Recombination is required for efficient HIV-1 replication and the maintenance of viral genome integrity

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ABSTRACT

Retroviruses package two complete RNA genomes into a viral particle but generate only one provirus after each infection. This pseudodiploid replication strategy facilitates frequent recombination, which occurs during DNA synthesis when reverse transcriptase switches templates between two co-packaged RNA genomes, generating chimeric DNA. Recombination has played an important role in shaping the current HIV-1 pandemic; however, whether recombination is required for HIV-1 replication is currently unknown. In this report, we examined viral replication when recombination was blocked in defined regions of the HIV-1 genome. We found that blocking recombination reduced viral titers. Furthermore, a significant proportion of the resulting proviruses contained large deletions. Analyses of the deletion junctions indicated that these deletions were the direct consequence of blocking recombination. Thus, our findings illustrate that recombination is a major mechanism to maintain HIV-1 genome integrity. Furthermore, recombination allows generation of the intact HIV-1 DNA genome and is required for efficient viral replication.

INTRODUCTION

Retroviruses are unique among all known virus families in which they package two complete copies of their genome into one viral particle although each infection event yields only a single DNA provirus (1). Thus, retroviruses are pseudodiploid (2). This distinct feature raises a key question that has not been answered yet: why do retroviruses contain two copies of the full-length RNA genome when only one DNA provirus is generated? Two hypotheses that are not mutually exclusive have been proposed. First, packaging two RNA genomes promotes homologous recombination, allowing the reassortment of mutations to increase diversity in the viral population (3). Alternatively, recombination acts as a repair mechanism by enabling the replication of viruses with damaged genomes, such as those containing RNA breaks (4). Retroviral recombination occurs frequently during reverse transcription of the viral genome (2,5–13). The viral enzyme reverse transcriptase (RT) switches between co-packaged RNA templates during minus-strand DNA synthesis, resulting in a hybrid DNA copy that contains a portion of information from each RNA genome (2,14). During infection by homozygous viruses containing two copies of identical RNA, recombination can occur but cannot be genetically detected. In contrast, recombination can be identified during infection by heterozygous particles that contain genetically distinct RNAs such as those from differ-
ent proviruses (22,15). Currently, there are two proposed mechanisms to describe how recombination occurs during minus-strand DNA synthesis. The forced copy-choice model proposes that recombination occurs when RT encounters a break in the RNA genome, which forces RT to switch to the copackaged RNA to continue DNA synthesis (4). This model is based primarily on the observation that DNA genomes isolated from defective viral particles often contain breaks. On the other hand, the dynamic copy-choice model posits that recombination is not necessarily caused by RNA breaks but depends on the dynamic process of DNA synthesis (16). As RT synthesizes DNA, RNase H degrades the RNA template, leaving the nascent DNA free to anneal to the copackaged RNA. If DNA synthesis slows down or RNase H degrades the RNA template more rapidly, the RT complex becomes less stable, dissociates from the template, and can switch to using the copackaged RNA as a template. Thus, recombination depends on the dynamics of DNA synthesis, is not necessarily an obligatory event, and can occur even in the absence of RNA breaks. This model is based on the finding that recombination is promoted by decreased polymerase or increased RNase H activity (16).

HIV-1 RT is estimated to switch templates 8 to 10 times during the synthesis of a single genome (11,17–21). Thus, HIV-1 recombines frequently during replication. Recombination has led to the emergence of more than 96 circulating recombinant forms (CRFs) of different HIV-1 group M subtypes, and these CRFs cause a substantial proportion of worldwide infections (22). Additionally, recombination has been shown to produce variants that confer multidrug resistance or evade the host immune response (23–25). Thus, the findings demonstrate that recombination plays an important role in generating viral diversity and shaping the current worldwide HIV-1 pandemic.

Despite the importance of HIV-1 recombination in generating viral diversity, whether it is required for HIV-1 replication has not yet been determined. If all recombination events are required to salvage genetic information, then recombination must be critical to HIV-1 replication, considering the high rate of crossovers. On the other hand, if only a small proportion of recombination events are obligatory, recombination may be dispensable for viral replication. In this report, we sought to answer this long-standing question by blocking homologous recombination in defined regions of the genome and monitoring the survival of progeny with intact genomes. Our results show that blocking recombination in a 2.5-kb region of the HIV-1 genome resulted in the loss of half of the proviruses with intact genomes. These findings indicate that HIV-1 recombination is required for efficient reverse transcription and maintenance of the intact genome structure.

