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Homologous Recombination Occurs in a Distinct Retroviral Subpopulation and Exhibits High Negative Interference

WEI-SHAU HU,[1,2] ELLA HARVEY BOWMAN,[1] KRISTA A. DELVIKS,[3] AND VINAY K. PATHAK[2,4]*

Department of Microbiology and Immunology,[1] Department of Biochemistry,[4] Department of Genetics and Developmental Biology,[3] and Mary Babb Randolph Cancer Center,[2] School of Medicine, West Virginia University, Morgantown, West Virginia 26506

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Homologous recombination and deletions occur during retroviral replication when reverse transcriptase switches templates. While recombination occurs solely by intermolecular template switching (between copackaged RNAs), deletions can occur by an intermolecular or an intramolecular template switch (within the same RNA). To directly compare the rates of intramolecular and intermolecular template switching, two spleen necrosis virus-based vectors were constructed. Each vector contained a 110-bp direct repeat that was previously shown to delete at a high rate. The 110-bp direct repeat was flanked by two different sets of restriction site markers. These vectors were used to form heterozygotic virions containing RNAs of each parental vector, from which recombinant viruses were generated. By analyses of the markers flanking the direct repeats in recombinant and nonrecombinant proviruses, the rates of intramolecular and intermolecular template switching were determined. The results of these analyses indicate that intramolecular template switching is much more efficient than intermolecular template switching and that direct repeat deletions occur primarily through intramolecular template switching events. These studies also indicate that retroviral recombination occurs within a distinct viral subpopulation and exhibits high negative interference, whereby the selection of one recombination event increases the probability that a second recombination event will be observed.

Retrovirus populations exhibit high variation, which allows them to evade the host immune response, acquire drug resistance, and frustrate efforts to develop vaccines (8, 14, 21, 51). The rates of genetic variation in retroviral populations depend upon the mutation and recombination rates per replication cycle, the replication rate (replication cycles per time), and the selective forces that act on the population. Recently, clinical isolates of the human immunodeficiency virus type 1 (HIV-1) were observed to harbor mutations in the virally encoded enzyme reverse transcriptase (RT) which conferred resistance to the antiretroviral drug (−)2′-deoxy-3′-thiacytidine (49). The mutant HIV-1 isolates exhibited a higher fidelity and a delay in the development of resistance to other antiretroviral drugs. These observations suggest that mechanisms contributing to variation in retroviral populations may be important determinants of HIV-1 pathogenesis and evolution.

One important mechanism for generating variation in retroviral genomes is reverse transcription. DNA synthesis by RT is error-prone in part because RT lacks a proofreading function. In addition, recombination can occur at a high rate during reverse transcription to further distribute mutations (5, 12, 17, 22, 26, 28, 30, 42, 44). The process of reverse transcription involves two obligatory template switches called minus-strand DNA transfer and plus-strand DNA transfer (11). It has been postulated that RTs have evolved to frequently switch templates to facilitate the two essential strand transfer steps during reverse transcription (45). Template switching events also occur during DNA synthesis of the rest of the viral genome and serve as an important mechanism for generating variation in retroviral populations. Because two copies of the RNA genome are packaged into each virion, template switching can be intramolecular or intermolecular. Intramolecular template switching involves dissociation and reassociation of the growing point of DNA synthesis on the same RNA template. In contrast, intermolecular template switching involves a transfer from one RNA template to a second copackaged RNA molecule. These template switches can result in genetic mutation and recombination, both of which increase retroviral variation.

Template switching is an important mechanism that plays a role in generating different types of mutations during retroviral replication. A broad spectrum of mutations occur during retroviral replication; among these mutations, simple deletions, deletions with insertions, and duplications all involve RT template switching events (9, 29, 32, 33, 35–37, 45, 47). Another consequence of template switching is the high-frequency deletion of direct repeats (20, 26, 34, 36–38, 46, 53). The in vivo frequencies of deletion were determined for direct repeats of different lengths during a single replication cycle of spleen necrosis virus (SNV). Direct repeats of 110, 383, and 788 bp and 1.3 kb deleted at frequencies of 41, 40, 85, and 93%, respectively (25, 36, 37). In addition, high frequency of direct repeat deletion has also been demonstrated for murine leukemia virus (8a). A template misalignment model was proposed to explain the high frequency of direct repeat deletions (25). As shown in Fig. 1A, it was hypothesized that during minus-strand DNA synthesis of the direct repeats, RT can dissociate from the viral RNA template and reassociate either with the 3′ copy of the direct repeat that was being transcribed or with the 5′ copy of the direct repeat. Association with the 5′ direct repeat may be favored by a substantial increase in base pairing between the nascent DNA and the template RNA (3, 43). Only an intramolecular template switch leading to direct repeat deletion is illustrated (Fig. 1A); however, the deletion could theoretically also occur by an intermolecular template switch.

Homologous and nonhomologous recombination between copackaged viral RNAs can reassort mutations and increase variation in retroviral populations (7, 8, 16, 17, 22, 48, 54). An

* Corresponding author. Mailing address: Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV 26506. Phone: (304) 293-5949. Fax: (304) 293-4667. E-mail: VPATHAK@WVUMBRCC1.hsc.wvu.edu.

