, Fig. 1). HIV-1 vector virus was harvested from the ICs and used to infect fresh 143B cells (step 5, Fig. 1). Steps 4 and 5 constitute the single cycle of replication (from provirus to provirus, IC to target cell). Finally, parallel sets of infections were placed under two types of drug selection: hygromycin B plus BrdU (Hyg + BrdU) and hygromycin B alone (Hyg) (step 6, Fig. 1). Mock infections, performed in parallel as a negative control, showed no colony formation under Hyg + BrdU selection. Viral titers were determined by counting the number of drug-resistant colonies in the linear range of the titration. The Hyg + BrdU titer revealed the number of mutants, while the Hyg titer revealed the number of viruses screened. The forward mutation frequency was calculated by dividing the total number of mutants detected by the total number of viruses screened (final step, Fig. 1 and Table 1).

3.3. Forward mutation rate of HIV-1

Forward mutation rates were calculated from 27 independent infections initiated from each of the three ICs (81 total infections; Figs. 1 and 3, Table 1). The rate was calculated by dividing the forward mutation frequency (Table 1) by the size of the *tk* sequence, which was 996 bases from start to stop codon. The average rate for HIV-1 was 2.2×10^{-5} mutations/base/cycle of replication (Table 1).

3.4. Mutation distribution

Twenty-seven of 349 mutants were randomly selected for sequencing in order to determine the types of mutations that resulted in loss of function for TK (Table 2). The majority of mutations (65%) were base substitutions with a preponderance of C → U (21%) and G → A (14%) mutations. One G → A and one C → A hypermutant were observed, as defined by nonconsecutive but repetitive substitution of adenosines. In this case, the hypermutation was not extensive; only two or three substitutions were observed in each hypermutant. Insertions were observed approximately twice as often as deletions (23% and 12%, respectively). Among the insertions, a predominance of single adenosine additions was observed.

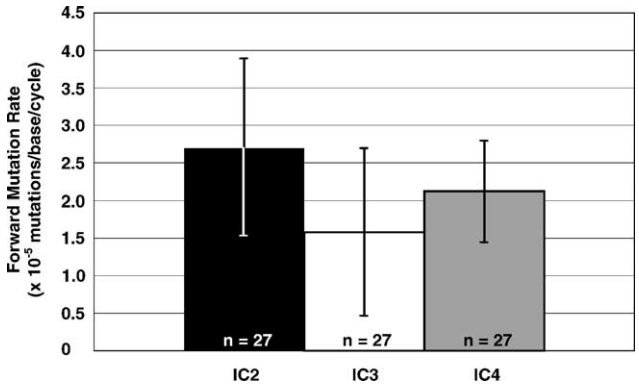

Fig. 3. Forward mutation rate of HIV-1. The forward mutation rate for HIV-1 was measured from three independent initiator cell clones (ICs). The number of infections is listed inside the column for each IC. The error bars represent 1 standard deviation from the mean.

4. Discussion

The successful development of a unique HIV-1 vector and cell-based assay for measuring the forward mutation rate of HIV-1 is described in this report. Using this assay, it was determined that the average forward mutation rate for HIV-1 was 2.2×10^{-5} mutations/base/cycle, which translates to a *tk*

Table 2
Types of mutations observed

Type of mutation[a]	No. of mutations observed (%)	
	New assay	Previous assay[b]
Substitutions		
C → U	9 (21)	6 (15)
*G → A	6 (14)	13 (33)
U → A	4 (9)	1 (3)
C → A	2 (5)	0
A → G	1 (2)	0
G → U	1 (2)	0
U → G	0	1 (3)
U → C	0	2 (5)
G → A hypermutant[c]	1 [+2 bases] (5)	2 [+2 bases] (10)
C → A hypermutant[c]	1 [+3 bases] (7)	0
Total	28 (65)	27 (68)
Insertions		
+A	6 (14)	1 (3)
+G	1 (2)	0
+U	3 (7)	1 (3)
*Total	10 (23)	2 (5)
Deletions		
Δ33	0	1 (3)
Δ21	0	1 (3)
Δ11	1 (2)	0
Δ10	1 (2)	0
Δ8	0	1 (3)
Δ5	1 (2)	0
Δ2	1 (2)	0
*Δ1	1 (2)	7 (18)
Total	5 (12)	10 (25)
Deletion with insertion		
Δ4, +15	0	1 (3)
Total mutations	43 (100)	40 (100)
Total mutants screened	27 [26,892 bases]	38 [10,640 bases]