MATERIALS AND METHODS

Construction of HIV-1 plasmids with heterologous sequences

HIV-1 control plasmids pT6S and pH0 were described previously as pWT

\[ \text{NL4-3} \]  and pWT

\[ \text{HXB2} \], respectively (26). Both plasmids express functional Tat and Rev but not Vif, Vpu, Vpr, Env or Nef. Additionally, pH0 expresses functional Gag/Gag-Pol, whereas pT6S does not express functional Gag/Gag-Pol due to an inactivating 4-bp insertion in the capsid-coding region of the gag gene. The pol genes of pT6S and pH0 were derived from NL4-3 and HXB2, respectively. Additionally, pT6S encodes a mouse thy (CD90.2) gene and a green fluorescence protein (gfp) gene that has an inactivating mutation at the 3′ end, whereas pH0 encodes a mouse heat stable antigen (hst; CD24) gene and a gfp gene that has an inactivating mutation at the 5′ end. To suppress recombination, the pol genes of pH0 and pT6S were modified to generate pH0-SynRT and pT6S-IN2, respectively. For simplicity, the RT- and integrase (IN)-coding region of the pol gene are referred to as rt and in region or rt and in sequence, respectively. HIV-1 plasmids with heterologous sequences were created from these control plasmids as follows. First, a synthetic HXB2 RT-coding sequence (SynRT) was engineered using a custom Python program based on Biopython (27) and developed in Enthought Canopy (v1.6.2). This program silently recoded HXB2 RT based on a human codon usage table but additionally forced codons to switch whenever possible to minimize sequence homology with the NL4-3 rt region. The sequence was further edited manually to remove several restriction enzyme sites and long runs of sequence homology. Although the NL4-3 and HXB2 rt regions share 97% homology at the nucleotide level, SynRT is only 63% homologous to the NL4-3 rt region. A DNA fragment containing the SynRT sequence was synthesized (GenScript) and cloned into pH0 using SpeI and Sall sites, generating pH0-SynRT. To create pT6S-IN2, a DNA fragment containing HIV-2 ROD in region with a silent XbaI site at the 5′ end was synthesized (IDT) and cloned into pT6S using XbaI and Ascl sites, generating pT6S-IN2. Single-virion analysis constructs for examining RNA copackaging were derived from the previously described vectors Gag-BglSL, GagCeFP-BglSL, Gag-MSSL and GagCeFP-MSSL (28). To insert the pol sequence into these vectors, small (∼200 bp) NL4-3 or HXB2 linkers containing pro sequence were inserted into the AgeI site of these plasmids. Next, fragments containing wild-type or modified rt and in regions were inserted using SgrAI and Ascl sites. All new constructs were verified by restriction digest mapping and sequencing of the cloned region. All helper plasmids have been described previously. Briefly, pHCMV-

\[ \text{G} \]  expresses the G glycoprotein of vesicular stomatitis virus (VSV-G) (29), pSYNGP encodes human codon-optimized HIV-1 gag and gag-pol (30) and pIIINL(AD8)env expresses the HIV-1 AD8 CCR5-tropic envelope (Env) (31).

Cell culture and flow cytometry

Human 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Hut/CCR5 cells are human T cells derived from the Hut78 cell line that stably express the chemokine receptor CCR5 and were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml puromycin and 500 μg/ml G418. Cells were analyzed by flow cytometry to determine viral titers as follows. Cells were collected, washed twice with Dulbecco’s...
phosphate-buffered saline (DPBS) containing 2.5% FBS, stained with 0.4 μg/ml phycoerythrin (PE)-conjugated anti-HSA and/or 2.0 μg/ml allophycocyanin (APC)-conjugated anti-Thy antibodies (BioLegend) for 30 min at 4°C, and washed two additional times with DPBS containing 2.5% FBS. Flow cytometry for PE-HSA, APC-Thy, and GFP was performed 72 h post-infection on an LSR II system (BD Biosciences), while cell sorting was performed on a FACSAria II system (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (TreeStar, LLC).

GFP recombination assay
Dually infected 293T producer cell lines were generated by sequential infection at low multiplicity of infection (MOI; <0.1). Virus stocks were produced by cotransferring 293T cells with an HIV-1 construct, pHCMV-G, and, in the case of constructs lacking functional Gag/Gag-Pol, pSYNGP, using TransIT-LT1 transfection reagent (Mirus). Virus stocks were harvested 48 h post-transfection, clarified through 0.45-μm pore size filters, and added to glass slides. Dually infected cells were enriched by multiple rounds of cell sorting until >93% of the cells were dual positive (HSA* and Thy*). Each producer cell line consisted of >62,000 independent infection events. As the serial infections were carried out at low MOI (<0.1), most of the cells in the final pool contained one copy of each provirus. Next, to determine GFP reconstitution frequencies, producer cell lines were transfected with pIIINL(AD8)env. Viruses were collected 48 h post-transfection, clarified through 0.45-μm pore-size filters, and used to infect cells. Dually infected cells were enriched by multiple rounds of cell sorting until >93% of the cells were dual positive (HSA* and Thy*). Each producer cell line consisted of >62,000 independent infection events. As the serial infections were carried out at low MOI (<0.1), most of the cells in the final pool contained one copy of each provirus. Next, to determine GFP reconstitution frequencies, producer cell lines were transfected with pIIINL(AD8)env. Viruses were collected 48 h post-transfection, clarified through 0.45-μm pore-size filters, and used to infect Hep2 cells. Infected cells were collected 72 h post-infection, stained and analyzed by flow cytometry. GFP reconstitution frequencies were calculated by dividing the percentage of GFP+ cells by the percentage of all infected (i.e. HSA, Thy and/or GFP+) cells.

