Proviral Progeny of Heterodimeric Virions Reveal a High Crossover Rate for Human Immunodeficiency Virus Type 2[▽]

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Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS in humans, exhibits a very high rate of recombination. Bearing in mind the significant epidemiological and clinical contrast between HIV-2 and HIV-1 as well as the critical role that recombination plays in viral evolution, we examined the nature of HIV-2 recombination. Towards this end, a strategy was devised to measure the rate of crossover of HIV-2 by evaluating recombinant progeny produced exclusively by heterodimeric virions. The results showed that HIV-2 exhibits a crossover rate similar to that of HIV-1 and murine leukemia virus, indicating that the extremely high rate of crossover is a common retroviral feature.

Human immunodeficiency virus type 1 (HIV-1) exhibits significant genetic diversity, which allows it to constantly evade the host immune response, to circumvent antiretroviral therapy, and to erect barriers to the development of an effective vaccine (3, 23). The molecular basis for such diversity lies in the error-prone nature of the virally encoded reverse transcriptase (RT) (16) and the ability of RT to switch templates (27). Together, these mechanisms generate viral variants at a high rate and represent a major force in driving HIV-1 evolution in infected populations worldwide. Previous studies from our laboratory and others have shown that HIV-1 exhibits a high rate of recombination, averaging between three and four crossovers per cycle of replication (10, 20, 29). It has been reported to be significantly higher when macrophages were used as targets (14). This high rate of recombination is not surprising in the context of the 16 circulating recombinant forms that have been recorded so far from patient samples worldwide (13).

It is hypothesized that, like HIV-1, HIV-2 arose by zoonotic transmission from simian immunodeficiency virus-infected primates to humans and while HIV-1 is responsible for the global AIDS epidemic, HIV-2 has remained largely restricted to West Africa, certain parts of Europe, and some parts of southwestern India (18, 26). Although they are very similar in the genomic organization and biological functions of their gene products, their sequences diverge by approximately 50% at the nucleotide level (8, 15, 22). Overall, HIV-2 differs from HIV-1 in having (i) lower rates of horizontal and vertical transmission (2, 11, 17), (ii) reduced pathogenicity (4), (iii) considerable CD4-independent tropism (19), and (iv) longer clinical latency periods (1, 5). In spite of its restricted geographical distribution, recombinant strains of HIV-2 have been reported in West Africa. These chimeric viruses include both intra- and intergroup (subtypes A and B) recombinants (7, 21). In this study

we aimed at measuring the crossover rate of HIV-2 by using a strategy that selectively scores for progeny of heterodimeric virions. Moreover, by using the same strategy, experiments with HIV-1 were conducted in parallel to further validate our findings. Also, the significance of a high crossover rate to the basic mechanism of retroviral reverse transcription is discussed.

Strategy to obtain recombinant progeny of heterodimeric virions. During retroviral replication, recombination can occur in cells infected with heterodimeric virions containing two genetically distinct RNAs. Heterodimeric virions are produced from cells harboring more than one provirus established as a consequence of double infection. It has been shown that dual infection occurs frequently in nature for HIV-1, thereby providing an opportunity to generate recombinant proviruses. In order to examine the intermolecular crossover rate between copackaged RNA templates in HIV-2, the recombinant progeny proviruses of only heterodimeric virions were analyzed. The reason for doing this was to avoid underestimating the rate of crossover because if HIV-2 preferentially forms homodimeric virions, as appears to be the case for murine leukemia virus (MLV) (6), the rate would be underestimated since crossovers between homodimeric RNAs could not be scored.

To implement the strategy used to determine the HIV-2 crossover rate, vectors containing expression cassettes that can serve as recombination templates were designed (Fig. 1A). The vectors were derived from the plasmid pSVRΔNB, which was based on the ROD strain of HIV-2 (9). Each expression cassette contains a heterologous promoter driving the expression of a mutated *gfp* linked to a unique selectable marker. The *gfp* mutations are complementary and are spaced 340 bp apart to enable crossovers to be scored during reverse transcription in the target cells. The *gfp* sequence is separated from the selectable marker by an internal ribosome entry site (IRES) sequence in both the vectors. Furthermore, each expression cassette contains a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence at the 3' end of the selectable marker to facilitate RNA export to the cell cytoplasm. The strategy as depicted in Fig. 1B utilizes two vectors,