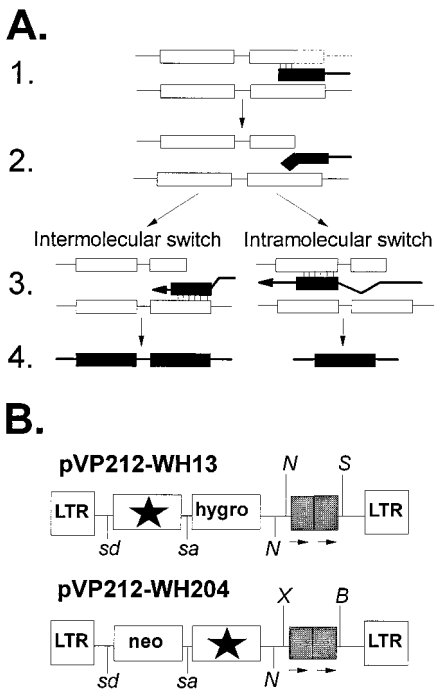

FIG. 1. (A) Intramolecular and intermolecular template switching by RT. RT and the growing point of minus-strand DNA synthesis can dissociate from the RNA template and undergo either an intermolecular or an intramolecular template switch. Both types of template switches can result in the deletion of a direct repeat. (Step 1) Minus-strand DNA synthesis (black box and bold line) begins to copy the region of the first direct repeat (white box). The dotted lines represent RNA template in RNA:DNA hybrids degraded by the virus-encoded RNase H. Vertical lines represent hydrogen bonds between the bases of the RNA template and the nascent strand of DNA. (Step 2) RT and the growing point of nascent DNA synthesis begin to dissociate from the RNA template. (Step 3). An intermolecular template switch results when the growing point of DNA synthesis associates with the homologous region on the second molecule of copackaged viral RNA (left side). An intramolecular template switch results when the growing point of DNA synthesis associates with the same molecule of RNA (right side). (Step 4) An intermolecular template switch results in a recombinant product and may or may not involve the deletion of one of the direct repeats. Only a recombinant without deletion is shown (left side). The deletion of a direct repeat results if realignment occurs with the homologous region in the 5′ copy of the direct repeat (right side). (B) Vectors used to determine rates of intramolecular and intermolecular template switching in one round of retroviral replication. Both vectors contain a neomycin phosphotransferase gene (*neo*) and a hygromycin phosphotransferase B gene (*hygro*). The stars represent the inactivating frameshift mutations in *neo* and *hygro* resulting from a 4-bp insertion. Gray boxes represent 110-bp direct repeats flanked by either an *Nsp*I-*Sal*I pair of restriction enzyme markers (pVP212-WH13) or an *Xba*I-*Bgl*I pair of markers (pVP212-WH204). The *Nsp*I sites shown below the lines are present in both vectors. Pairs of arrows indicate direct repeat sequences; N, *Nsp*I; S, *Sal*I; X, *Xba*I; B, *Bgl*I; LTR, long terminal repeat; sd, splice donor site; sa, splice acceptor site.

intermolecular template switch between copackaged RNAs leading to homologous recombination is illustrated in Fig. 1A. Recent experiments support the copy choice model for homologous recombination, suggesting that during minus-strand DNA synthesis, the growing point of DNA synthesis can switch from one RNA to the copackaged viral RNA (7, 12, 18, 19, 42, 48). This generates a chimeric DNA product containing sequences derived from each of the parental RNAs. While it was thought that a break in the viral RNA was required to cause the template switch (7), it is now believed that a break is not necessary to initiate such switches (25, 53). The rate of homologous recombination in SNV was previously determined to be 4% per replication cycle for two markers separated by a distance of 1 kb (17). While nonhomologous recombination can also serve to increase variation, the rate of nonhomologous recombination is approximately 1,000-fold lower than the rate of homologous recombination (54).

A comparison of the rates of homologous recombination and direct repeat deletions indicates that these two apparently similar events occur at very different rates (4%/kb/cycle versus 85%/0.8-kb/cycle, respectively) (17, 25). Recombination requires an intermolecular template switch, whereas direct repeat deletions may occur by an intramolecular or an intermolecular template switch. The difference in the frequencies of direct repeat deletion and recombination suggests that these two events utilize different acceptor templates. We postulated that direct repeat deletions occur primarily by intramolecular template switches. If this is so, then the frequency of intramolecular template switches will be much higher than the frequency of intermolecular template switches. Little is known about the organization of the two viral RNAs during reverse transcription. If some structural hindrance was to prevent the two RNAs from being equally accessible to the growing point of the nascent DNA strand, then recombination would occur much less frequently than direct repeat deletions. To test this hypothesis, we directly determined the rates of intramolecular and intermolecular template switching for identical sequences in a single replication cycle.

MATERIALS AND METHODS

Definitions. pVP212-WH13 and pVP212-WH204 refer to plasmids, and VP212-WH13 and VP212-WH204 refer to the viruses derived from these plasmids, respectively.

Plasmid construction. pVP212-WH13 and pVP212-WH204 were constructed by modifications of plasmids pVP212 (36), pWH13 (17), and pWH204 (18). Standard molecular techniques were used (41). pVP212 was partially digested with *Bgl*I, treated with T4 DNA polymerase to generate blunt ends, and ligated to a *Sal*I linker to generate pVP212-Sal. pVP212 was digested with *Hin*dIII and self-ligated to generate pVP212-neo, which lacked the *neo* gene. pVP212-neo was partially digested with *Nsp*I, treated with T4 DNA polymerase to generate blunt ends, and ligated to an *Xba*I linker to generate pVP212-neo-Xba. pVP212-Sal and pVP212-neo-Xba were digested with *Hin*dIII and *Hin*dIII plus *Asp*718, respectively; pWH13 and pWH204 were digested with *Asp*718 plus *Cla*I, and their respective *neo*-splice site-*hygro* fragments were isolated; the *neo*-splice site-*hygro* fragments were ligated to pVP212-Sal or pVP212-neo-Xba to generate pVP212-WH13 and pVP212-WH204. A complete description of the cloning steps taken to generate these vectors is available upon request.

