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Correlated Template-Switching Events during Minus-Strand DNA Synthesis: a Mechanism for High Negative Interference during Retroviral Recombination

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Two models for the mechanism of retroviral recombination have been proposed: forced copy choice (minus-strand recombination) and strand displacement-assimilation (plus-strand recombination). Each minus-strand recombination event results in one template switch, whereas each plus-strand recombination event results in two template switches. Recombinant proviruses with one and more than one template switches were previously observed. Recombinants with one template switch were generated by minus-strand recombination, while recombinants containing more than one template switch may have been generated by plus-strand recombination or by correlated minus-strand recombination. We recently observed that retroviral recombination exhibits high negative interference whereby the frequency of recombinants containing multiple template-switching events is higher than expected. To delineate the mechanism that generates recombinants with more than one template switch, we devised a system that permits only minus-strand recombination. Two highly homologous vectors, WH204 and WH221, containing eight different restriction site markers were used. The primer binding site (PBS) of WH221 was deleted; although reverse transcription cannot initiate from WH221 RNA, it can serve as a template for DNA synthesis in heterozygotic virions. After one round of retroviral replication, the structures of the recombinant proviruses were examined. Recombinants containing two, three, four, and five template switches were observed at 1.4-, 10-, 65-, and 50-fold-higher frequencies, respectively, than expected. This indicates that minus-strand recombination events are correlated and can generate proviruses with multiple template switches efficiently. The frequencies of recombinants containing multiple template switches were similar to those observed in the previous system, which allowed both minus- and plus-strand recombination. Thus, the previously reported high negative interference during retroviral recombination can be caused by correlated template switches during minus-strand DNA synthesis. In addition, all examined recombinants contained an intact PBS, indicating that most of the plus-strand DNA transfer occurs after completion of the strong-stop DNA.

Frequent homologous recombination has been reported in all examined retroviruses, including avian, murine, and human viruses (2, 20, 21, 23, 25, 28, 29, 41–43). Recombination increases retroviral genetic fitness by increasing the variation in the viral population through reassortment of mutations generated during viral replication by the error-prone, virally encoded reverse transcriptase (RT). This allows the development of viral strains that escape host immune systems (31, 34, 36) or strains that are resistant to one or more antiviral drugs (8, 14, 15, 23, 24, 26, 40). In addition, recombination allows the completion of reverse transcription in virions with damaged RNA genomes (4, 12). Therefore, recombination plays an important role in retroviral pathogenesis, treatment, and vaccine development.

Frequent recombination occurs during reverse transcription between two copackaged RNAs (11). Two models to explain the mechanism of retroviral recombination have been proposed: forced copy choice (4) and strand displacement-assimilation (19) (Fig. 1). The forced copy choice model proposes that recombination occurs during minus-strand DNA synthesis. When RT encounters breaks in the RNA genome, it can switch templates and use the other copackaged RNA for DNA synthesis (Fig. 1A). Therefore, a viral DNA containing genetic information from both parental RNAs is generated. It was later suggested that breaks in the viral RNA are not required for template switching (18, 46). This model is referred to as "minus-strand recombination" to avoid reference to the structure of the packaged viral RNA. In contrast, the strand displacement-assimilation model proposes that both RNAs undergo initiation of reverse transcription to form two minus-strand DNAs (Fig. 1B). An internally initiated plus-strand DNA fragment can be displaced from one minus-strand DNA molecule and be assimilated with the other minus-strand DNA. A recombinant is formed after DNA mismatch repair. This model is referred to as "plus-strand recombination."

The structures of the recombinants differ in these two proposed models. Each template-switching event during minus-strand DNA synthesis results in one template switch (a crossover) in the viral DNA. In contrast, each strand displacement-assimilation event during plus-strand DNA synthesis results in two crossovers in the viral DNA. Thus, recombinants containing one crossover are generated by minus-strand recombination; however, recombinants with two crossovers can be generated by one plus-strand recombination event or two minus-strand recombination events.

Previously, by using two highly homologous spleen necrosis virus (SNV)-based vectors that contained eight different sets of restriction enzyme markers, the frequencies of recombinants

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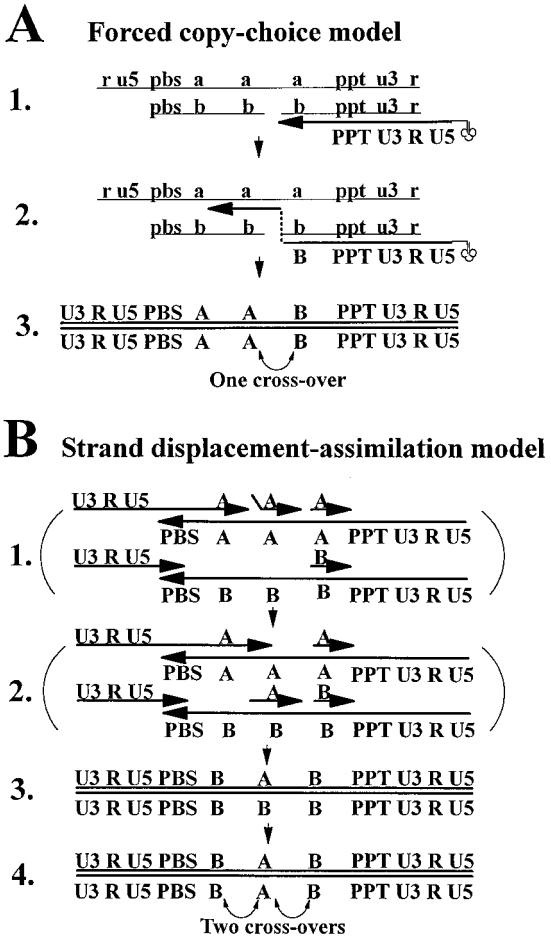