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FIG. 1. Vector constructs and strategy. (A) Plasmids used for the study. HIV2SGpt is an HIV-2-based vector with deleted *gag-pol* and *env* genes and an expression cassette consisting of an SV40 promoter driving expression of a mutant *gfp* (frameshift mutation introduced at nucleotide position 364) and a *gpt* resistance gene separated by an IRES and followed by the WPRE sequence. HIV2ΔCPuro is similar to HIV2SGpt except for the expression cassette consisting of a CMV promoter driving expression of a mutant *gfp* (a complementary frameshift mutation introduced at nucleotide position 24) and a puromycin resistance gene (*puro*) separated by an IRES. Further, the 5' LTR has been replaced with a CMV promoter, and the 3' U5 has been replaced with a bovine growth hormone-derived polyadenylation signal (pA). Asterisks indicate the positions of complementary frameshift mutations. (B) Strategy to obtain recombinant progeny of heterozygous virions. HIV2ΔCPuro and HIV2SGpt genomic RNA templates are packaged together in a single virion in the producer cell. In the target cells infected with this heterozygous virion, reverse transcription begins utilizing both the templates. Without r, the HIV2ΔCPuro RNA strong-stop DNA primer (upper strand) fails to effectively strand-transfer due to lack of homology with 3' r. An intermolecular primer strand transfer from HIV2SGpt RNA to HIV2ΔCPuro RNA is shown as only *puro*-resistant cells will be scored. A crossover is depicted to indicate an example of recombination. Gray lines represent viral RNAs, and black lines represent DNA. Abbreviations: IRES, encephalomyocarditis virus internal ribosome entry site; ψ , viral packaging signal; CMV, CMV immediate-early gene promoter; *gfp*, green fluorescence protein gene; SV40, SV40 derived enhancer-promoter; pbs, primer binding site. Lowercase indicates sequences in the RNA; corresponding uppercase indicates sequences in the DNA derived from the RNA.

HIV2SGpt and HIV2ΔCPuro. HIV2SGpt is a minimal HIV-2-derived vector with intact 5' and 3' long terminal repeats (LTRs) and *cis*-acting sequences. Additionally, it contains a simian virus 40 (SV40)-derived promoter driving the expression of the mutant *gfp* which is linked to the guanine phospho-

ribosyltransferase resistance gene (*gpt*). In HIV2ΔCPuro the 5' LTR was replaced with the cytomegalovirus (CMV) promoter, and the 3' U5 was replaced with the bovine growth hormone poly(A) signal due to partial deletion of the viral poly(A). The expression cassette of HIV2ΔCPuro differs from that of

HIV2SGpt in (i) the replacement of SV40 by the CMV-derived promoter-enhancer as the internal promoter, (ii) the position of the mutation in *gfp*, and (iii) the selectable marker being the puromycin resistance gene (*puro*) instead of *gpt*. It is noteworthy to point out that the primer binding site and the encapsidation signal (ψ) of HIV2ACPuro and HIV2SGpt were kept the same so that the efficiency of tRNA priming and RNA packaging would be equivalent for the two vectors. Moreover, the 3' U5 of HIV2ACPuro was deleted to prevent the regeneration of a functional 5'LTR during transfection via recombination.

Homodimers of HIV2ACPuro RNA virions should not be able to produce any progeny due to the lack of a 5' R to carry out successful minus-strand primer transfer (Fig. 1B). Although homodimers of HIV2SGpt RNA virions are capable of carrying out minus-strand primer transfers due to intact LTRs, none of the progeny are capable of surviving *puro* selection due to the presence of *gpt* instead of the *puro* selection marker within the HIV2SGpt vector (Fig. 1A). Only heterodimeric virions (HIV2SGpt/HIV2ACPuro), which have undergone an intermolecular minus-strand primer transfer, can confer puromycin resistance (Fig. 1B), and the crossover frequency is given by the ratio of *gfp*-positive, *puro*-resistant colonies to the overall number of *puro*-resistant colonies.