Cells, transfections, and virus propagation. D17 (obtained from the American Type Culture Collection) is a canine osteosarcoma cell line permissive for SNV infection (39). This cell line was used as a target for infection experiments. DSDh and C3A2 are D17-derived helper cell lines that express SNV- and reticuloendotheliosis virus-encoded proteins, respectively. Reticuloendotheliosis virus is closely related to SNV, and these helper cell lines efficiently package SNV-based retroviral vectors (10, 17, 50).

All cells were grown in Dulbecco's modified Eagle's medium with 6% calf serum at 37°C with 5% CO_2. G418 selection was performed at 400 μg/ml for single-drug selection and 300 μg/ml for double-drug selection. Hygromycin selection was performed at 240 μg/ml for single-drug selection and 160 μg/ml for double-drug selection. Helper cell clones containing the proviruses of interest were propagated in the presence of chicken anti-SNV antibodies to suppress reinfection.

Transfections were done by the dimethyl sulfoxide-Polybrene method (27). Viral infections were performed immediately following viral harvest. Virus collected from helper cells was centrifuged at 3,000 × *g* for 10 min to remove cellular debris. Ten-fold serial dilutions of each viral stock were used for infection of D17 cells to determine viral titers.

Analysis of proviral DNAs. Proviral DNAs were amplified by PCR (15, 40) in an automated thermal cycler (OmniGene) with two primers that annealed to the regions flanking the direct repeats in each of the proviral constructs. The 5′ primer annealed to the pBRori region of each provirus and was comprised of the sequence 5′-GGACAGGTATCCGGTAAGCGGCAGGGTC-3′. The 3′ primer annealed to the U3 region of each provirus and was comprised of the sequence 5′-GCTTCTCGAATCGGCTGCATTTCTCGGCATC-3′. Following PCR amplification, a 1,761-bp PCR product should be obtained for all proviruses that have not undergone a 110-bp direct repeat deletion. Restriction enzyme analysis of markers flanking the direct repeats was performed for each of the 1,761-bp PCR products to verify that one intact copy of each vector provirus was present in each of the helper cell clones. DNA purification, PCR amplification, and restriction enzyme analysis were performed by standard techniques (41). *Bgl*I,

*Nsp*I, *Sal*I, and *Xba*I restriction enzyme digests were performed for each PCR product to confirm the presence or absence of the four restriction site markers.

RESULTS

Structure of retroviral vectors used to measure rates of intramolecular and intermolecular template switching. Two SNV-based vectors, pVP212-WH13 and pVP212-WH204, were constructed to directly compare the rates of intramolecular and intermolecular template switching during retroviral replication (Fig. 1B). Both vectors contained a neomycin phosphotransferase gene (*neo*) (24) and a hygromycin phosphotransferase B gene (*hygro*) (13) (Fig. 1B). The *neo* gene was expressed from the long terminal repeat as a direct transcript, and the *hygro* gene was expressed as a spliced transcript. Both vectors were constructed with an inactivating frameshift mutation (4-bp insertion) in either *neo* or *hygro*; hence, each vector conferred resistance to only one drug. Vector pVP212-WH13 contained an inactivating frameshift mutation in *neo* and thus conferred resistance only to hygromycin. In contrast, pVP212-WH204 contained an inactivating frameshift mutation in *hygro* and conferred resistance only to the neomycin analog, G418. The recombination rate between the mutations in *neo* and *hygro* has been previously measured as 4% per replication cycle (17). In addition to *neo* and *hygro*, these two vectors contained two tandem copies of a 110-bp region forming a direct repeat. The direct repeats were previously shown to delete at a rate of 41% per replication cycle (36). In each experimental vector, these direct repeats were flanked by a different pair of restriction enzyme sites. The vector pVP212-WH13 had an *Nsp*I and an *Sal*I restriction site flanking the two 110-bp direct repeats, whereas the vector pVP212-WH204 had an *Xba*I site and a *Bgl*I site flanking the direct repeats. In addition, these two vectors differ in three sets of restriction site markers located in the noncoding regions of *neo* and *hygro*; these three sets of markers do not interfere with virus replication or expression of the drug resistance genes. Thus, the two vectors used in these experiments were designed to be highly homologous to each other to avoid any possible bias in packaging efficiencies; the vectors differed only by the 4-bp insertion mutations that inactivated *neo* or *hygro*, the two sets of restriction site markers flanking the direct repeats, and the three sets of restriction site markers in *neo* or *hygro*.

Protocol for analysis of intramolecular and intermolecular template switching in a single replication cycle. The experimental approach taken to directly compare the rates of intramolecular and intermolecular template switching during one round of retroviral replication is outlined in Fig. 2. One replication cycle is defined as the steps required to generate a provirus in the target cell from a provirus in the virus-producing helper cell. This process includes RNA transcription in the producer cells and reverse transcription in the target cells. The vectors pVP212-WH13 and pVP212-WH204 were separately transfected into DSDh helper cells, and each was placed on appropriate drug selection. Next, colonies of hygromycin- or G418-resistant transfected cells were each pooled and expanded, and viruses produced from the cells were used to infect fresh DSDh cells by either coinfection or step infection. In the coinfection protocol, viruses from each of the transfected pools were mixed together and this mixture was used to infect the helper cells. The infected cells were selected for G418 plus hygromycin resistance. In the step-infection protocol, DSDh helper cells were first infected with one of the viruses. Following the appropriate drug selection, resistant cells were pooled and infected with the other virus. The infected cells were again selected for resistance to the appropriate drug. Regardless of the protocol used, single-cell clones resistant to G418 plus hygromycin were isolated and expanded to establish helper cell clones containing a provirus derived from pVP212-WH13 and a provirus derived from pVP212-WH204.