FIG. 1. Models for retroviral recombination: forced copy choice (A) and strand displacement-assimilation (B). Viral RNA sequences are indicated by thin lines and lowercase letters, whereas viral DNA sequences are indicated by thick lines and uppercase letters. a and b represent two copackaged viral RNAs, while A and B represent DNA sequences generated from the two copackaged RNAs. Cloverleaf, tRNA primer; dotted line, template-switching event; ppt/PPT, polypurine tract. The direction of DNA synthesis is indicated by the arrows. (A) In step 1, RT uses RNA b as a template for DNA synthesis until a break in the RNA is encountered. In step 2, RT switches to use RNA a as a template for DNA synthesis. In step 3, the resulting DNA is a recombinant with one crossover event. (B) Two DNA copies are generated from the two copackaged RNAs. In step 1, an internally initiated plus-strand DNA fragment from DNA A is displaced by an upstream-initiated plus-strand DNA. In step 2, this displaced DNA is assimilated to the complementary region of minus-strand DNA B. In step 3, the resulting DNA has a mismatched region. In step 4, a recombinant with two crossovers is formed after mismatch repair.

with one crossover and more than one crossover were determined (10). Ten of 22 recombinant proviruses contained one crossover, indicating that they were generated by minus-strand recombination. In addition, 12 of 22 recombinant proviruses contained more than one crossover; however, the mechanism(s) used to generate these recombinants was not clear.

We recently demonstrated that retroviral recombination exhibits high negative interference (13). The relative rates of inter- and intramolecular template-switching events were examined. It was found that if a recombination event was selected, the probability of a second recombination event was significantly higher than expected by random chance; this is defined as high negative interference (1a, 45). In order to delineate the mechanism(s) generating recombinants with more than one crossover, we designed a system that allows

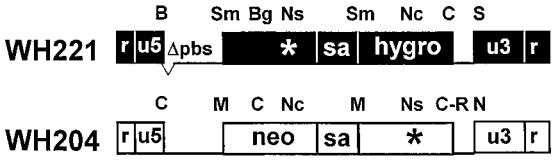

FIG. 2. SNV-based retroviral vectors used to study recombination during minus-strand DNA synthesis. WH221 RNA is shown in black, and WH204 RNA is shown in white. Restriction enzyme sites are indicated above the viral RNAs. Δpbs, deletion of the PBS; neo, neomycin phosphotransferase gene; sa, splice acceptor site derived from reticuloendotheliosis virus strain A; hygro, hygromycin phosphotransferase B gene; *, inactivating frameshift mutation generating a unique *Nsi*I restriction enzyme site; B, *Bam*HI; C, *Cla*I; Sm, *Sma*I; M, *Mlu*I; Bg, *Bgl*II, Ns, *Nsi*I; Nc, *Nco*I; C-R, *Cla*I followed by *Eco*RI; S, *Sac*I; N, *Not*I.

recombination to occur only during minus-strand DNA synthesis. The molecular nature of the recombinants was examined and compared with those from the previous study (10).

MATERIALS AND METHODS

Definitions and plasmid construction. pWH204 and pWH221 refer to plasmids, whereas WH204 and WH221 refer to the viruses derived from these plasmids. pWH204 has been described previously (10). pWH221 was constructed from pWH13 (11) with the deletion of 104 bp between the *Bsp*MII and *Nar*I restriction enzyme sites. This deletion removed the primer binding site (PBS), 27 bp 5′ to the PBS, and 59 bp 3′ to the PBS. A detailed description of the vector constructions is available upon request.

Cell culture, DNA transfection, and virus infection. D17 (obtained from the American Type Culture Collection) is a dog osteosarcoma cell line permissive for SNV infection (37). DSDh is a D17-derived helper cell line that expresses SNV-encoded proteins (10). All cells were grown in Dulbecco's modified Eagle's medium with 6% calf serum at 37°C with 5% CO_2. G418, a neomycin analog, and hygromycin selections were performed at concentrations of 400 and 80 μg/ml, respectively. DSDh helper cell clones were propagated in the presence of chicken anti-SNV polyclonal antibodies to suppress reinfection.

Transfections were performed by the dimethyl sulfoxide-Polybrene method (22). Viral infections were performed immediately after viral harvest. Viruses were collected from helper cells and centrifuged at 3,000 × *g* for 10 min to remove cellular debris. Tenfold serial dilutions were made from each viral stock, and viral titers were determined by infecting 2×10^5 D17 cells per 60-mm-diameter dish.

Mapping of recombinant proviruses. Recombinant proviruses were mapped by PCR. DNA lysates were prepared from cell clones resistant to G418 plus hygromycin and used as a substrate for PCR (9). Proviral genomes were amplified with different sets of primers and analyzed by restriction enzyme digestion (see Fig. 5). The sequences for the primers have been described previously (10) and are as follows: U3Sac, 5′-TGGGAGGGAGCTCTGGGGGGA-3′; U3Not, 5′-TGGGAGGGGCGGCCGCCTGGG-3′; 1934OEN, 5′-ACACCCAGCCGG CCACAGTCG-3′; Neo1115, 5′-GGCGATAGCTAGACTGGGCGG-3′; 3288yH, 5′-TGCCTCCGCTCGAAGTAGCGC-3′; Neo2007, 5′-CCGCTTCCTCGTGC TTTACGG-3′; U5HMAB, 5′-CGGATTCAGTCCGGATCCCTG-3′; U5ALC, 5′-CGGATTCAGTCCGGATCGATC-3′. All PCRs were carried out in a Hybaid Omnigene thermal cycler for 40 cycles.

The amplified DNAs were analyzed with various restriction enzyme digestions. All DNA manipulations were performed by standard procedures (30).

RESULTS

Retroviral vectors used to examine minus-strand recombination events. Two SNV-based vectors, pWH221 and pWH204, were used to examine the nature of the recombinants generated during minus-strand DNA synthesis. pWH221 and pWH204 are very similar in sequence; both vectors contain a neomycin phosphotransferase gene (*neo*) and a hygromycin phosphotransferase B gene (*hygro*) (Fig. 2) (7, 10, 17). In both vectors, *neo* is expressed from a full-length transcript from the retroviral long terminal repeat, whereas *hygro* is expressed from a spliced message. These vectors, however, differ in several aspects. pWH221 contains a functional *hygro* and an inactivated *neo*, whereas pWH204 contains a functional *neo* and an inactivated *hygro*. A 4-bp frameshift mutation inactivated *hygro* or *neo*; this mutation also destroyed an *Nco*I restriction enzyme site and generated a unique *Nsi*I restriction enzyme site.