[a] The asterisk indicates statistically significant differences between the new and previous assays, as determined using a one-tailed Fisher's exact test.

[b] Mansky and Temin (1995).

[c] Each mutation counted separately toward the total number of mutations.

inactivation rate of 2.2% per kbp per cycle and extrapolates to one mutation for every 4.5 genomes per cycle. The new assay has many advantages and improvements over previous assays (Gao et al., 2004; Mansky and Temin, 1995). It is easier, cheaper, and faster to perform. It provides a direct measurement of the forward mutation rate, a known number of mutants detected, and a known number of viruses screened. The new assay incorporates a larger target sequence that yields higher forward mutation frequencies. Selective agents are added after the single cycle of replication, thus avoiding any potential mutagenic or undesired effects. Mutants survive the selection process and can be further studied. Lastly, the new assay has the potential for mechanization.

The mutation rate of 2.2×10^{-5} mutations/base/cycle is a conservative measurement because the entire *tk* sequence (996 bp) was used in the calculation, and some mutant proviruses had more than one mutation. Although every base in the *tk* gene is subject to a potential deletion or insertion, not every base is subject to a non-synonymous base change. For *tk*, the probability of whether a base substitution will result in an amino acid replacement is 74%. That is, if random mutations occurred throughout the gene, 737 out of the 996 base changes would result in an amino acid change, but not all amino acid substitutions would result in loss of function for TK. Amino acid substitutions that do not knock out TK function would be susceptible to selection by BudR and would not be detected in the assay. There are no reports on saturation mutagenesis of *tk*. Therefore, a comprehensive catalog of inactivating *tk* mutations is not available. If the rate in this study had been calculated using 737 instead of 996 bases for *tk*, then the rate would be 3.0×10^{-5} mutations/base/cycle. The decision to include all 996 bases in the calculation considers all types of mutations: deletions, insertions, and substitutions. Mutant proviruses were counted as one in the rate calculation, even though some mutants contained multiple genetic alterations. Table 2 shows that there were 43 mutations among 27 mutants screened. The decision to count the mutants as one considers the possibility that each mutation, on its own, may not have inactivated *tk*. If all of the mutations were inactivating mutations and the rate was adjusted to account for these multiple mutations, then the rate would be 3.5×10^{-5} mutations/base/cycle. Thus, as stated, the forward mutation rate reported for this study is a conservative one and could be as much as 1.6-fold higher.

The overall forward mutation rate reported in the current study was slightly higher and the *tk* inactivation rate was slightly lower in comparison to a cell-based study on Moloney murine leukemia virus (MoMLV), in which *tk* was part of a larger mutational target sequence (Parthasarathi et al., 1995). In the MoMLV study, an overall forward mutation rate of 1.6×10^{-5} mutations/base/cycle and a *tk* inactivation rate of 3.0% per kbp were reported, based on calculations similar to those in the current study (the gene inactivation frequency was divided by the total number of bases in the target sequence). One striking difference between the two studies is the types of mutations observed. The majority of mutations in the current study were base substitutions, while the majority of mutations in the MoMLV study were labeled as "gross rearrangements" (deletions, deletions with insertions, duplications, and complex hypermutations). The majority of gross rearrangements in the MoMLV study were found at locations corresponding to open regions of RNA: hairpin loops, internal loops, and bulges. This underscores the importance of sequence context in interpreting patterns of mutations. The different patterns of mutation observed between MoMLV (a gammaretrovirus) and HIV-1 (a lentivirus) also emphasizes the caution that must be exercised when extrapolating results from one retroviral genus to another.