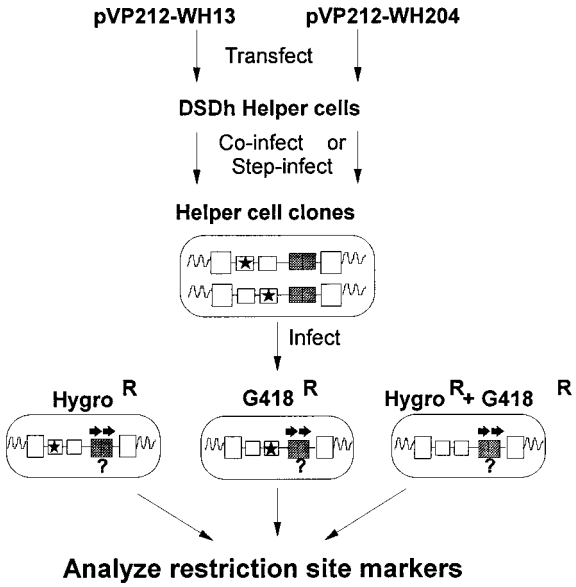

FIG. 2. Protocol for isolating target cell clones to determine rates of intramolecular and intermolecular template switching. DSDh helper cell clones containing one intact copy of the VP212-WH13 and the VP212-WH204 proviruses were isolated by using G418 plus hygromycin double-drug selection. Viruses were harvested from these double-drug-resistant helper cells and used to infect D17 target cells. The D17 target cells were selected by using both single-drug selection and double-drug selection protocols. Zigzag lines, host genomic sequences flanking integrated provirus. Other symbols and abbreviations are as described in the legend for Fig. 1.

Since the 110-bp direct repeat was previously shown to delete at a high frequency, it was important to establish that the direct repeat deletion did not occur during transfection or infection of the DSDh helper cells. The structures of proviruses in the helper cell clones were characterized by PCR and restriction enzyme digestion. The helper cell clones that were selected and used had one copy of each provirus, and both proviruses contained a direct repeat of the 110-bp sequence (data not shown).

It was possible for deletion of a direct repeat to occur during the PCR amplification and thus to complicate the analysis. To eliminate this possibility, the accuracy of the PCR analysis was tested. Six helper cell clones that contained one copy of each undeleted provirus were plated at a low cell density, and a total of 43 subclones were isolated. PCR amplification and restriction enzyme digestion were performed on these 43 cell clones; all of the cell clones generated the expected DNA fragments and correct restriction enzyme digestion patterns (data not shown). This indicated that under the condition used, PCR amplification did not result in detectable levels of direct repeat deletion.

PCR amplification and restriction analysis of 73 helper cell clones were performed. Among these cell clones, 14 helper cell clones contained a VP212-WH13 provirus and a VP212-WH204 provirus, and both proviruses retained two copies of the 110-bp sequence (data not shown). Ten of these fourteen cell clones were used for analysis of template switching events.

Viruses were harvested from the DSDh helper cell clones

containing undeleted VP212-WH13 and VP212-WH204 proviruses and were used to infect D17 target cells (Fig. 2). The infected target cells were selected for resistance to hygromycin, G418, or hygromycin plus G418. VP212-WH13 and VP212-WH204 each contained only one functional drug resistance gene and conferred resistance to a single drug. However, a recombinant virus containing two functional resistance genes could confer resistance to both drugs. This recombinant virus could be generated by an intermolecular template switch between the inactivating frameshift mutations in *neo* and *hygro* during reverse transcription of the copackaged VP212-WH13 and VP212-WH204 RNAs. Theoretically, coinfection with VP212-WH13 and VP212-WH204 could also confer resistance to both hygromycin and G418. However, because of the low multiplicities of infection used in these experiments (<0.001), very few cells were expected to be infected with two or more viruses.

Both single-drug-resistant and double-drug-resistant target D17 cell clones were isolated. The nature and frequencies of intramolecular and intermolecular template switches within the direct repeat regions were analyzed by PCR amplification and restriction analysis. Proviruses in the double-drug-resistant cells were recombinants generated from heterozygotic virions and contained two functional genes. Proviruses in the single-drug-resistant colonies could have been generated from either heterozygotic or homozygotic virions. In most of the helper cell clones analyzed, the two viral vectors were expressed at similar levels, and it was expected that three populations of viruses would be formed at a ratio of 1:2:1 (VP212-WH13/VP212-WH13, VP212-WH13/VP212-WH204, and VP212-WH204/VP212-WH204). Random viral RNA packaging is assumed because these two vectors are almost identical in nucleotide sequences. Therefore, we expect that half of the proviruses in the single-drug-resistant colonies were generated from heterozygotic virions and that an intermolecular template switch in these proviruses could be observed.

Protocol for PCR amplification and restriction analysis of intramolecular and intermolecular template switching. The protocol utilized to analyze the nature and frequency of template switching is illustrated in Fig. 3. The D17 target cell clones were individually propagated, and proviral genomes were amplified by PCR (Fig. 3). Amplification of the proviral DNAs with and without direct repeat deletions were expected to yield 1,651- and 1,761-bp fragments, respectively. Some proviruses would generate fragments containing the same *Nsp*I-*Sal*I or *Xba*I-*Bgl*I restriction site markers as expected for the VP212-WH13 and VP212-WH204 parental vectors, respectively. The remaining proviruses would generate fragments containing *Nsp*I-*Bgl*I or *Xba*I-*Sal*I restriction site markers, indicative of an intermolecular template switch between the restriction site markers flanking the direct repeats. Thus, the size of the PCR product and the restriction site markers could be used to determine whether an intramolecular or an intermolecular template switch occurred.