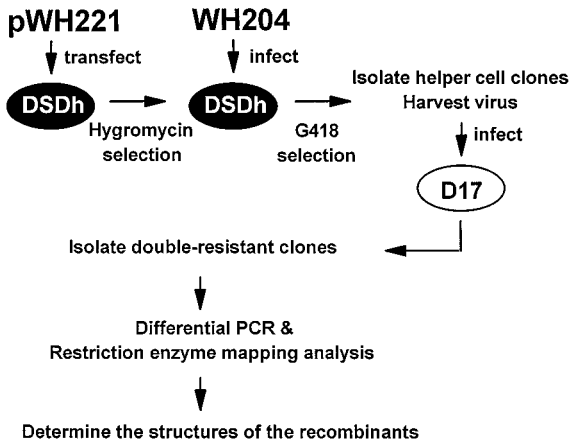

FIG. 3. Experimental protocol for studying recombination during minus-strand DNA synthesis.

The entire PBS of pWH221 was deleted. In addition to the PBS deletion in pWH221 and the inactivating mutations, these two highly homologous vector RNAs differ at six other restriction enzyme markers. These markers are located in U5 (*Bam*HI/*Cla*I), between encapsidation sequences (E) and *neo* (*Sma*I/*Mlu*I), in the 5′ noncoding region of *neo* (*Bgl*II/*Cla*I), between the splice acceptor and *hygro* (*Sma*I/*Mlu*I), 3′ to *hygro* (*Cla*I/*Cla*I-*Eco*RI), and in U3 (*Sac*I/*Not*I) (Fig. 2). The natures of these six sets of markers are 4- to 8-bp insertions; furthermore, these markers do not interfere with viral replication or with the expression of *neo* or *hygro* (10).

Based on the current model of reverse transcription (6), minus-strand DNA synthesis is not expected to be initiated from WH221 RNA because it lacks a PBS. However, reverse transcription can initiate from WH204 RNA. Therefore, in a heterozygotic virion containing a copy of WH221 RNA and a copy of WH204 RNA, WH221 can be used as a template for DNA synthesis after reverse transcription initiates from the PBS of WH204. Recombination during plus-strand DNA synthesis, such as in the strand displacement-assimilation model, requires the presence of two minus-strand DNAs. Since only one minus-strand DNA is synthesized, plus-strand recombination cannot occur in these heterozygotes. Therefore, recombinants from WH221 and WH204 can be generated only during minus-strand DNA synthesis.

Experimental protocol and virus titers. The protocol used to study the mechanism of recombination is outlined in Fig. 3. Retroviral vector pWH221 was first introduced into SNV-based DSDh helper cells by transfection because of its inability to complete reverse transcription. These transfected cells were placed on hygromycin selection, and resistant cells were pooled. WH204 was then introduced into hygromycin-resistant DSDh cells by infection. Cells were placed on G418 selection, and the resistant cell clones were isolated. Viruses were harvested from these double-drug-resistant DSDh cell clones and used to infect D17 cells. D17 cells were placed on single-drug selection (G418 or hygromycin) or double-drug selection (G418 plus hygromycin). After drug selection, cell clones resistant to G418 plus hygromycin were isolated and the molecular nature of the proviruses in these cell clones was characterized by PCR and restriction enzyme mapping. The multiplicities of infection used to generate these double-drug-resistant cell clones were very low (WH204, <0.05; WH221 or recombinant, <0.00004), and each cell clone should represent only one infection event. Because each parental virus contains only one functional drug resistance gene, the double-drug-resistant cells most likely contain a recombinant provirus expressing a functional *neo* and a functional *hygro*.

TABLE 1. D17 viral titers obtained with WH221 and WH204[a]

Expt	Viral titer (CFU/ml)		
	G418 (10^3)	Hygromycin (10^0)	G418 + hygromycin (10^0)
1	6	5	2
2	10	7	5
3	6	0	1
4	8	1	1
5	4	2	1
Avg	7	3	2

[a] Virus was harvested from DSDh cells containing WH221 and WH204.

Viral titers generated from five different DSDh helper cell clones are shown in Table 1. The G418 titers varied from 4×10^3 to 1×10^4 CFU/ml of virus, with an average of 7×10^3 CFU/ml. The hygromycin titers varied from 0 to 7×10^0 CFU/ml, with an average of 3×10^0 CFU/ml. The G418-plus-hygromycin titers varied from 1 to 5×10^0 CFU/ml, with an average of 2×10^0 CFU/ml.

Effect of minus-strand DNA transfer on the number of template switches in double-drug-resistant proviruses. In this system, recombinants are selected by the ability to confer resistance to G418-plus-hygromycin selection. In order to obtain a functional *neo* and a functional *hygro*, RT has to avoid the two inactivating mutations and copy the *Nco*I marker in the *hygro* of WH221 as well as the *Nco*I marker in the *neo* of WH204 (Fig. 4). Thus, these two restriction enzyme sites are selectable markers; recombination between these two markers is the selected recombination event.

The minimum number of template switches needed to generate a G418-plus-hygromycin-resistant provirus is dependent upon the type of minus-strand strong-stop DNA transfer during reverse transcription. The two possibilities are shown in Fig. 4. In a heterozygotic virion, reverse transcription is expected to initiate only from WH204, with a $tRNA^{Pro}$ primer bound to the PBS (27), because WH221 lacks a PBS. Thus, the resulting minus-strand strong-stop DNA contains sequences from WH204 including the U5 *Cla*I marker that is located 34 nucleotides (nt) upstream of the 5′ end of the PBS. Minus-strand DNA can be transferred intermolecularly to the 3′ R region of WH221 (Fig. 4A) or intramolecularly to the 3′ R of WH204 (Fig. 4B) (10, 16, 33). After an intermolecular minus-strand DNA transfer, only one recombination event is required to generate a provirus with two functional drug resistance genes (Fig. 4A). In contrast, if an intramolecular minus-strand DNA transfer occurs, then at least two recombination events are required (Fig. 4B). The first template switch must occur before the inactivating *Nsi*I mutation of *hygro* in WH204, and the second template switch must occur between the two *Nco*I markers.