Single-genome sequencing (SGS) to determine provirus structures
Genomic DNA from GFP+ cell pools was first purified using the QIAamp DNA blood kit (Qiagen). To achieve single-genome amplification, genomic DNA was serially diluted in 96-well plates to identify a dilution in which ~20% of wells were positive by polymerase chain reaction (PCR). At this dilution, most amplicons are derived from single templates. PCR was performed in 20-μl reactions containing 1 × High Fidelity Buffer, 2 mM MgSO4, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 0.2 μM of each primer and 0.025 U/μl Platinum Taq High Fidelity DNA Polymerase (Invitrogen). For the first round of PCR, sense primer HIV.BK5.F1 5′ CCT TGA CT T TTC CAA GTA GTG TGT GCC CGT CT GTG and antisense primer pTS-R1: 5′ GTC GAC ACC CAA TTC TGA AAT GG were used. PCR was performed with the following parameters: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 15 s, 55°C for 30 s and 68°C for 4 min; and a final extension of 68°C for 10 min. For the second round of PCR, 1 μl of first-round PCR product was used with sense primer HIV.BK5.F2: 5′ GTA GTG TGT GCC CGT CTG TTG TGT GAC and antisense primer pTS-R2: 5′ GCC TTC CAA TCC CAT ATG GTG. Second-round PCRs were performed with the same conditions as the first-round PCR, but with a total of 45 cycles. The final amplicon was ~4.5 kb in size and included the 5′ U5, 5′ UTR, gag and pol regions. All procedures were performed under PCR clean-room conditions with additional safeguards against sample contamination, such as preAliquoting of reagents, use of dedicated equipment and physical separation of pre- and post-PCR sample processing. Amplicons were directly sequenced using BigDye terminator chemistry according to the manufacturer’s recommendations (Applied Biosystems). Individual sequences for each amplicon were assembled and edited using Sequencher (Gene Codes). Chromatograms were inspected for the absence of mixed bases at each nucleotide position throughout the entire amplicon; this quality-control measure confirmed that the amplicons analyzed were derived from single-genome amplification and allowed amplicons that contained PCR-generated recombination events or errors to be excluded from the analysis.

Sequence and statistical analyses
Assembled single-genome sequences were aligned to appropriate parenteral reference sequences using MAFFT v7 (32). Sequence alignments were manually edited and trimmed to remove primer regions in AliView v1.18 (33). Final Alignments were visualized using Highlighter (34). After trimming, sequences included the last 61 bp of the 5′ UTR, all of gag, and all of pol except the last 64 bp of the in sequence. Crossovers were quantified using a custom Python program based on Biopython (27) and developed in Enthought Canopy (v1.6.2). This program was used to quantify the number of crossovers and reference bases within entire sequences as well as defined subregions such as the pro, rt and in sequences. To distinguish crossovers from mutations or sequencing errors, crossovers were required to switch at least two successive markers. Crossovers that occurred at gene junctions were assigned to the more likely
gene. As an example, for the H0/T6S vector pair, the marker segment at the rt/in junction is 31 bp in length, with 28 bp in rt and 3 bp in the in region; crossovers in this segment were thus assigned to rt. Deletions were quantified and characterized manually using AliView. Sequence duplicates containing deletions were collapsed into a single sequence. All statistical analyses were performed in GraphPad Prism v7.01 (GraphPad Software, Inc.).

Calculation of recombination events

We performed the following calculation to estimate the proportion of viruses that would be lost when recombination is blocked in the entire genome. The control virus pair (H0/T6S) and the test virus pair (H0-SynRT/T6S-IN2) generated 5.6 and 2.8% of infected cells with GFP+ intact proviruses, respectively. Therefore, blocking recombination in 28% of the viral genome (2.5 out of 9.2 kb) resulted in the loss of 51% of GFP+ intact proviruses. These results indicate that the rate of forced crossovers is at least 0.51/sequence in the rt and in region. However, some viruses likely would have undergone multiple force crossovers in this region. Recombination events have been shown to be distributed throughout the HIV-1 genome (11,35) and do not exhibit high negative interference (18). Assuming that forced crossovers are randomly distributed, the rate of forced crossovers is 0.71/sequence in the rt and in region and 2.55/sequence in the ∼9.2 kb HIV-1 genome. Under these conditions, ∼92% of viruses would undergo at least one forced crossover during reverse transcription that, when blocked, would lead to a noninfectious virus.

The proportion of obligatory (forced) crossover events was calculated as followed. The observed rate of crossovers in the rt and in region was 1.78/sequence for the control vector pair (H0/T6S) and the estimated rate of forced crossover is 0.71/sequence (see above). Thus, ∼40% (0.71/1.78) of crossover events are forced whereas ∼60% of crossover events are not forced.