Measurement of the HIV-2 crossover rate. Initially, to ensure that HIV2ACPuro was incapable of producing infectious vector virus, an experiment was performed to determine whether virus conferring puromycin resistance to target cells could be generated. 293T cells were transiently transfected with HIV2ACPuro along with helper plasmids, and viral supernatant was harvested followed by infection of HeLa T4 cells. These cells were subjected to puromycin selection, and as anticipated, we obtained a puromycin resistance titer of 0.0 IU/ml. As a positive control for viral propagation, vector virus was derived from transient transfection of HIV2CPuro (identical to HIV2ACPuro but with intact LTRs [data not shown]) with helper plasmids, and HeLa T4 cells were infected and subjected to puromycin selection. A *puro*-resistant titer of $3.8 \times 10^6 \pm 0.4 \times 10^6$ IU/ml was obtained, and all the *puro*-resistant clones were found to be *gfp* negative.

To implement the strategy described above (Fig. 2), a HeLa cell clone harboring a HIV2SGpt provirus was established. The reason for establishing a producer cell clone harboring a HIV2SGpt provirus was that it eliminates the need for cotransfecting HIV2SGpt (with intact LTRs) and HIV2ACPuro (with defective LTRs) when producing heterodimeric vector virus, which prevents the possibility of restoring the HIV2ACPuro 5' LTR and/or the wild-type *gfp* via recombination during transfection.

Next, it was crucial to ascertain conditions for confining replication to a single round of replication involving one cycle of RNA transcription in the producer cells and one cycle of reverse transcription in the target cells. Since vesicular stomatitis virus G envelope protein (VSV-G) would be used to pseudotype vector virus, there was a chance that the clonal population harboring the HIV2SGpt provirus when transfected with helper plasmids and HIV2ACPuro would be reinfected with progeny virus which would not confine replication to a single cycle. To ensure that viral replication was restricted to a single cycle, viral titers were evaluated at 6, 12, 19, 25, 36,

FIG. 2. Experimental protocol for measurement of crossover rates in HIV-2 and HIV-1. HeLa cells were infected with HIV2SGpt vector virus pseudotyped with VSV-G, and *gpt*-resistant clones were selected and expanded. A HeLa cell clone harboring a HIV2SGpt provirus and yielding the highest *gpt*-resistant titer was transiently transfected separately with HIV2ACPuro vector construct along with either HIV-1-based or HIV-2-based packaging constructs using a standard Lipofectamine transfection protocol. Viral supernatant was harvested 24 h posttransfection and used to infect HeLa T4 target cells. Target cells were placed under puromycin selection, and green fluorescent protein (GFP)-positive clones were manually counted via fluorescence microscopy after selection was complete.

and 48 h posttransfection by sampling supernatant and infecting target cells followed by selection for *puro* resistance. This was done to determine the earliest time point at which a significant viral titer could be generated while avoiding reinfection of the producer cells. A substantive *puro*-resistant titer on HeLa T4 target cells was recorded with viral supernatant harvested 24 h posttransfection, during which time no reinfection of the producer cells was noted. This was evaluated by subjecting the producer cells to puromycin selection after the viral supernatant was harvested. *puro*-resistant producer cells were not detected up to 24 h posttransfection, while on average, *puro*-resistant titers of 1.1×10^1 IU/ml and 2.8×10^3 IU/ml were derived at the 36 and 48 h time points, respectively. Thus, by using vector virus harvested 24 h posttransfection, which prevents significant recycling of virus within the producer cell population, and by infecting target cells that do not express the viral proteins, one can effectively restrict replication to a single cycle with this system.

TABLE 1. Crossover rates^a

Packaging plasmid	Expt no.	GFP titer (IU/ml)	<i>puro</i> titer (IU/ml)	Crossover rate ^b	Extrapolated crossover rate ^c
HIV-2-based	1	28	2.3×10^2	5.2×10^{-4}	5.0
	2	15	1.3×10^2	4.9×10^{-4}	4.7
	3	17	1.6×10^2	4.5×10^{-4}	4.3
Average				4.86×10^{-4}	4.7
HIV-1-based	1	15	1.4×10^2	4.3×10^{-4}	4.2
	2	11	1.5×10^2	2.9×10^{-4}	2.8
	3	19	2.1×10^2	3.6×10^{-4}	3.5
Average				3.6×10^{-4}	3.5

^a All experiments were repeated at least thrice, and infections were done in duplicate.