Table 3
Comparison of mutation data between assays

	New assay	Previous assay[a]
Number of mutants detected	349	38[b]
Number of viruses screened	15,930	8678[c]
Forward mutation frequency[d] ($\times 10^{-2}$ mutations/cycle)	2.2	0.44
Forward mutation rate ($\times 10^{-5}$ mutations/base/cycle)	2.2[e]	3.5

[a] Mansky and Temin (1995).
[b] Interpreted from the number of mutant bacterial colonies.
[c] Interpreted from the total number of bacterial colonies.
[d] Forward mutation frequency = number of mutants detected/number of viruses screened.
[e] Forward mutation rate = forward mutation frequency/number of bases in target sequence.

The average forward mutation rate reported in this study is 1.6-fold lower than the rate reported for the previous study, which was 3.5×10^{-5} mutations/base/cycle (Table 3; Mansky and Temin, 1995). This previous rate was based on a lower frequency of forward mutation detection (Table 3), which may be due, at least in part, to the smaller mutational target sequence that was used. The previous assay used the *lacZα* peptide sequence, which is 3.6 times smaller than the *tk* sequence used in the current study. The number of viruses screened cannot be directly compared because this number is unknown for this previous assay. A total number of 15,930 viruses were screened in the current study, while a total number of 8678 bacterial colonies were screened in the previous assay.

Differences were found in the types of forward mutations observed in the new assay versus this previous assay (Table 2; Mansky and Temin, 1995). In both assays, the majority of forward mutations were base substitutions. However, there were significantly less G → A transitions observed in the new assay as compared to the previous assay ($p = 0.04$). There were significantly more insertions observed in the new assay as compared to the previous assay ($p = 0.02$). There were significantly less single nucleotide deletions observed in the new assay as compared to the previous assay ($p = 0.02$). Lastly, there were significantly less transition mutations, and conversely more transversion mutations, observed in the new assay as compared to the previous assay ($p = 0.01$). Frequencies were used in a one-tailed Fisher's exact test to determine

the statistical significance with an alpha level of 0.05. For some categories of mutants, the numbers were too small for statistical analyses. These differences in the types of mutations could be explained by procedural differences between the two assays or sequence context of the target genes.

The phenomenon of G → A hypermutation in HIV-1 genomes, as defined by nonconsecutive but repetitive substitution of adenosines, has been well documented. The occurrence of these mutations in HIV-1, predominantly in the context of GpA and GpG dinucleotides, is caused by nucleotide pool imbalances or by deamination of cytidine by certain members of the APOBEC family that are resistant to Vif (Bishop et al., 2004; Lecossier et al., 2003; Liddament et al., 2004; Mangeat et al., 2003; Martinez et al., 1995; Vartanian et al., 1991, 1994; Zhang et al., 2003). The G → A hypermutations observed in both assays (Table 2) were not extensive. However, as mentioned above, there was a significant difference in the total number of G → A substitutions observed between these studies. There was also a significant difference in the fraction of G → A substitutions occurring within GpA and GpG dinucleotide contexts; in the current study, only 3 of 8 (38%) G → A substitutions occurred in this context, while in the previous study, 16 of 17 (89%) G → A substitutions occurred in this context ($p = 0.006$, one tailed Fisher's exact test; Mansky and Temin, 1995). The higher number of G → A substitutions observed in the previous study could be due to nucleotide pool imbalances caused by mitomycin C (Das et al., 1983) or due to differential expression of APOBEC proteins in the cells (COS-1 and HeLa in the previous study versus 143B in the present study).