PCR amplification and restriction enzyme analysis of the recombinant proviruses. By using viruses harvested from 5 different DSDh helper cell clones, a total of 47 double-drug-resistant D17 target cell clones were generated. To ensure that independent recombination events were studied, all cell clones were isolated from different cell culture dishes. The nature of the proviruses in these cell clones was analyzed by PCR and restriction enzyme mapping. The approximate locations of primers used for PCR and the expected sizes of PCR products

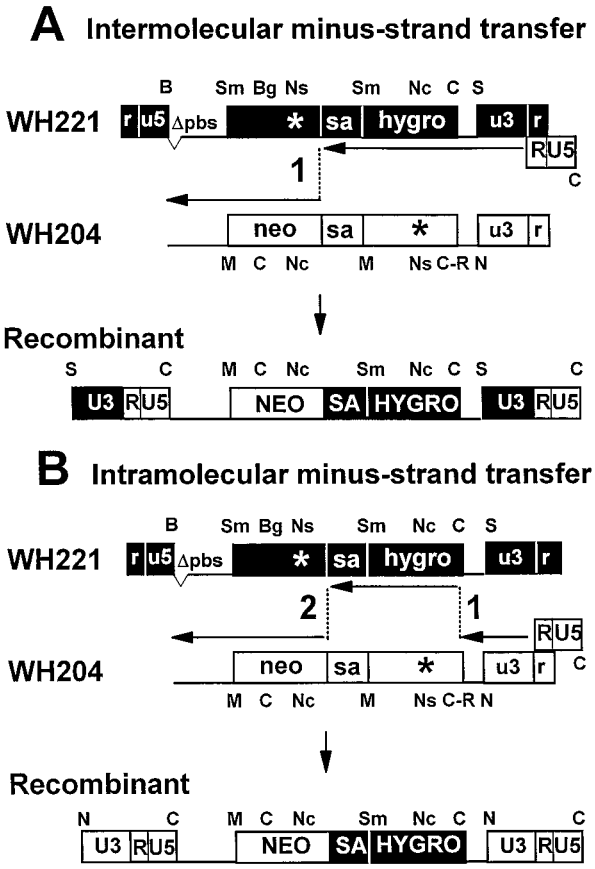

FIG. 4. The minimum number of template switches required to produce a recombinant provirus with a functional *neo* and a functional *hygro* is dependent on the type of minus-strand strong-stop DNA transfer. (A) Intermolecular transfer. (B) Intramolecular transfer. Symbols and abbreviations are the same as in Fig. 1 and 2.

are shown in Fig. 5A. The 5′ ends of the recombinant proviral genomes were amplified with primer sets 1A and 1B. Primer sets 1A and 1B utilize the same 3′ primer, 1934OEN, whereas the 5′ primers, U3Sac (1A) and U3Not (1B), are specific to the U3 regions of WH221 and WH204, respectively. The center portions of the proviruses were amplified with primer set 3, primers Neo1115 and 3288yH. The 3′ ends of the recombinant proviral genomes were amplified with primer sets 2A and 2B. Primer sets 2A and 2B contain the same 5′ primer, Neo2007, whereas the 3′ primers, U5HMAB (2A) and U5ALC (2B), are specific to the U5 regions of WH221 and WH204, respectively. WH221 contains a deletion in the PBS and was not expected to initiate minus-strand DNA synthesis. Therefore, primer set 2A was not expected to generate an amplified DNA fragment in the recombinant cell clones.

A representative differential PCR analysis is shown in Fig. 5B. Primer sets 1A and 1B were used to amplify two cell clone lysates, AQ2 and W12, that each contained a recombinant provirus. Using AQ2 DNA lysate as a template, a 1.93-kb PCR product was obtained with primer set 1A but not primer set 1B, indicating that this provirus contains the U3 *Sac*I marker from WH221. In contrast, clone W12 DNA produced a 1.93-kb amplification product with primer set 1B but not primer set 1A, indicating that the W12 provirus contains the U3 *Not*I marker from WH204. Reverse transcription initiates only from WH204 RNA; thus, AQ2 was generated by an intermolecular minus-strand DNA transfer event, whereas W12 was generated by an intramolecular minus-strand DNA transfer event.

Restriction enzyme digestion analysis was performed on PCR products to determine the molecular nature of the recombinant proviruses. Representative restriction mapping of the 5′ proviral genome for clones AQ2 and W12 is shown in Fig. 5C and D, respectively. Restriction enzyme digestion analysis of amplified DNA from AQ2 revealed 1.03- and 0.90-kb bands when digested with *Bam*HI, 1.38- and 0.55-kb bands when digested with *Cla*I, 1.03- and 0.90-kb bands when digested with *Sma*I, an undigested 1.93-kb band with *Mlu*I, 1.23- and 0.70-kb bands when digested with *Bgl*II, a 1.83-kb band when digested with *Nco*I, and an undigested 1.93-kb band when digested with *Nsi*I (Fig. 5C). Besides the *Bam*HI marker in the U5 region of WH221, there is an additional *Bam*HI site located 5′ to *neo* in both WH221 and WH204 (shown in Fig. 5A). PCR products from proviruses that contain the U5 *Cla*I marker should generate 1.03- and 0.90-kb bands when digested with *Bam*HI, whereas the PCR products from proviruses that contain the U5 *Bam*HI markers should generate 0.35-, 0.55-, and 1.03-kb bands (not shown). These results indicate that the provirus in clone AQ2 contained the U5 *Cla*I and *Nco*I markers from WH204 and the *Sac*I, *Sma*I, and *Bgl*II markers from WH221. A partial map derived from this analysis is illustrated in Fig. 5C. This analysis indicates that there were at least two recombination events in the 5′ portion of the AQ2 proviral genome; one occurred between *Nco*I of WH204 and *Bgl*II of WH221, and the other occurred in E between the *Sma*I of WH221 and the PBS of WH204.