Representative restriction analysis of a provirus without deletion of the direct repeat and a provirus with deletion of the direct repeat occurring through an intermolecular template switch are shown in Fig. 3. Both PCR products were analyzed by gel electrophoresis without digestion or with digestion with *Bgl*I, *Nsp*I, *Sal*I, and *Xba*I restriction enzymes. The provirus analyzed in the left panel retained the 110-bp direct repeat, since the undigested PCR product was 1,761 bp long. Also, the provirus was not a recombinant, since digestion of the PCR product with *Xba*I and *Bgl*I yielded smaller restriction fragments expected of the parental VP212-WH204 vector (825 and 936 bp for *Xba*I and 1,109 and 652 bp for *Bgl*I). Digestion of the same PCR product with *Nsp*I generated a 1.5-kb fragment and a 220-bp fragment, as expected for the parental VP212-WH204 vector. In both vectors, an *Nsp*I site is located 0.6 kb upstream of the direct repeats, and digestion at this site generates a 220-bp fragment and a 1.5-kb fragment (Fig. 1B and 3). An additional *Nsp*I site present in VP212-WH13 upstream of the direct repeats cleaves the 1.5-kb fragment into smaller fragments. Thus, the presence of the 1.5-kb fragment indicated that the *Nsp*I site upstream of the direct repeats was not present in the PCR product. Finally, digestion with *Sal*I did not cleave the 1,761-bp PCR product, confirming that all of the restriction site markers were derived from VP212-WH204 and not from VP212-WH13. Therefore, this provirus was not a recombinant between VP212-WH13 and VP212-WH204. Since the single-drug-resistant and double-drug-resistant D17 target cell clones each represent a single infectious event, only one provirus should be present in each of the target cell clones. Thus, the D17 target cell clones should contain only two of the four restriction site markers.

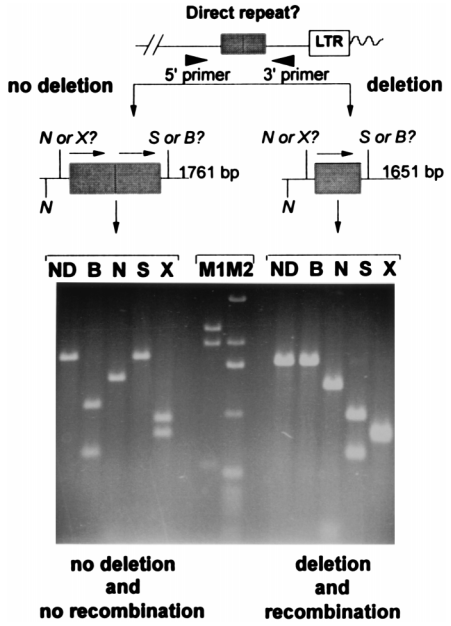

FIG. 3. Protocol for PCR amplification and restriction enzyme mapping of proviral region containing direct repeats. A 1,761-bp fragment or a 1,651-bp fragment was obtained upon PCR amplification of proviruses containing both copies of the direct repeat and a deletion of one of the copies, respectively. The left side of the photo depicts an example of the restriction enzyme mapping for a wild-type provirus (labelled "no deletion and no recombination"). The right side of the photo depicts an example of the restriction enzyme mapping for a recombinant clone that deleted one of the direct repeats (labelled "deletion and recombination"). Arrowheads, 5′ and 3′ PCR primers; ND, not digested; B, *Bgl*I; N, *Nsp*I; S, *Sal*I; X, *Xba*I; M1, λ*Hin*dIII marker; M2, 1-kb ladder. Other symbols and abbreviations are as described in the legend for Fig. 1.

Similar PCR and restriction analysis of a recombinant provirus with a deletion of the direct repeat are shown in the right panel of Fig. 3. The undigested PCR product is 1,651 bp long, indicating that the provirus had deleted one copy of the 110-bp direct repeat. Digestion of the PCR product with *Bgl*I did not generate additional restriction fragments, and digestion with *Nsp*I generated the 1.5- and 0.2-kb fragments, indicating that these restriction site markers were not flanking the direct repeat. In contrast, digestion of the PCR product with *Sal*I and *Xba*I generated the expected restriction fragments, indicating that these restriction site markers were present in the provirus (999 plus 652 bp for *Sal*I and 825 plus 826 bp for *Xba*I). Since

TABLE 1. Analysis of proviruses from single-drug-resistant and double-drug-resistant target cell clones

Cell clone type	Provirus type	No. without deletion	No. with deletion
Single-drug resistant[a]			
G418 resistant	Parental	34	9
G418 resistant	Recombinant	0	0
Hygromycin resistant	Parental	29	18
Hygromycin resistant	Recombinant	0	0
Total		63	27
Double-drug resistant[b]			
G418 plus hygromycin resistant	Parental	29	15
G418 plus hygromycin resistant	Recombinant	5	4
Total		34	19

[a] Recombination frequencies for single-drug-resistant proviruses were calculated as follows: total number of recombinants/total number of proviruses × 100% (0/90 × 100% = 0%). Deletion frequencies for single-drug-resistant cell clones were calculated as follows: total number of deletions/total number of proviruses × 100% (27/90 × 100% = 30%).

[b] Recombination frequencies for double-drug-resistant proviruses were calculated as follows: total number of recombinants/total number of proviruses × 100% (9/53 × 100% = 17%). Deletion frequencies for double-drug-resistant cell clones were calculated as follows: total number of deletions/total number of proviruses × 100% (19/53 × 100% = 36%).

the *Sal*I restriction site marker is present in VP212-WH13, and the *Xba*I restriction site marker is present in VP212-WH204, an intermolecular template switch must have occurred between these two restriction site markers to generate this recombinant provirus. In this manner, PCR amplification and analysis of the restriction site markers flanking the direct repeats were performed to determine the nature and frequency of template switches during a single replication cycle.