The HIV-1 mutation rate of 5.4×10^{-5} mutations/base/cycle reported by Gao et al. (2004) is 2.5-fold higher than the average rate reported in the current study and 1.5-fold higher than the average rate reported by Mansky and Temin (1995). Since this assay did not select for mutations or establish initiator cells, it is difficult to make comparisons with this study.

In conclusion, the new assay described in this report will be useful for studying HIV-1 mutation rate with respect to development of drugs, vaccines, and personalized medicine. This assay may provide a novel phenotypic test that can measure the rate at which HIV-1 is mutating at a given time in an infected individual. Thus, it could predict the future pattern of mutation and could assist in making treatment decisions, especially for newly infected people and those who are changing drugs due to treatment failure.

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References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59, 284–291.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), 1995. Current Protocols in Molecular Biology. John Wiley & Sons Inc., USA.

Bacchetti, S., Graham, F.L., 1977. Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA. Proc. Natl. Acad. Sci. U.S.A. 74, 1590–1594.

Bebenek, K., Abbotts, J., Roberts, J.D., Wilson, S.H., Kunkel, T.A., 1989. Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase. J. Biol. Chem. 264, 16948–16956.

Bebenek, K., Abbotts, J., Wilson, S.H., Kunkel, T.A., 1993. Error-prone polymerization by HIV-1 reverse transcriptase. Contribution of template-primer misalignment, miscoding, and termination probability to mutational hot spots. J. Biol. Chem. 268, 10324–10334.

Bishop, K.N., Holmes, R.K., Sheehy, A.M., Davidson, N.O., Cho, S.J., Malim, M.H., 2004. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Curr. Biol. 14, 1392–1396.

Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I.M., Gage, F.H., 1997. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. J. Virol. 71, 6641–6649.

Bolzan, A.D., Bianchi, M.S., 2001. Genotoxicity of streptonigrin: a review. Mutat. Res. 488, 25–37.

Butner, K.A., Lo, C.W., 1986. High frequency DNA rearrangements associated with mouse centromeric satellite DNA. J. Mol. Biol. 187, 547–556.

Chen, D., Patton, J.T., 2001. Reverse transcriptase adds nontemplated nucleotides to cDNAs during 5′-RACE and primer extension. Biotechniques 30 (574–580), 582.

Das, S.K., Benditt, E.P., Loeb, L.A., 1983. Rapid changes in deoxynucleoside triphosphate pools in mammalian cells treated with mutagens. Biochem. Biophys. Res. Commun. 114, 458–464.

DeStefano, J.J., Raja, A., Cristofaro, J.V., 2000. In vitro strand transfer from broken RNAs results in mismatch but not frameshift mutations. Virology 276, 7–15.

Gao, F., Chen, Y., Levy, D.N., Conway, J.A., Kepler, T.B., Hui, H., 2004. Unselected mutations in the human immunodeficiency virus type 1 genome are mostly nonsynonymous and often deleterious. J. Virol. 78, 2426–2433.

Gibbs, J.S., Regier, D.A., Desrosiers, R.C., 1994. Construction and in vitro properties of HIV-1 mutants with deletions in the "nonessential" genes. AIDS Res. Hum. Retrov. 10, 343–350.

Heartlein, M.W., Knoll, J.H., Latt, S.A., 1988. Chromosome instability associated with human alphoid DNA transfected into the Chinese hamster genome. Mol. Cell. Biol. 8, 3611–3618.

Ji, J., Loeb, L.A., 1994. Fidelity of HIV-1 reverse transcriptase copying a hypervariable region of the HIV-1 env gene. Virology 199, 323–330.

Kawai, S., Nishizawa, M., 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. 4, 1172–1174.

Lecossier, D., Bouchonnet, F., Clavel, F., Hance, A.J., 2003. Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science 300, 1112.

Liddament, M.T., Brown, W.L., Schumacher, A.J., Harris, R.S., 2004. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. Curr. Biol. 14, 1385–1391.