PCR and restriction enzyme analysis revealed that the provirus in clone W12 differed from the provirus in clone AQ2 in the U3 marker and the marker between E and *neo* (Fig. 5D). The provirus in W12 contained the *Mlu*I marker of WH204, whereas the provirus in AQ2 contained the *Sma*I marker of WH221. Similar to the provirus in AQ2, the provirus in W12 also underwent at least two recombination events in the 5′ portion of the proviral genome. One template switch occurred between the *Nco*I site of WH204 and the *Bgl*II site of WH221, and the other occurred in the 5′ noncoding region of *neo* between the *Bgl*II marker of WH221 and *Mlu*I of WH204.

Molecular nature of the recombinant proviruses. The genomes of 47 recombinant proviruses were amplified by PCR and mapped with restriction enzymes; their structures are shown in Fig. 6. All of the recombinant clones contained the U5 *Cla*I marker derived from WH204. This indicates that the PBS deletion in the WH221 RNA prevents initiation of minus-strand DNA synthesis. Of the 47 proviruses analyzed, 29 contained the U3 *Sac*I marker from WH221, indicating an intermolecular minus-strand DNA transfer event, whereas 18 contained the U3 *Not*I marker from WH204, indicating an intramolecular minus-strand DNA transfer event. These data are consistent with the previous observation that minus-strand DNA transfer occurs both intermolecularly and intramolecularly in recombinant proviruses (10).

All of the recombinants that had an intermolecular minus-strand DNA transfer also contained an odd number of recombination events (see Discussion for explanation). Of these 29 proviruses, 18 had one recombination event, 10 had three recombination events, and 1 had five recombination events (Fig. 6A). Thus, 11 proviruses contained template switches in addition to the obligatory recombination event between the two selectable markers. Ten proviruses had two additional template-switching events upstream of the selectable markers. The first template switch occurred either between the WH204 *Nco*I marker and the WH221 *Bgl*II marker (nine proviruses) or between the WH204 *Cla*I marker and the WH221 *Sma*I

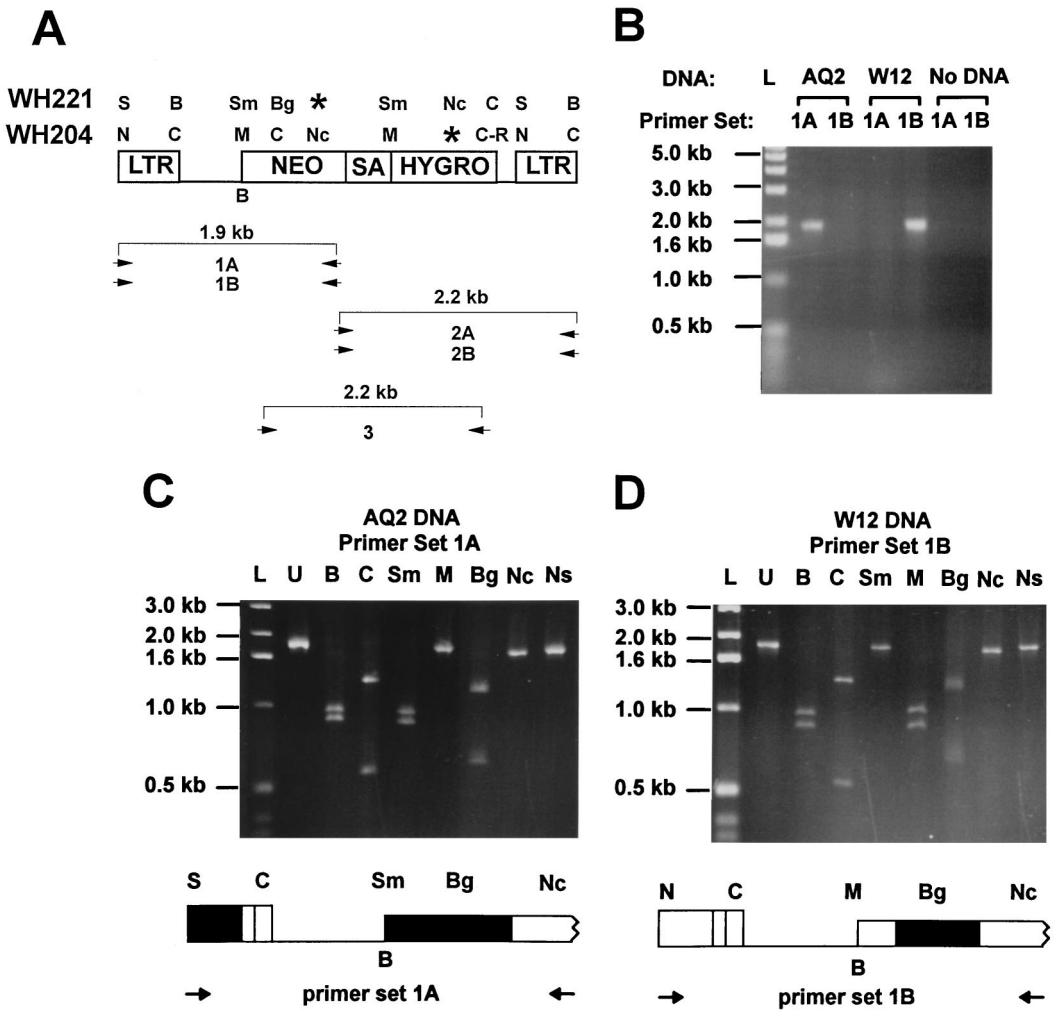

FIG. 5. Strategy for mapping recombinant proviruses in cell clones. (A) Generic structure of a recombinant provirus. Above the recombinant, restriction enzyme markers are listed, and a *Bam*HI site contained in both WH221 and WH204 is indicated beneath the recombinant. Primer sets for differential PCR analysis and the expected sizes of the amplification products are shown below the recombinant. Arrows indicate primer directions. (B) Representative differential PCR analysis of recombinant cell clones AQ2 and W12 with primer sets 1A and 1B. L; 1-kb ladder. (C) Restriction enzyme digestion analysis of AQ2 DNA amplified with primer set 1A. (D) Restriction enzyme digestion analysis of W12 DNA amplified with primer set 1B. U, undigested DNA. The 5′ structure of each recombinant proviral DNA derived from the analysis is illustrated below the restriction enzyme mapping gel in panels C and D. Symbols and abbreviations are the same as in Fig. 1 and 2.

marker (one provirus). The second template switch occurred either between the WH221 *Bgl*II marker and the WH204 *Mlu*I marker (seven proviruses) or between the WH221 *Sma*I marker and the PBS of WH204 (three proviruses). Two proviruses contained two additional recombination events downstream of the selectable markers. The first occurred between the U3 *Sac*I marker of WH221 and the *Cla*I-*Eco*RI marker 3′ to *hygro* in WH204, and the second occurred between the WH204 *Cla*I-*Eco*RI marker and the *Nco*I marker in *hygro* of WH221.