RESULTS

Experimental strategy to suppress homologous recombination

During reverse transcription, two template-switching events (minus- and plus-strand DNA transfers) are required to complete viral DNA synthesis (1,36). Therefore, the role of recombination in HIV-1 replication cannot be addressed by blocking all template-switching events. Instead, we adopted a strategy to suppress recombination in part of the viral genome, based on previous observations that frequent HIV-1 recombination depends on high sequence homology (37–39). To suppress recombination, we introduced sequences with reduced homology to replace the wild-type genes in two previously described HIV-1 constructs, H0 and T6S (26) (Figure 1A). These near-full-length constructs contain all the cis-acting elements required for viral replication and encode gag-pol, tat and rev. However, T6S-based constructs do not express Gag or Gag-Pol due to a 4-nt frameshift insertion in the capsid region of gag. Both H0 and T6S are derived from the NL4-3 molecular clone; however, H0 contains the pol gene from HXB2, whereas T6S retains the pol gene from NL4-3. The pol genes from HXB2 and NL4-3 share 97% sequence homology, which is sufficient to mediate frequent recombination while still allowing for the detection of crossovers using polymorphic sites (26). We selected the rt region for sequence alteration because it lacks known cis-acting elements that are essential for viral replication. To suppress homologous recombination, we replaced the rt region in H0 with a synthetic sequence (SynRT; shown as a blue box in Figure 1B) that encodes the same protein but contains multiple silent mutations so that it has ∼63% homology to the rt region in T6S. Additionally, we replaced the in region, which contains the central pyrimidine tract (cPPT). Previous studies have shown that disruption of the cPPT does not affect reverse transcription kinetics or viral titers (40). Nonetheless, to preserve the cPPT, we replaced the in region in T6S with the in sequence from HIV-2 ROD (IN2; shown as a red box in Figure 1B), which has 67% homology to the in region in H0. Previous studies indicate that this strategy should block >95% of the homologous recombination events in this region (37–39). Similar to T6S, T6S-IN2 contains a frameshift mutation in gag and does not express Gag/Gag-Pol, thereby avoiding potential incompatibility of proteins from different viruses (41).

In this strategy, heterozygous viruses with one copy of each RNA (H0-SynRT and T6S-IN2) contain two RNAs with different rt and in sequences, thus suppressing recombination in this region. However, recombination in the rt and in regions is not blocked in homologous viruses containing two copies of the same RNA. To examine the effect of suppressing recombination on viral replication, we iden-
HIV-1 constructs contain either a mouse *hsa* or a mouse *thy* gene; additionally, both constructs contain an internal ribosomal entry site (I) and a defective green fluorescence protein (gfp) gene (Figure 1A and B). HSA and Thy are cell surface markers that can be detected by flow cytometry and used to identify infected cells. The gfp genes in the two vectors contain inactivating mutations at the 5′ (H0) or 3′ (T68) end (asterisks in Figure 1A and B). In cells infected by heterozygous particles, a functional gfp gene can be restored by recombination during reverse transcription.

To study the replication of heterozygous particles, we first generated producer cell lines that contain one copy of each parental provirus (Figure 1C). For this purpose, we generated H0- or T68-derived viruses that were pseudotyped with VSV-G and serially infected human 293T cells with these viruses at low MOI (>0.1). Dually infected cells were enriched by multiple rounds of cell sorting until >93% of the cell populations were HSA^+ and Thy^+. To examine viral replication, we transfected 293T producer cells with a plasmid that expresses HIV-1 Env and used the harvested virus to infect Hut/CCR5 T cells. Infected cells were analyzed by flow cytometry to determine the proportions of cells expressing HSA, Thy and/or GFP. Infected cells express HSA or Thy markers, whereas only cells infected by heterozygous particles can express GFP. Therefore, we could monitor the replication of heterozygous particles in the entire viral population by determining the proportion of GFP^+ cells in the infected cells (HSA^+ or Thy^+). If recombination was not required for replication, RT would be able to complete DNA synthesis regardless of whether recombination was blocked and GFP titers would not be significantly affected. In contrast, if recombination was required to complete DNA synthesis, blocking recombination would inhibit the replication of heterozygous particles, thereby reducing GFP titers.

Based on our previous study, most viruses should have at least one crossover in the *rt* and *in* regions (26), and we should be able to distinguish between these two possible outcomes. Additionally, it has been shown that HIV-1 recombination events do not exhibit high negative interference; that is, the occurrence of one crossover event does not significantly increase the chance of a second crossover event (18). Therefore, results obtained from studying the GFP^+ subpopulation should accurately reflect the entire viral population.