^b The crossover frequency is expressed as GFP-positive titer/*puro* titer, which averages 11% when HIV-2 packaging constructs are used and 9% when HIV-1 packaging constructs are used. To determine a more accurate rate of crossover, the denominator should be slightly adjusted because some of the *puro* progeny would have undergone crossovers before reaching the position of the 3'-*gfp* frameshift mutation, so these progeny would not be able to contribute to the population that can score for crossovers since even if they crossed over between the frameshift mutations they would result in a GFP-negative phenotype. To obtain a minimum correction the denominator should be decreased by the crossover frequency times the distance between *puro* and the 3' frameshift mutation (965 bases) divided by the distance between the frameshift mutations (340 bases) or by 31% ($11\% \times 965/340$) for HIV-2 packaging constructs and 26% ($9\% \times 965/340$) for HIV-1 packaging constructs. Thus the crossover rate is equal to GFP-positive titer/(*puro* titer $\times$ 0.69)/340 bases/replication cycle for HIV-2-based packaging constructs and GFP-positive titer/(*puro* titer $\times$ 0.74)/340 bases/replication cycle for HIV-1-based packaging constructs.

^c Calculated by multiplying the crossover rate by the corresponding RNA genome size. For HIV-1, the genomic size is 9,709 bases (strain NL4-3, accession number M19921). For HIV-2, the genomic size is 9,671 bases (strain ROD, accession number X05291). Values indicate numbers of crossovers per genome per cycle of replication.

To measure the HIV-2 rate of crossover, the HeLa cell clone harboring the HIV2SGpt provirus was transfected with HIV2ΔCPuro along with HIV-2 packaging constructs and VSV-G expression plasmid using a Lipofectamine transfection protocol (Fig. 2). Viral supernatant was harvested 24 h post-transfection and used to infect HeLa T4 cells in serial dilutions. The cells were subjected to puromycin selection, and *gfp* and *puro* titers were determined after selection was complete (Table 1, top). The frequency of crossover between the two complementary mutations was given by the *gfp* titer, while the *puro* titer, obtained from HIV2SGpt/HIV2ΔCPuro heterodimeric virions, constituted the overall viral titer. The crossover rate per cycle of replication was expressed as *gfp* titer/*puro* titer/340 bp. It was found that the HIV-2 rate was 4.86×10^{-4} /base/replication cycle, and when extrapolated to the entire genome, it averaged 4.7 crossovers per genome per cycle (Table 1).

Measurement of the HIV-1 crossover rate using the same HIV-2 vector templates. Parallel experiments were also conducted with HIV-1-based packaging plasmids instead of HIV-2-based packaging constructs to generate VSV-G pseudotyped vector virus (Fig. 2). This serves as a control since we have previously determined the HIV-1 rate of recombination by using a different experimental approach. A nonreciprocal packaging relationship between HIV-1 and HIV-2 exists in which the HIV-1 Gag protein is capable of packaging HIV-2-

derived genomic RNA into its viral core with a high efficiency (12). Since the crossover phenomenon is a direct outcome of the processivity of reverse transcriptase, employing an HIV-1-derived *gag-pol* packaging construct would allow for a direct comparison of the crossover rates of HIV-1 and HIV-2 using the same sets of HIV-2-derived crossover constructs (HIV2SGpt and HIV2ΔCPuro). The results from this experiment are shown in Table 1 (bottom). The rate of crossover was found to be 3.6×10^{-4} /base/replication cycle, which corresponds to 3.5 crossovers per genome per cycle and correlates very nicely with our previous results confirming the validity of this approach.

Significance. In this study, we have shown that the HIV-2 rate of crossover averages 4.7 per genome per cycle of replication and is similar to the HIV-1 rate (Table 1). The results were obtained using an approach which eliminated the possibility that nonrandom copackaging might result in an underestimation of the crossover rate (Fig. 1). Moreover, the fact that HIV-1 proteins can package HIV-2 genomic RNA allowed testing whether this approach would yield a crossover rate similar to that already determined for HIV-1 using the same RNA template. The crossover rate obtained with HIV-1 RT was 3.5 crossovers per genome per cycle, which is in agreement with our previous results using a more conventional but more labor-intensive approach.