All of the recombinants that had an intramolecular minus-strand transfer had an even number of recombination events (see Discussion for explanation). Of these 18 proviruses, 9 had two recombination events and 9 had four recombination events (Fig. 6B). Thus, nine proviruses contained two template switches in addition to the two obligatory recombination events required to obtain a functional *hygro* and a functional *neo*. The additional template switches occurred upstream of the selected *Nco*I in *neo* of WH204. The first of these occurred either between the WH204 *Nco*I and the WH221 *Bgl*II markers (eight proviruses) or between the WH204 *Cla*I and WH221 *Sma*I markers (one provirus). The second occurred either between the WH221 *Bgl*II and WH204 *Mlu*I markers (four proviruses) or between the WH221 *Sma*I marker and the PBS of WH204 (five proviruses).

The majority of recombination events occur during minus-strand DNA synthesis. Two results indicate that in this system recombination occurs only during minus-strand DNA synthesis. First, virus harvested from DSDh cells transfected with pWH221 was unable to transfer hygromycin resistance to D17 cells (data not shown). In addition, none of the 47 recombinants contained the U5 *Bam*HI marker from WH221. These data confirm that reverse transcription is not initiated from the PBS-deleted WH221. Therefore, plus-strand recombination cannot occur in this system because only one minus-strand DNA molecule can be formed.

In addition to the minimum recombination events needed to generate proviruses with a functional *neo* and *hygro*, other recombination events were frequently observed in the proviruses analyzed in this system (20 of 47; 42%). In the previous

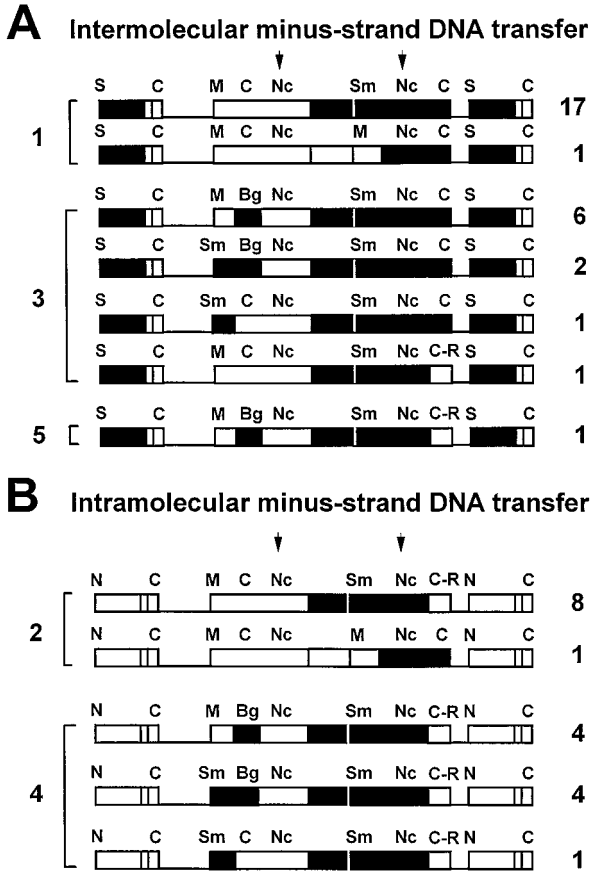

FIG. 6. Restriction enzyme maps of recombinant proviruses. Maps are shown with an intermolecular minus-strand DNA transfer (A) and an intramolecular minus-strand DNA transfer (B). WH221-derived sequences are shown in black, whereas WH204-derived sequences are shown in white. Restriction enzyme sites are listed above each genotype, and arrows indicate the selectable markers in *neo* and *hygro*. The number to the left of each genotype represents the number of crossovers, and the number to the right indicates the number of recombinants of the same genotype observed among the 47 recombinant proviruses analyzed. Restriction enzyme abbreviations are as given in the legend to Fig. 2.

system, which allowed recombination during minus- and plus-strand DNA synthesis, a similar frequency of proviruses undergoing additional recombination events was observed (11 of 22; 50%) (10). The frequencies of additional recombination events (other than the selected events) are similar in these two systems ($P > 0.562$ [Pearson's chi-square test]). Minus-strand recombination generates recombinants with one crossover. The results in this study demonstrate that minus-strand recombination can also efficiently generate recombinants with more than one crossover. Therefore, it is concluded that template switching during minus-strand DNA synthesis is the major recombination mechanism.

DISCUSSION

Correlated template switching during minus-strand DNA synthesis causes high negative interference in retroviral recombination. With a vector system similar to WH221 and WH204, except that both vectors contained a PBS, the observable recombination rate between the two *Nco*I sites of *neo* and *hygro* was previously measured to be 2% per replication cycle (11). It was estimated that if all retroviruses underwent recombination, then the theoretical observable recombination rate would be 14% (see reference 11 for a detailed calculation). By using the ratio of the measured rate to the theoretical observable rate, it was estimated that one in seven retroviruses would undergo recombination. Because the viruses selected in this study were recombinants, all proviruses contained at least one recombination event (the selected event). If recombination events are independent, then it was expected that 1 in 7 proviruses would have two recombination events, and 1 in 49, 343, and 2401 proviruses would have three, four, and five recombination events, respectively.