**Suppressing recombination reduces the proportion of GFP^+ cells in the infected population**

To determine whether recombination is required for HIV-1 replication, we generated producer cell lines containing pairs of proviruses that suppress recombination in 0- (control), 0.9-,, 1.7- or 2.5-kb regions (Figure 2). We then produced viruses, infected Hut/CCR5 T cells, and performed flow cytometry to determine HSA, Thy and GFP titers (Figure 1C). To determine whether replacing HIV-1 sequences affects viral replication, we compared HSA and Thy viral titers in Hut/CCR5 cells. We found that relative HSA and Thy titers were similar for viruses from all four producer cell lines (Supplementary Figure S1). These results show that replacing the *rt* or *in* sequence in H0-SynRT or T68-IN2, respectively, did not affect the expression or replication of these vectors. Furthermore, as viruses from these cell lines generated similar relative HSA and Thy titers, we could directly compare their GFP titers. When using virus produced from cells containing the control vector pair, ~5.7% of infected cells were GFP^+ (Figure 2). However, when using viruses generated from vector pairs containing 0.9-, 1.7- or 2.5-kb heterologous sequences, ~4.1%, ~3.9% or ~3.1% of the infected cells were GFP^+; these titers were significantly lower than that of the control (*P* < 0.05, *P* < 0.01 and *P* < 0.001, respectively; one-way ANOVA with Dunnett's post-test). Furthermore, the decrease in GFP titers correlated with the lengths of heterologous sequences that suppressed recombination (Pearson’s *r* = -0.96, *P* = 0.04). These results indicate that GFP titers progressively declined as the region with suppressed recombination was extended.

Proviruses expressing GFP arise from heterozygous particles (Figure 1A and B). Thus, we hypothesized that decreased GFP titers were caused by inhibiting recombination in heterozygous particles. Alternatively, it is also possible that viral RNAs with different *rt* and *in* sequences did not copackate efficiently. We consider this possibility unlikely because the *pol* sequence does not contribute to RNA genome packaging (28,42), a finding that is supported by the viral titers in this study (Supplementary Figure S1). However, to rule out this possibility, we performed single-virion analysis to detect viral RNAs in individual particles as previously described (28). In this assay, some of the Gag molecules were tagged with cerulean fluorescent protein (CeFP) to identify viral particles, whereas viral RNAs were detected using RNA-binding proteins fused to fluorescent proteins (Bgl-mCherry and MS2-YFP). These RNA-binding proteins specifically recognize stem–loops engineered into the constructs. Assuming there would be equal expression and random copackaging of RNAs from two viral constructs, ~50% of the particles should be heterozygous, whereas each type of homozigous particles should be ~25% of the viral population. For the control, we found that Gag-CeFP^+ particles were ~25% MS2-YFP^+, ~26% Bgl-mCherry^+ and ~45% MS2-YFP^+/Bgl-mCherry^+ (Supplementary Figure S2), consistent with near-random copackaging of viral RNA (28). For the vector pair containing altered *rt* and *in* sequences, the Gag-CeFP^+ particles were ~27% MS2-YFP^+, ~25% Bgl-mCherry^+ and ~43% MS2-YFP^+/Bgl-mCherry^+ (Supplementary Figure S2), which was not significantly different from the control (*P* = 0.27, unpaired t-test). These results indicate that the RNAs from viral constructs containing SynRT and HIV-2 IN copackate efficiently; thus, decreased GFP titers cannot be explained by reduced copackaging of viral RNAs into particles.

**Blocking recombination results in progeny with large deletions in the viral genome**

During DNA synthesis of the region where homologous recombination is blocked, RT is unable to switch to the corresponding region in the copackaged RNA when encountering a break in the template (4) or when the RT complex becomes unstable and dissociates from template (16).
In this case, RT may not be able to continue DNA synthesis, resulting in premature termination. Alternatively, RT may switch templates in a nonhomologous manner, leading to deletions. To characterize progeny generated from heterozygous particles, we performed single genome sequencing (SGS) of proviruses from GFP+ cells (43). Briefly, we infected Hut/CCR5 T cells as described (Figure 1C) and sorted out GFP+ cell pools that each contained >5000 infection events. We then purified cellular DNA and performed nested, limiting-dilution PCR of a 4.5-kb amplicon that included 5′ U5, 5′ UTR, gag and pol. DNA recombination occurs frequently during PCR (44–46). To ensure that our study is not complicated by PCR-mediated recombination, we used experimental conditions in which only ∼20% of amplification reactions were positive. Furthermore, PCR products were directly sequenced, and sequences with mixed bases were discarded. Thus, most, if not all, of the resulting sequences were derived from single proviruses.

To validate our experimental strategy, we first examined whether introducing heterologous sequences indeed suppressed recombination during viral replication. In our control constructs (H0 and T6S), the region including the pro, rt and in sequences shared 97% homology. In the 86 sequences that we analyzed, 181 homologous recombination events were identified, resulting in an average of 2.1 crossovers per sequence (Table 1), similar to the previously observed rate with this vector pair (26). Within the analyzed sequences, there were 28, 113 and 40 crossovers in the pro, rt and in regions, respectively, indicating that recombination occurred frequently throughout these regions. Furthermore, 78% of the sequences had at least one crossover in the rt or in region.