Is the high crossover rate idiosyncratic to lentiviruses, or is it a general retroviral phenomenon? At this juncture, our laboratory has also measured the crossover rate for murine leukemia virus, a member of the gammaretrovirus genus, and has found that its crossover rate is similar to those of both HIV-1 and HIV-2 (30). Thus, members of two different genera which diverge in sequence and genome structure exhibit similar crossover rates, so it is quite possible that multiple crossovers are a common property of all or most retroviruses during each cycle of replication. This seems to be the case for HIV-1. In a previous experiment, producer cells harboring two HIV-1 vector proviruses with most of the autologous viral sequence intact were established (10). The autologous sequence between the two vectors differed by approximately 3% throughout the genome, providing genetic markers to allow direct examination of crossovers by employing DNA sequencing, heteroduplex tracking, and restriction mapping. Viral RNA from one of the vectors was expressed in a 100-fold-greater amount than the other. To focus upon progeny of heterodimeric virions, proviruses that contained the marker from the limiting viral RNA were analyzed, and it was found that all 86 proviruses were recombinant. Thus, given the similar crossover rates for MLV, HIV-2, and HIV-1, it is quite possible that full-length MLV and HIV-2 genomes also undergo crossovers essentially during every cycle of replication, but at this time we are extrapolating the crossover rate for MLV and HIV-2 to the entire genome and cannot formally rule out the existence of a population that is not subjected to crossovers.

It is noteworthy that although the crossover rate of MLV is similar to that of HIV-1, it does not produce genetic recombinants at the same rate. A contributing factor is the fact that MLV preferentially packages homodimers in contrast to HIV-1 (6), so even though the crossover rates for HIV-1 and MLV are similar, crossovers will not as effectively result in novel MLV recombinant genomes. However, even though

FIG. 3. Model of reverse transcription showing multiple crossovers. (i) tRNA primes minus-strand DNA synthesis. tRNA priming from only one strand is depicted here although dual initiation might occur. (ii) r-u5 digested by RNase H activity of RT; the minus-strand DNA primer transfers to the 3' end of the RNA genome (only intramolecular strand transfer is shown). (iii) Synthesis of minus-strand DNA continues with concomitant synthesis of the plus-strand DNA primer. (iv) Reverse transcriptase along with the nascent DNA strand undergoes template switching between the copackaged RNA genomes multiple times. (v) Plus-strand strong-stop transfer to the 5' end of the newly synthesized minus-strand DNA occurs, utilizing sequence homology to the primer binding site (PBS). (vi) Completion of both minus- and plus-strand DNA synthesis leads to the formation of a full-length double-stranded viral DNA. Thin lines represent RNA. Thick lines represent DNA. Dotted lines represent RNase H-digested RNA. PPT, polypurine tract; r-u5, sequences in the RNA. Lowercase indicates sequences in the RNA; the corresponding uppercase indicates sequences in the DNA derived from the RNA.

crossovers between identical RNA templates do not yield genetic recombinant genomes, they might still contribute to the pseudodiploid nature of retroviruses. Retroviral virions contain two RNA genomes, yet they yield a single DNA genome.

It is possible that DNA synthesis can be primed on both viral RNA templates (24, 25). Nevertheless, even in cases in which DNA synthesis was primed on both RNA templates, the high crossover rate would restrain synthesis of a second proviral

genome because once the first crossover occurred, it would disrupt the synthesis of the second DNA genome. The reason for this is that the RNase H activity of reverse transcriptase would digest the viral RNA template for the second DNA strand, preventing its continued synthesis and resulting in the production of a single viral DNA genome.

Current models show continuous minus-strand DNA synthesis occurring during reverse transcription. However, given that the high crossover rates of HIV-1, HIV-2, and MLV indicate that template switching is occurring multiple times during each cycle of replication, it would seem to be more accurate to indicate this in models of retroviral reverse transcription. In Fig. 3, step iv represents a model depicting multiple intermolecular crossovers during minus-strand DNA synthesis. For simplicity, the model shows minus-strand strong-stop synthesis occurring on only one strand (Fig. 3, step i); however, as noted above, it is possible that DNA synthesis can be primed on both virion RNAs (24, 25). Also, an intramolecular minus-strand-strong primer transfer is depicted, but as was previously shown, it can be either intra- or intermolecular (28). The model implies that crossovers are a basic aspect of the mechanism of retroviral reverse transcription and that retroviral genes are essentially genetically unlinked.

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