Intramolecular template switching occurs much more frequently than intermolecular template switching. The PCR amplification and restriction analysis of the PCR products illustrated in Fig. 3 were performed on 43 G418-resistant cell clones and 47 hygromycin-resistant cell clones (Table 1). A 30% deletion frequency was observed (27 of 90), confirming that the 110-bp direct repeat deleted at a high frequency in a single replication cycle (37). All proviruses in the G418-resistant clones with deletions contained the *Xba*I-*Bgl*I restriction site markers, and all proviruses in the hygromycin-resistant clones with deletions contained the *Nsp*I-*Sal*I restriction site markers. None of the proviruses generated PCR products with *Nsp*I-*Bgl*I or *Xba*I-*Sal*I restriction site markers, indicating that all of the observed direct repeat deletions most likely occurred through an intramolecular template switch. In addition, none of the 63 clones that retained the direct repeat had an observable intermolecular template switch between the restriction site markers. No intermolecular template switches were observed among the 90 proviruses; approximately 45 proviruses (half of 90) are expected to be derived from heterozygotes, which can undergo observable intermolecular template switches. Therefore, the recombination frequency for the proviruses selected for single-drug resistance was less than 2% (1/45 × 100%). These results indicated that in the population of cell clones selected for resistance to either G418 or hygromycin, the rate of intramolecular template switching was much higher than the rate of intermolecular template switching.

The rate of intermolecular template switching is much higher in proviruses selected for resistance to G418 plus hygromycin. Identical PCR amplification and restriction analysis were also performed on 53 cell clones that were resistant to G418 plus hygromycin (Table 1). Since the cell clones were resistant to both drugs, the proviruses present were recombinants between the parental vectors VP212-WH13 and VP212-WH204. A total of 19 of the 53 proviruses (36%) had deleted one copy of the direct repeat, indicating that the deletion frequency was similar to the frequency observed for single-drug-resistant proviruses. In sharp contrast to the result obtained for the single-drug-resistant cell clones, a high proportion of the proviruses had undergone an intermolecular template switch between the restriction site markers. Of the 53 proviruses, 9 had either the *Nsp*I-*Bgl*I or the *Xba*I-*Sal*I restriction site markers, indicating that 17% of the proviruses underwent an intermolecular template switch between the restriction site markers. Four of the nine proviruses that recombined between the restriction site markers had also deleted one copy of the direct repeat, indicating that about half of the time, the intermolecular template switch resulted in a direct repeat deletion.

Retroviral recombination occurs in a distinct subpopulation and exhibits negative interference. The deletion and recombination frequencies of proviruses selected for single-drug resistance were compared to the deletion and recombination frequencies of proviruses selected for resistance to G418 plus hygromycin (Table 2). The results indicated that the selection of cell clones for one recombination event (resistance to both G418 and hygromycin) dramatically increased the probability that a second recombination event would be observed (*Nsp*I-*Bgl*I or *Xba*I-*Sal*I restriction site markers) ($P < 0.003$). Therefore, retroviral recombination exhibits high negative interference.

The overall rates of intramolecular and intermolecular template switching were also estimated by adjusting for the proportion of total viruses that conferred resistance to either single- or double-drug selection (Table 2). The recombination rate between the inactivating frameshift mutations in *neo* and *hygro* was measured previously (17). Based on this recombination rate, approximately 2% of the cells should contain double-drug-resistant proviruses and the other 98% of the cells should contain single-drug-resistant proviruses. Adjusting for these proportions, the overall deletion frequency was 30% for the 110-bp direct repeat. Similarly, the recombination frequencies were adjusted for the proportions and pooled. The overall recombination frequency was <2.3% between the two markers flanking the direct repeats. These frequencies confirmed that

TABLE 2. Comparison of deletion and recombination frequencies in single-drug-resistant and double-drug-resistant populations

Type of resistance	Frequency (%) of:	
	Recombination	Deletion
G418 or hygromycin[a]	<2	30
G418 plus hygromycin[b]	17	36
Total	<2.3[c]	30[d]

[a] Single-drug-resistant cell clones represented 98% of the total population.

[b] Double-drug-resistant cell clones represented 2% of the total population.

[c] Recombination frequency, normalized to the total population, was calculated as follows: (<2% × 98%) + (17% × 2%) = <2.3%.

[d] Deletion frequency, normalized to the total population, was calculated as follows: (30% × 98%) + (36% × 2%) = 30%.

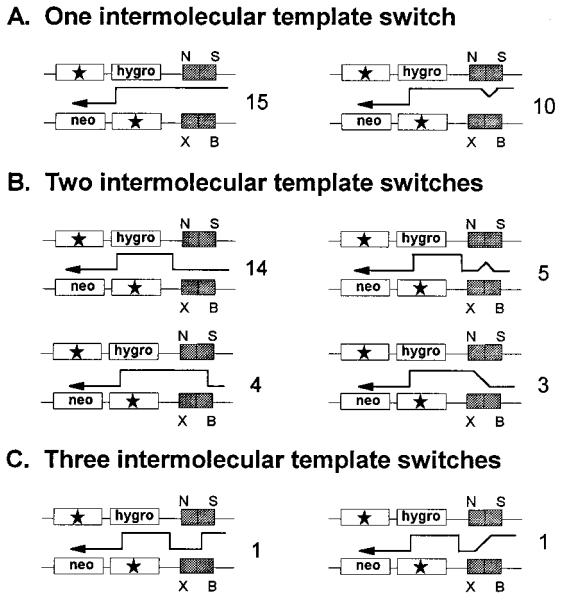

FIG. 4. Analysis of template switches in proviruses of double-drug-resistant cell clones. Only the middle portion of the viral genome structure is shown. Proviruses with one observable intermolecular template switch (A), two observable intermolecular template switches (B), and three observable intermolecular template switches (C). All abbreviations are the same as those in Fig. 1B. Numbers of recombinants with the observed phenotypes are listed after the deduced template switching events.

intramolecular template switching occurs more frequently than intermolecular template switching.