We next analyzed 130 sequences from GFP+ cell pools generated by the H0-SynRT/T6S virus pair, in which the rt sequences shared 63% homology. Of the 74 homologous recombination events that we identified, we observed 18, 0 and 56 crossovers in pro, rt and in, respectively. Additionally, we analyzed 120 sequences from GFP+ cell pools for the H0-SynRT/T6S-IN2 virus pair, in which both rt and in regions had decreased homology. Of the 16 homologous recombination events that we identified, we observed 14, 0 and 2 recombination events in pro, rt and in, respectively. Therefore, we did not observe homologous crossovers between the wild-type rt and synthetic rt regions for either pair of constructs, indicating that more than 99% of the recombination events were blocked. However, we did detect two homologous crossovers between the HIV-1 and HIV-2 in regions; both events occurred in a conserved stretch of 13 nucleotides. We estimate that ∼96% of crossovers were blocked in the in region. Taken together, these results demonstrate that our strategy of recoding the genome to block recombination was successful.

To determine the consequences of blocking recombination, we next analyzed proviral genome structures. We first examined whether there were deletions outside the region that we altered (rt and in), as these would likely not be due to blocked recombination. Of the 336 sequences that we obtained, there were only two deletions located entirely outside the rt and in regions: one deletion was observed among the progeny of the control virus pair H0/T6S and one deletion was observed among the progeny of the virus pair H0-SynRT/T6S-IN2 (Supplementary Figure S3). Therefore, deletions outside the rt and in regions occurred at similar rates among the progeny from different virus pairs. However, we observed strikingly higher levels of deletions involving rt and in sequences in the progeny of the noncontrol virus pairs. Among progeny from the control, we observed one deletion in 86 sequences, resulting in a deletion frequency of ∼1.2% (Figure 3). In contrast, we observed 9 deletions in 130 progeny sequences from H0-SynRT/T6S (∼6.9%; \( P = 0.05 \); Fisher's exact test; Figure 3) and 16 dele-
Table 1. Decreased nucleotide sequence homology blocks homologous recombination

| Vector pair       | Seq | pro Crossovers | pro Crossovers/seqa | rt Crossovers | rt Crossovers/seqa | in Crossovers | in Crossovers/seqa | Total Crossovers | Total Crossovers/seqa |
|-------------------|-----|----------------|---------------------|--------------|---------------------|--------------|---------------------|-------------------|-----------------------|
| H0/T6S            | 86  | 28             | 0.33                | 113          | 1.31                | 40           | 0.47                | 181               | 2.10                  |
| H0-SynRT/T6S      | 130 | 18             | 0.14                | 0            | < 0.01b             | 56           | 0.43                | 74                | 0.57                  |
| H0-SynRT/T6S-IN2  | 120 | 14             | 0.12                | 0            | < 0.01b             | 2            | 0.02                | 16                | 0.13                  |

*a* The total number of crossovers within each region was divided by the total number of sequences.

*b* No crossovers were observed between wild-type and synthetic rt for either vector pair, so the rate of crossovers in rt is less than 1/130 or 1/120 (<0.01).

Figure 3. Blocking recombination leads to large deletions. SGS was performed to generate and analyze a 4.5-kb amplicon including the gag, pro, rt and in regions. The total numbers of sequences analyzed are indicated, but only sequences with deletions are shown. Deletions are depicted as dark gray boxes; deletion frequencies, calculated as deletion events per sequence, are also indicated.

Deletions are mediated by nonhomologous recombination

To examine the mechanism(s) of deletions, we analyzed their locations, sizes and junction sequences (Table 2). Among progeny sequences from the control virus pair (H0/T6S), only one deletion was observed in the region of interest (rt and in). Sequence analysis revealed that RT used a 7-bp direct-repeat sequence to perform an intermolecular template switch, resulting in the removal of a 1.4-kb fragment from pro to rt region. For progeny sequences from H0-SynRT/T6S and from H0-SynRT/T6S-IN2, the sizes of the deletions ranged from 0.1 to 3.3 kb, with an average of 1.7 kb. Importantly, all the 3’ ends of the deletion junctions were located in the region where recombination was blocked due to low sequence homology. For H0-SynRT/T6S, all 9 deletions had 3’ ends in the rt sequence; for H0-SynRT/T6S-IN2, 9 deletions had 3’ ends in the rt sequence and 7 deletions had 3’ ends in the in sequence. These results demonstrate that these deletions were the direct consequence of blocking homologous recombination. Furthermore, direct repeats were observed at most deletion junctions, and ranged in size from 1 to 11 bp (Table 2). The presence of short direct repeats at deletion junctions is a known hallmark of RT-mediated nonhomologous recombination (47,48). Taken together, these results show that, when homologous recombination is blocked, HIV-1 undergoes nonhomologous recombination and loses genome integrity.

Recombination is required for efficient HIV-1 replication

To assess the importance of recombination to HIV-1 replication, we determined the proportion of GFP+ intact proviruses remaining after blocking recombination in 2.5 kb of the viral genome. For the control virus pair (H0/T6S), 5.7% of infected cells expressed GFP and 99% of GFP+
proviruses had intact gag-pol sequences. Therefore, 5.6% of infected cells contained GFP+ intact proviruses. For the virus pair with altered rt and in regions (H0-SynRT/T6S-IN2), 3.1% of infected cells expressed GFP and only 88% of GFP+ proviruses had intact gag-pol sequences. Overall, 2.8% of infected cells contained GFP+ intact proviruses. Thus, blocking recombination in the rt and in regions resulted in the loss of 51% of GFP+ intact proviruses. In these experiments, we blocked recombination in only 28% of the viral genome (2.5 out of 9.2 kb). Recombination events have been shown to occur throughout the HIV-1 genome (11,35). Based on our results and the assumption that the events that eliminated GFP+ intact proviruses are randomly distributed, we predict that ∼92% of GFP+ intact proviruses would be lost if homologous recombination was blocked across the entire 9.2-kb genome (‘Materials and Methods’ section). Thus, recombination is a major mechanism to maintain full-length genome structure and promote efficient HIV-1 replication.