Of the 47 recombinant proviruses analyzed in this study, 18 proviruses had one recombination event, and 9, 10, 9, and 1 proviruses had two, three, four, and five recombination events, respectively. The frequencies at which the proviruses with two, three, four, and five recombination events occurred were 1.4-, 10-, 65-, and 50-fold higher, respectively, than expected. Statistical analysis revealed a significant difference between the observed and expected frequencies at which proviruses with one to five recombination events occurred ($P < 0.0001$ [Pearson's chi-square test]). This result indicates that recombination events are not independent. Because only minus-strand recombination can occur in this system, the template-switching events in minus-strand DNA synthesis are correlated. These events can result in the previously observed high negative interference in retroviral recombination (13).

The mechanism for correlated template switching is not known. Recent experimental data has indicated that intramolecular template switching occurs far more frequently than intermolecular template switching (13). This suggested that the two RNAs are not equally accessible to the RT. We have hypothesized that a subpopulation of viruses has an altered structure in the reverse transcription complex which allows the RT to access and switch frequently between the two RNAs (13).

Plus-strand DNA transfer occurs primarily after the completion of strong-stop DNA synthesis. The current view of reverse transcription suggests that the 18-nt complementarity between the PBS in minus-strand DNA and the 3′ end of plus-strand strong-stop DNA is used to mediate plus-strand DNA transfer (6). In this study, two types of minus-strand DNA with different 3′ ends were generated prior to plus-strand DNA transfer. After copying the *Nco*I in *neo* in WH204, minus-strand DNA synthesis could either continue to use WH204 as a template or switch to WH221 RNA. Minus-strand DNA synthesis of WH204 stops after copying the PBS, whereas minus-strand DNA synthesis of WH221 stops after copying the R and U5 regions. Since reverse transcription cannot initiate on WH221, the R and U5 regions were not expected to be degraded by RNase H.

If plus-strand DNA transfer occurs with R-U5 complementarity prior to the completion of strong-stop DNA synthesis, the resulting viral DNA will lack a PBS but have the correct sequences in both ends for integration. Alternatively, R-U5 complementarity can be used to mediate plus-strand strong-stop DNA transfer. If this occurs, there will be a mismatch between the 3′ end of strong-stop DNA that contains a PBS and the minus-strand DNA from WH221 that lacks a PBS. Although mismatch extension can occur with small regions of mismatches, such as 1 to 3 nt (35), this DNA will contain a much larger region of mismatch (45 nt) and is unlikely to carry out efficient extension of plus-strand DNA synthesis. Therefore, these products were not likely to be observed in our provirus studies.

We have examined 19 proviruses that switched to use WH221 as a template during minus-strand DNA synthesis af-

ter copying the selectable marker in WH204. During minus-strand DNA synthesis, 11 of the 19 proviruses switched back to use WH204 RNA as a template prior to copying the *Sma*I marker from WH221, whereas 8 of the 19 proviruses copied the *Sma*I marker from WH221 between E and *neo*. To determine whether these eight proviruses contained a PBS, restriction enzyme mapping of the PCR products was performed to examine the presence of a *Ban*II site, which locates in the PBS. It was found that all eight proviruses contained a PBS (data not shown). Therefore, there is a strong selection for the presence of a PBS in minus-strand DNA. If most plus-strand DNA transfer occurs before completion of strong-stop DNA synthesis with R-U5 complementarity, then there should not be selection pressure for the presence of a PBS in the minus-strand DNA. Since all of the analyzed proviruses contained a PBS, it was concluded that the major substrate for plus-strand DNA transfer is strong-stop DNA. This is consistent with the current view that plus-strand DNA transfer occurs by displacement DNA synthesis (6), and it is expected that strong-stop DNA is mostly used.

The requirement of a PBS in plus-strand DNA transfer determines the number of template switches at the 5′ end of the viral genome. If template switching occurs after copying of the *Nco*I marker in *neo* of WH204, then a second switch back to WH204 must occur to copy the PBS. Therefore, an even number of template switches is always observed if template switching occurs upstream of the selectable markers. The minimum number of template-switching events required to generate a recombinant with two functional drug resistance genes is one and two for intermolecular and intramolecular minus-strand DNA transfer, respectively. Thus, an odd number of template switches is always observed for proviruses generated by intermolecular minus-strand DNA transfer (Fig. 6A). In contrast, an even number of template switches is always observed for proviruses generated by intramolecular minus-strand DNA transfer (Fig. 6B).

Effects of marker distance on recombination. The eight restriction enzyme markers located throughout the viral RNA genome of WH204 and WH221 allowed us to determine the effects of marker distance on recombination events. The *Sma*I/*Mlu*I markers are located between the two selected markers, 0.66 kb downstream of *Nco*I in *neo* and 0.37 kb upstream of *Nco*I in *hygro*. If recombination is proportional to marker distance, then it was expected that among the 47 proviruses analyzed, 30 would recombine in the 0.66-kb region and 17 would recombine in the 0.37-kb region. However, 45 recombined in the 0.66-kb region, whereas 2 recombined in the 0.37-kb region. Therefore, template switching between the *Sma*I of WH221 and the *Nco*I of WH204 occurred at a significantly greater rate than expected ($P < 0.0001$ [Pearson's chi-square test]).

In addition to the selected recombination events, other recombination events also occurred in some of the analyzed proviruses either upstream of *Nco*I in *neo* or downstream of *Nco*I in *hygro*. In the upstream region, 19 proviruses had two recombination events over a 1.17-kb distance from *Nco*I in *neo* to the deletion in WH221. Two sets of markers divide this area into three smaller portions, a 0.24-kb region between the deletion junction of WH221 and the *Sma*I/*Mlu*I markers, a 0.33-kb region between the *Sma*I/*Mlu*I markers and the *Bgl*II/*Cla*I markers, and a 0.60-kb region between the *Bgl*II/*Cla*I markers and the *Nco*I marker of WH204. Because each virus underwent 2 template-switching events, there were a total of 38 template-switching events in the 1.17-kb region. Assuming that recombination events are proportional to marker distance, we expected 8, 11, and 19 template-switching events in the 0.24-, 0.33-, and 0.60-kb regions, respectively. Similar to the expected values, we observed 8, 13, and 17 template-switching events in the 0.24-, 0.33-, and 0.60-kb regions, respectively. The observed frequencies were not significantly different from the expected frequencies ($P > 0.75$ [Pearson's chi-square test]); therefore, the distribution of the unselected recombination events is proportional to the distances between markers in the upstream region.