A high proportion of recombinant proviruses have multiple intermolecular template switches. Analysis of the intermolecular template switches that occurred in the 53 proviruses selected for double-drug resistance is shown in Fig. 4. Selection for resistance to both G418 and hygromycin indicated that at least one intermolecular template switch occurred between the inactivating frameshift mutations in *neo* and *hygro* in all 53 recombinant proviruses. The presence of *Nsp*I-*Sal*I restriction site markers in 25 of the 53 proviruses suggested that only one intermolecular template switch between the mutations in *neo* and *hygro* was required for the formation of these proviruses; 10 of these 25 proviruses also underwent direct repeat deletion through an intramolecular template switch.

The presence of *Xba*I-*Bgl*I restriction site markers in 19 of the 53 proviruses suggested that at least two intermolecular template switches occurred during the formation of these proviruses. One intermolecular template switch occurred between the *Xba*I restriction site marker and the inactivating frameshift mutation in *hygro*, and a second intermolecular template switch occurred between the mutations in *neo* and *hygro*. Five of these nineteen proviruses also underwent direct repeat deletion through an intramolecular template switch.

Seven of the fifty-three proviruses had the *Nsp*I-*Bgl*I restriction site markers indicative of an intermolecular template switch between the restriction site markers flanking the direct repeats. Therefore, one template switch occurred between the restriction site markers, and a second template switch occurred between the mutations in *neo* and *hygro* during the formation of these proviruses. Three of these seven proviruses also underwent direct repeat deletion through the intermolecular template switch between the restriction site markers flanking the direct repeats.

Two of the fifty-three proviruses had the *Xba*I-*Sal*I restriction site markers indicative of an intermolecular template switch; one of these two proviruses also underwent direct repeat deletion through the intermolecular template switch between the restriction site markers. A second intermolecular template switch occurred between the *Xba*I restriction site marker and the inactivating mutation in *hygro*, and a third intermolecular template switch occurred between the inactivating mutations in *neo* and *hygro*. Thus, at least three intermolecular template switches occurred in the formation of these proviruses.

Based on this analysis, 25 of the 53 recombinant proviruses (47%) underwent at least one intermolecular template switch, 26 of the 53 proviruses (49%) underwent at least two intermolecular template switches, and 2 of the 53 proviruses (4%) underwent at least three intermolecular template switches. Therefore, the probability of observing a second intermolecular template switch (53%) was considerably higher than the probability of observing one intermolecular template switch (2%).

DISCUSSION

Intramolecular template switching is much more efficient than intermolecular template switching. In this study, we directly compared the rates of intramolecular and intermolecular template switching. The results clearly show that intramolecular template switching occurs much more frequently than intermolecular template switching. Intramolecular template switching occurred at a rate of 30% for the 110-bp direct repeat; the rate of intermolecular template switching for a 250-bp region encompassing the 110-bp direct repeat was <2.3%. These results suggest that when RT dissociates from the template, it does not have equal access to both copackaged molecules within the nucleoprotein complex and it is most likely to reassociate with the same template RNA.

Direct repeat deletions occur primarily through intramolecular template switching. The results obtained in this study demonstrate that deletion of directly repeated sequences primarily occurs through intramolecular template switching by RT. None of the 27 proviruses that were resistant to a single drug and underwent direct repeat deletion underwent an observable intermolecular template switch. Direct repeat deletion is mechanistically similar to simple deletion, a type of mutation that frequently occurs during reverse transcription (37). Simple deletions characteristically have short direct repeats at their deletion junctions. The observation that direct repeat deletions primarily occur through intramolecular template switching strongly suggests that all simple deletions probably also occur through intramolecular template switches (32, 33, 35, 37).

Recombination occurs in a distinct subpopulation of retroviruses. The results obtained in this study indicate the existence of two viral subpopulations. In one subpopulation, the rate of both intramolecular and intermolecular template switching was high. Analysis of proviruses from double-drug-resistant cell colonies indicated that 17% of the viruses underwent an intermolecular template switch between restriction site markers flanking the direct repeats. In the second subpopulation, the rate of intramolecular template switching was high but no intermolecular template switching was observed between the same restriction site markers. It is expected that half of the 90 proviruses generated from the single-drug-resistant cell clones were generated from heterozygotic virions. If intermolecular template switching occurred at the same frequency in these viruses, then 7.7 proviruses would be expected

to undergo an observable intermolecular template switch between the restriction site markers; however, no such intermolecular template switches were observed ($P < 0.003$). The absence of any detectable recombination in the population of cell clones selected for resistance to a single drug indicates that recombination occurs in a distinct subpopulation of viruses. The two subpopulations of viruses coexisted in the same virus pool because the same virus pools were used to generate the single-drug-resistant and the double-drug-resistant cell clones.

In these two subpopulations, the overall rates of direct repeat deletion were not significantly different (30% for single-drug-resistant cell clones and 36% for double-drug-resistant cell clones). Within the viral subpopulation that underwent intermolecular template switching, the rate of intramolecular template switches leading to direct repeat deletion (15 of 53, 28%) was four times that of intermolecular template switches leading to the same deletion (4 of 53, 7.5%).