**DISCUSSION**

A critical step in viral replication is the transfer of genetic information to progeny. Retroviral particles package RNA genomes and must convert the genetic information into a DNA form before generating a provirus in the new target cell. To successfully complete DNA synthesis, RT performs two obligatory strand-transfer events: minus- and plus-strand transfers. In this report, we demonstrate that, most of the time, HIV-1 RT must undergo additional template-switching events via homologous recombination to complete synthesis of full-length DNA. This conclusion comes from our observation that blocking homologous recombination in less than one-third of the HIV-1 genome re-

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**Table 2.** Features of deletions observed upon blocking homologous recombination

| Vector pair | # | 5' end location | 3' end location* | Size (kb) | Type† | Junction sequence‡ | Repeat length (bp) |
|-------------|---|----------------|------------------|-----------|-------|-------------------|-------------------|
| H0/T6S      | 1 | pro            | rt (H0)          | 1.4       | Inter | CAAAGTGGAGGCA     | 7                 |
|             | 2 | rt             | rt (H0)          | 0.7       | Intra | GTAGCCAAAT         | 3                 |
|             | 3 | rt             | rt (T6S)         | 1.1       | Intra | AGGAAGAAAACAT     | 7                 |
|             | 4 | gag            | rt (H0)          | 1.7       | Inter | CTTCAAGC          | 2                 |
|             | 5 | rt             | rt (H0)          | 1.2       | Intra | AAAAGAAGGTTG       | 6                 |
|             | 6 | rt             | rt (H0)          | 0.7       | Intra | GTCGCTTTT          | 2                 |
|             | 7 | rt             | rt (H0)          | 0.6       | Intra | ACCAAAAAGGAGCCATT  | 11                |
|             | 8 | gag            | rt (T6S)         | 1.5       | ND    | TTAGGAAACAAAGC     | 10                |
|             | 9 | gag            | rt (T6S)         | 2.6       | ND    | AGACAGTG           | 2                 |
|             | 10*| gag           | rt (H0)          | 1.5       | Inter | N/A               | N/A               |
|             | 11‡| gag           | rt (H0)          | 0.1       | Intra | CAGGCTACGCAC       | 8                 |
|             | 12‡| gag           | rt (H0)          | 1.5       | ND    | TAGCTCT            | 1                 |
|             | 13 | rt             | rt (H0)          | 0.7       | Inter | AAAATAGA           | 2                 |
|             | 14 | gag            | rt (H0)          | 2.5       | ND    | CAGGGACAAG         | 4                 |
|             | 15 | rt             | rt (H0)          | 0.2       | Intra | GCCGAAAG           | 1                 |
|             | 16 | rt             | rt (T6S)         | 0.4       | Intra | CTAAAGAACCAT       | 8                 |
|             | 17 | gag            | rt (T6S)         | 2.1       | ND    | C TGCCCTTTAGAAA    | 10                |
|             | 18 | gag            | rt (T6S)         | 2.6       | ND    | ATAGTCAT           | 1                 |
|             | 19 | gag            | in (H0)          | 2.7       | ND    | GCCAATAG           | 2                 |
|             | 20 | gag            | in (T6S)         | 2.7       | ND    | ATAGCCCAAT         | 4                 |
|             | 21 | rt             | in (H0)          | 1.7       | Intra | AGG.ATA            | 0                 |
|             | 22 | gag            | in (H0)          | 3.1       | ND    | CATIAAG            | 1                 |
|             | 23 | gag            | in (T6S)         | 2.8       | ND    | ACC. ACT           | 0                 |
|             | 24 | gag            | in (H0)          | 3.3       | ND    | GACAAAGGACCAGC     | 8                 |
|             | 25 | rt             | in (H0)          | 1.5       | Intra | ATCTCTCT           | 2                 |

* The origins of the donor sequences are indicated in parentheses, with H0 representing H0 or H0-SynRT and T6S representing T6S or T6S-IN2.
† Deletion type (intra- or intermolecular) was determined only when both ends of the deletion were <70 bp away from a genetic marker, which corresponds to less than a 5% chance of recombination between the deletion and marker; ND, not determined.
‡ Deletion junction sequences, with direct repeats underlined and mutations shown in blue. Only one copy of each direct repeat is shown.
§ This sequence contains a complex insertion (≥17 bp).
¶ This sequence contains two deletions.
sulted in the loss of half of proviruses with intact genomes. Although frequent retroviral recombination has been observed for four decades, our study provides a clear demonstration of its role in maintaining genome integrity. Thus, in addition to generating viral diversity, recombination is critical for viral replication. This study answers a long-standing question in the field of retrovirology and reveals previously unknown aspects of HIV-1 replication.