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., Trono, D., 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature 424, 99–103.

Mansky, L.M., 1996. The mutation rate of human immunodeficiency virus type 1 is influenced by the *vpr* gene. Virology 222, 391–400.

Mansky, L.M., Le Rouzic, E., Benichou, S., Gajary, L.C., 2003. Influence of reverse transcriptase variants, drugs, and Vpr on human immunodeficiency virus type 1 mutant frequencies. J. Virol. 77, 2071–2080.

Mansky, L.M., Temin, H.M., 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J. Virol. 69, 5087–5094.

Martinez, M.A., Sala, M., Vartanian, J.P., Wain-Hobson, S., 1995. Reverse transcriptase and substrate dependence of the RNA hypermutagenesis reaction. Nucleic Acids Res. 23, 2573–2578.

Murnane, J.P., Yezzi, M.J., Young, B.R., 1990. Recombination events during integration of transfected DNA into normal human cells. Nucleic Acids Res. 18, 2733–2738.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D., 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263–267.

Parthasarathi, S., Varela-Echavarria, A., Ron, Y., Preston, B.D., Dougherty, J.P., 1995. Genetic rearrangements occurring during a single cycle of murine leukemia virus vector replication: characterization and implications. J. Virol. 69, 7991–8000.

Patel, P.H., Preston, B.D., 1994. Marked infidelity of human immunodeficiency virus type 1 reverse transcriptase at RNA and DNA template ends. Proc. Natl. Acad. Sci. U.S.A. 91, 549–553.

Peliska, J.A., Benkovic, S.J., 1992. Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. Science 258, 1112–1118.

Perrino, F.W., Preston, B.D., Sandell, L.L., Loeb, L.A., 1989. Extension of mismatched 3′ termini of DNA is a major determinant of the infidelity of human immunodeficiency virus type 1 reverse transcriptase. Proc. Natl. Acad. Sci. U.S.A. 86, 8343–8347.

Preston, B.D., Poiesz, B.J., Loeb, L.A., 1988. Fidelity of HIV-1 reverse transcriptase. Science 242, 1168–1171.

Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway Jr., S.R., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., Wong-Staal, F., 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313, 277–284.

Roberts, J.D., Bebenek, K., Kunkel, T.A., 1988. The accuracy of reverse transcriptase from HIV-1. Science 242, 1171–1173.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Swanstrom, R., Varmus, H.E., Bishop, J.M., 1981. The terminal redundancy of the retrovirus genome facilitates chain elongation by reverse transcriptase. J. Biol. Chem. 256, 1115–1121.

Varela-Echavarría, A., Garvey, N., Preston, B.D., Dougherty, J.P., 1992. Comparison of Moloney murine leukemia virus mutation rate with the fidelity of its reverse transcriptase in vitro. J. Biol. Chem. 267, 24681–24688.

Vartanian, J.P., Meyerhans, A., Asjo, B., Wain-Hobson, S., 1991. Selection, recombination, and G → A hypermutation of human immunodeficiency virus type 1 genomes. J. Virol. 65, 1779–1788.

Vartanian, J.P., Meyerhans, A., Sala, M., Wain-Hobson, S., 1994. G → A hypermutation of the human immunodeficiency virus type 1 genome: evidence for dCTP pool imbalance during reverse transcription. Proc. Natl. Acad. Sci. U.S.A. 91, 3092–3096.

Weiss, K.K., Chen, R., Skasko, M., Reynolds, H.M., Lee, K., Bambara, R.A., Mansky, L.M., Kim, B., 2004. A role for dNTP binding of human immunodeficiency virus type 1 reverse transcriptase in viral mutagenesis. Biochemistry 43, 4490–4500.

Zhang, H., Yang, B., Pomerantz, R.J., Zhang, C., Arunachalam, S.C., Gao, L., 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature 424, 94–98.