The nature of the differences between the two subpopulations is not known. We hypothesize that a subpopulation of retroviruses have an altered structure of the reverse transcription complex. This change in the reverse transcription complex allows the RT access to both genomic template RNAs and frequently switches templates between them. The putative alteration in the reverse transcription complex does not affect the rate of intramolecular template switching. RNA breaks have long been postulated to play a role in retroviral recombination, and one possibility is that breaks in the viral RNA constitute the structural alteration that lead to high rates of recombination. However, it is unlikely that RNA breaks are necessary for intermolecular template switching. The rate of intermolecular template switching within the subpopulation of viruses appears to be very high (17% per 250 bp). The length of the vector RNA is approximately 6 kb, which is 24-fold longer than the 250-bp region analyzed (6,000 bp/250 bp). Therefore, the rate of intermolecular template switching within the subpopulation of viruses can be extrapolated to the complete vector genome as 24 times 17%, or 408%. This suggests that an average of four breaks would be present in each viral RNA if the breaks were required for template switching. One possibility is that RNAs that contain one break are more likely to contain additional breaks. Breaks in the viral RNA may lead to other structural alterations that promote intermolecular template switching. A second possibility is that the first intermolecular template switch causes a structural alteration in the reverse transcription complex, which in turn increases the probability that additional intermolecular template switching events will be observed. Another possibility is that the failure of copackaged RNAs to dimerize or the packaging of two RNA dimers in one virion may result in structural alterations which promote recombination.

Alternatively, the nearly identical RNAs of the two vectors may not be copackaged randomly, and the subpopulation of viruses that undergo recombination could simply be the heterozygotic viruses. Recent analysis of RNA movements in living cells suggests the formation of RNA granules and their active transport along microtubules (1). Further, localized RNA distribution has been observed for cytoskeletal protein mRNAs and several drosophila embryonic mRNAs (1). These studies have suggested the hypothesis that newly synthesized RNAs may be transported along cytoskeletal elements to their destination. It is conceivable that RNAs that are transcribed from two proviruses integrated at different chromosomal sites exit the nucleus at different locations and are thus transported along microtubules to different locations in the cell. If this is so, then the two RNAs would rarely colocalize and their copackaging frequency would be less than 50% of the virus population. However, eight helper cell clones with random proviral integrations have been analyzed, and the rates of recombination for the clones are nearly identical (range, 1 to 2.6%; mean ± standard deviation, 1.8 ± 0.6) (17), suggesting that the proviral integration sites do not strongly influence the rates of recombination. Second, the RNA localization signals appear to be present in the 3′ untranslated regions of mRNAs (2, 31); since the two mRNAs derived from VP212-WH13 and VP212-WH204 are identical in their 3′ untranslated regions, this cytoplasmic localization mechanism cannot distinguish between the two mRNAs.

The results of this study also confirm and extend some observations from previous studies of minus-strand DNA transfer events. It was observed that minus-strand transfer could occur both intramolecularly and intermolecularly in a population of viruses undergoing recombination (18). Later, it was observed that although both types of transfers can be observed in recombinant viruses, minus-strand DNA transfer primarily occurs through an intramolecular template switch in viruses that are not selected for recombination events (23). Our result that selection for one recombination event increases the probability that a second intermolecular template switch will be observed parallels these studies. This result confirms previous observations that minus-strand DNA transfer, a specific template switch during reverse transcription, is more likely to occur intermolecularly when the virus is selected for a recombination event.

Retroviral recombination exhibits high negative interference. Within the subpopulation of recombining viruses, multiple intermolecular template switches occurred at a greater-than-expected probability; this is defined as high negative interference (4, 52). The expected rate of multiple recombination events can be estimated by utilizing the previously measured rate of one intermolecular template switch (4%/kb/replication cycle) and assuming that the rate of recombination is directly proportional to the distance between the markers. The distance between the inactivating frameshift mutations in *neo* and *hygro* is 1 kb; the distance between the inactivating frameshift mutation in *hygro* and the *Xba*I restriction site marker is approximately 2.48 kb, and the distance between the inactivating frameshift mutation in *hygro* and the *Nsp*I/*Xba*I and the *Sal*I/*Bgl*I restriction site markers is approximately 0.29 kb. Based on these considerations, the expected rate of intermolecular template switches between the mutation in *hygro* and the *Nsp*I/*Xba*I restriction site markers was approximately 10% (5 of 53 proviruses). Contrary to this expectation, we observed that at least 40% of the proviruses had an intermolecular template switch within this region (21 of 53 proviruses) ($P < 0.005$). Similarly, the expected rate of intermolecular template switches between the restriction site markers was approximately 1% (0.5 of 53 proviruses). In contrast, we observed that at least 17% of the proviruses had an intermolecular template switch between the restriction site markers (9 of 53 proviruses) ($P < 0.05$). These observed frequencies are at least fourfold higher than expected for the region between the mutation in *hygro* and the *Xba*I marker and 17-fold higher than expected for the distance between the restriction site markers flanking the direct repeat. The observed frequency of three intermolecular template switches is also higher than expected. We expected that approximately 0.1% of the proviruses (10% × 1%, or 0.05 of the 53 proviruses analyzed) would have three intermolecular template switches. Thus, the observed 4% frequency of three intermolecular template switches (2 of 53 proviruses) is higher than the expected frequency. Taken together, these analyses clearly indicate that the rate of multiple recombina-

tion events is higher than the product of the rates of single recombination events.

Implications for the evolutionary potential of retroviruses. These results have important implications for the evolutionary potential of retroviruses. Since retroviral recombination exhibits high negative interference, a subpopulation of viruses can undergo multiple intermolecular template switching events to repair extensive mutational damage and generate wild-type replication-competent virus. The same high frequency of multiple template switching can result in faster generation of multiple advantageous mutations in a single viral genome. For example, HIV variants that have multiple mutations conferring resistance to a combination of antiprotease and anti-RT drugs should arise at much higher rates than were previously predicted. Multiple mutations in the V3 loop of the HIV envelope gene must accumulate for the virus to interact with HIV-1 coreceptors and alter their target cell specificity (6). The rate of the accumulation of these mutations may also be much higher than previously predicted. Clearly, selection pressures will also be important in determining the rate of accumulation and the proportion of these genotypes in the viral population. The high frequency of multiple template switching events suggests that the evolutionary potential of retroviruses is likely to be much greater than previously anticipated. Therefore, these results have important implications for HIV pathogenesis, evolution, and drug resistance.

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