Our results also shed light on the mechanistic basis of HIV-1 recombination. We found that when homologous recombination was blocked in the rt and in regions, only 49% of intact proviruses survived. In regions of the genome where recombination was blocked, deletion frequencies increased significantly (Figure 3), and deletion junctions often contained short stretches of homology between donor and acceptor templates (Table 2). Thus, when homologous recombination is not possible and RT cannot continue synthesis of the same template, RT uses small regions of homology to switch elsewhere in the genome. These findings are consistent with the central thesis of the forced copy-choice model, which proposes that recombination serves as a repair mechanism to salvage damaged genomes (4). However, in our experiments, when recombination was not blocked, 78% of proviruses had at least one crossover in the rt or in region. If all crossovers were obligatory or ‘forced’ — that is, caused by RNA breaks (4) or irreversible kinetic events during DNA synthesis (16) — then only ~22% of intact proviruses would survive. We observed that 49% of intact proviruses survived, far more than the predicted 22%, indicating that some, but not all, crossovers are forced. These findings are consistent with the central thesis of the dynamic copy-choice model, which proposes that recombination depends on the kinetic stability of the RT complex and that crossovers can be forced or not forced (16). If we assume that the events that force recombination are randomly distributed, our data are consistent with ~40% of crossovers being forced (See ‘Materials and Methods’ section for calculation). Therefore, we conclude that HIV-1 recombination occurs through both forced and dynamic copy-choice models.

Whether both copackaged retroviral genomes are required for DNA synthesis has been studied previously with varied conclusions. Using spleen necrosis virus, one study concluded that only one RNA genome is required for DNA synthesis, because minus-strand DNA transfer was found to be mostly intramolecular in nature (49). However, spleen necrosis virus, like murine leukemia virus, may not form heterozygous particles efficiently (50–54). Thus, most of the analyzed progeny may have been from homzygous particles, in which case intra- and intermolecular minus-strand transfers cannot be distinguished. Using HIV-1 vectors, another study concluded that one infection event can generate two proviruses, because plaque assays gave rise to rare plaques containing more than one provirus (55). However, these experiments only measured rare recombinants resulting from DNA recombination during cotransfection. This limitation would result in severe underestimation of parental virus titers, such that the observed events were likely due to coinfection. Lastly, using HIV-1 vectors, it was reported that a marker gene was inactivated in target cells more frequently when cotransfected with an empty vector lacking the marker gene (56). However, further analyses revealed that the internal promoter sequence in the vector contained cryptic splice sites, with most inactivation coming from the packaging of aberrantly spliced RNAs lacking the complete marker gene. Therefore, these previous studies did not address whether both packaged RNA genomes are required for DNA synthesis in retroviral replication. In contrast, in our study, we specifically blocked recombination in only part of the viral population—the heterozygous particles—and we observed a significant decline in the progeny of heterozygous particles (Figure 2). Proviral structure analyses showed that deletions were significantly increased specifically in the regions of the genome in which recombination was blocked (Figure 3). Wild-type and altered sequences were used as deletion donors at similar rates (Table 2), suggesting that deletions were not caused by abnormal features of the engineered sequences. Furthermore, the deletions were marked by small regions of homology (Table 2), a known hallmark of nonhomologous recombination. Taken together, these results demonstrate that both copies of the RNA genome must be used during reverse transcription of most HIV-1 particles, although only a single provirus is generated.

Retroviruses transfer their genetic information between RNA and DNA forms, and it is intriguing to contemplate how this transfer evolved to use a pseudodiploid replication strategy. Retroviruses need to perform two strand-transfer steps during DNA synthesis to reconstitute the long terminal repeats. Therefore, RT must be able to switch templates to some extent, and this ability likely enabled template switching elsewhere in the genome through the process of homologous recombination. It is possible that retroviruses evolved to package two copies of the RNA genome to compensate for the tendency of RT to switch templates, thereby preserving genome integrity. On the other hand, it is also possible that because retroviruses package two copies of the RNA genome, they have less selective pressure to evolve a more processive RT or a mechanism to better protect the RNA genome. Regardless of the evolutionary path, the pseudodiploid replication strategy has obviously been highly successful, resulting in hundreds of different retroviral species, including HIV-1.

Our study reveals that efficient HIV-1 replication requires both copackaged RNAs and recombination to maintain genome integrity. These findings unveil the possibility that novel antiviral strategies could be developed to control HIV-1 replication by targeting the essential recombination process or the packaging of two RNA genomes. Such novel strategies could be used in combination with other antivirals such as approved RT inhibitors. Although reverse transcription is a major target for current antiretrovirals, these compounds do not specifically target the essential recombination process during DNA synthesis. Approaches that block different aspects of reverse transcription, such as DNA synthesis and homologous recombination, could potentially achieve synergistic antiviral effects.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.
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