Twenty proviruses underwent template switching in a 0.77-kb region downstream from the *Nco*I site of *hygro*. This region can be divided into two smaller sections, a 0.70-kb region between *Nco*I in *hygro* and the *Cla*I/*Cla*I-*Eco*RI markers and a 0.07-kb region between the *Cla*I/*Cla*I-*Eco*RI markers and the U3 *Sac*I/*Not*I markers. Among these 20 proviruses, 18 had one template-switching event and 2 had two template-switching events, for a total of 22 recombination events. If recombination is proportional to marker distances, it was expected that 20 recombination events would occur in the 0.70-kb region, whereas 2 events would occur in the 0.07-kb region. Similar to the expected values, we observed 19 and 3 template-switching events in the 0.70- and 0.07-kb regions, respectively. There was no statistical difference between the observed and expected frequencies ($P > 0.459$ [Pearson's chi-square test]); therefore, the recombination events occurring 3′ to the *Nco*I site in *hygro* were proportional to the marker distances.

Taken together, these data suggest that the frequencies of template switching are generally proportional to marker distances from 0.07 to 0.70 kb. The only notable exception is the template-switching events between the two selectable markers. In contrast, when greater marker distances are analyzed, such as 2 and 7 kb, we have observed that the recombination rate reaches a plateau and is no longer proportional to the marker distances (1). Therefore, the relationship between marker distance and recombination rate appears to be proportional within small marker distances under 0.7 kb. The exact nature of this relationship has yet to be determined.

In a previous report, it was postulated that the dimerization linkage structure of murine leukemia virus (MLV) is a recombination hot spot because the close RNA-RNA interactions facilitate template switching (32). The packaging signals of MLV and SNV have little homology in primary sequences but contain conserved double-hairpin secondary structures. The putative dimer linkage structure (DLS) is present in both WH204 and WH221 RNA and is located in the 0.24-kb region upstream of the *Sma*I/*Mlu*I markers. As the analysis indicated, we did not observe significantly frequent template switching in this region. The reason for this observed difference is unclear. One possible explanation is that different viruses were used (MLV versus SNV). It is also possible that additional RNA interactions outside the DLS may facilitate template switching events. Although the DLS was retained in WH221, the PBS and flanking regions were deleted, which could cause this difference.

Among the 47 recombinants analyzed, 3 recombinants contained template-switching events between a set of markers 0.07 kb apart. This indicates that recombination can be observed between very small marker distances. This also brings up the possibility that recombination events may occur more frequently than we have measured because an even number of template-switching events between two markers is not detected.

Comparison of viral titers. In the previous studies, recombination rates were measured by comparing the recombinant titers to the parental titers (11). The two parental viruses were introduced into the helper cell clones by infection; as a result,

the parental viruses generated similar titers in most of the cell clones.

In this system, the two parental vectors were introduced into the helper cells by transfection (WH221) and infection (WH204). Therefore, the different routes of introduction may have affected the level of expression of these two vectors in the helper cells. Furthermore, the hygromycin-resistant titers do not reflect the expression of WH221, because minus-strand DNA synthesis cannot be initiated in WH221. Therefore, recombination rates cannot be directly measured in these experiments.

This, however, should not affect the results and the conclusions of these experiments. In these experiments, the patterns of the recombinants were examined, and conclusions were drawn from the genotypes of the recombinants. Regardless of the viral titers, all recombinants were generated from heterozygotic virions. The level of expression of the two parental vectors would have influenced the percentage of heterozygotic virions formed, but once the heterozygotic virions were formed, reverse transcription and recombination within these heterozygotic virions should not have been affected. Therefore, the patterns of the recombinants should not have been altered by the level of the expression of the parental viruses in producer cells.

Implications of high negative interference during minus-strand DNA synthesis. These results and a previous study (13) indicate that retroviruses can undergo multiple recombination events during a single infectious cycle at a frequency much greater than previously estimated. This high negative interference is relevant to drug treatment and pathogenesis of retroviral infections. For example, human immunodeficiency virus type 1-infected patients may be treated with the antiviral drugs saquinavir, zidovudine (AZT), and (−)2′,3′-dideoxy-3′-thiacytadine (3TC). Mutations in the viral genome that lead to resistance to these drugs have been identified (8, 14, 15, 23, 24, 26, 40). One mutation (L90M) in protease can confer resistance to saquinavir (14, 15), whereas one mutation (M184V) in RT can confer resistance to 3TC (40). Five mutations in RT can confer high-level resistance to AZT (M41L, D67B, K70R, T215Y or -F, and K219Q) (24, 26). With high negative interference, recombination can assort all of these mutations and generate a virus resistant to all three drugs much more frequently than we have previously calculated based on the recombination rate. For example, a heterozygote can form and contain a copy of the RNA with the five mutations that confer resistance to AZT and a copy of the RNA with the two mutations that confer resistance to saquinavir and 3TC. Three recombination events would be needed to generate a virus that contains all of these mutations: one template switch between codons 215 and 184 in RT (93 nt), a second between codons 184 and 70 in RT (342 nt), and a third between codon 41 in RT and codon 90 of protease (150 nt). If recombination events are independent, the probability of observing three recombination events within a 0.6-kb region is low. However, three recombination events within a small region were observed quite frequently in the proviruses analyzed in this study. Therefore, given the presence of the different mutations that confer drug resistance in the viral population, a multi-drug-resistant strain may be produced through correlated recombination events. By the same rationale, recombination can occur in the V3 loop-coding region and the flanking regions to generate viruses with different tropisms or pathogenicity (3, 5, 38, 39, 44). Therefore, in addition to the significant effects on the generation of variation in the retroviral population, correlated template-switching events during minus-strand DNA synthesis also have an impact on the pathogenesis and treatment of retrovirus infections.

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