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Effect of Gamma Radiation on Retroviral Recombination

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To elucidate the mechanism(s) of retroviral recombination, we exposed virions to gamma radiation prior to infecting target cells. By using previously described spleen necrosis virus-based vectors containing multiple markers, recombinant proviruses were studied after a single round of retrovirus replication. The current models of retroviral recombination predict that breaking virion RNA should promote minus-strand recombination (forced copy-choice model), decrease or not affect plus-strand recombination (strand displacement/assimilation model), and shift plus-strand recombination towards the 3′ end of the genome. However, we found that while gamma irradiation of virions reduced the amount of recoverable viral RNA, it did not primarily cause breaks. Thus, the frequency of selected recombinants was not significantly altered with greater doses of radiation. In spite of this, the irradiation did decrease the number of recombinants with only one internal template switch. As a result, the average number of additional internal template switches in the recombinant proviruses increased from 0.7 to 1.4 as infectivity decreased to 6%. The unselected internal template switches tended to be 5′ of the selected crossover even in the recombinants from irradiated viruses, inconsistent with a plus-strand recombination mechanism.

Frequent recombination during retrovirus replication increases the genetic diversity in the virus population and repairs lethal mutations (4, 14, 16, 20). Virions that contain two copies of genetically different RNA are obligatory intermediates for observing this recombination (9, 23–25).

Two models have been proposed for the mechanism of retroviral recombination: copy-choice or minus-strand recombination (4, 21, 24) and strand displacement/assimilation or plus-strand recombination (11, 13, 19). The copy-choice model proposes that the reverse transcription growing point switches between the two copackaged viral RNAs during minus-strand DNA synthesis. An extension of the copy-choice model, called the forced copy-choice model, proposes that the switches occur at breaks in the viral RNA. The strand displacement/assimilation model proposes that internally initiated plus-strand DNA is displaced from one DNA molecule by the synthesis of other plus-strand DNA; the displaced DNA fragment assimilates into the plus-strand DNA being copied from the second DNA molecule, and recombinants are formed after DNA repair (1, 2, 8, 15).

We previously described a system using vectors containing several markers to study retroviral recombination in a single round of viral replication (10). In this system, recombinants resulting from more than one internal template switch occurred more frequently than expected from the overall rate of recombination. In addition, the number of internal template switches per recombinant was correlated with the type of minus-strand strong stop DNA transfer: intermolecular minus-strand strong stop DNA transfers occurred primarily in recombinants with one internal template switch, and intramolecular minus-strand strong stop DNA transfers occurred primarily in recombinants with more than one internal template switch. To explain the fact that recombination was more frequent than expected, we proposed that retroviral recombination occurs during the synthesis of both minus-strand and plus-strand DNAs (two-pathway hypothesis). To explain the correlation of the type of minus-strand strong stop DNA transfer and the number of internal template switches in the recombinants, we suggested that those recombinants that underwent intermolecular transfer of minus-strand strong stop DNA recombined during the synthesis of minus-strand DNA and that those recombinants that underwent intramolecular transfer of the minus-strand strong stop DNA recombined during the synthesis of plus-strand DNA.

To examine further the mechanism(s) of retroviral recombination and to test the two-pathway hypothesis, we attempted to alter the pathway(s) of retroviral recombination by gamma irradiation. We reasoned that breaks in the template RNA would increase minus-strand recombination; would decrease or not affect plus-strand recombination, depending on the number of plus-strand initiation sites; and would shift plus-strand recombination towards the 3′ end of the genome. In the experiments reported here, virions were exposed to gamma radiation to damage the viral RNA, and recombinants generated from the irradiated virions were analyzed. Although the major effect of the irradiation was not to break viral RNA, the frequency of recombination was somewhat altered. In addition, unselected internal template switches tended to be 5′ of the selected crossover even in the recombinants from irradiated viruses, inconsistent with a plus-strand recombination mechanism.

MATERIALS AND METHODS

Viral constructs and cell culture. WH13 and WH204 have been described previously (10). Briefly, these two spleen necrosis virus-based constructs contain two drug resistance genes: the neomycin resistance gene (*neo*) and the hygromycin phosphotransferase B gene (*hyg*). *neo* is expressed as a direct transcript from the viral long terminal repeat, whereas *hyg* is expressed by a spliced message. A 4-bp insertion was introduced into WH13 to abolish the function of *neo*, and a 4-bp insertion was similarly introduced into WH204 to abolish the function of *hyg*. As a result, each vector contains

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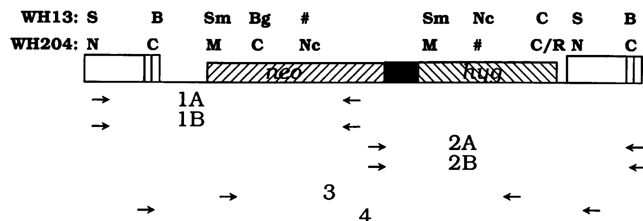

FIG. 1. Structures of two parental constructs and strategy for PCR amplification of proviruses. The structures of the two parental constructs are shown in their DNA form: open boxes with two lines in the boxes, spleen necrosis virus long terminal repeats; left hatched box, *neo* gene; black box, splice acceptor fragment derived from reticuloendotheliosis virus strain A; right hatched box, *hyg* gene. The two parental constructs are identical except for the eight sets of markers shown above the viral structure. The markers are small insertions. Because the viral structure is shown in its DNA form, the two sets of markers in the long terminal repeats are shown twice. B, *Bam*HI cleavage site; Bg, *Bgl*II; C, *Cla*I; C/R, *Cla*I followed by *Eco*RI; M, *Min*I; N, *Not*I; Nc, *Nco*I; S, *Sac*I; Sm, *Sma*I. # represents the frameshift mutations generated by polymerase filling reactions at the *Nco*I sites which abolished the function of the drug resistance genes. Markers from WH13 are listed on the top line, and markers from WH204 are listed on the lower line. The strategy for PCR amplification of the proviruses is shown below the viral structure. Arrows represent the directions and approximate locations of the primers. Individual reactions are designated 1A, 1B, 2A, 2B, 3, and 4. The 5′ ends of the proviruses were amplified by reaction 1A or 1B by using 5′ primer U3Sac or U3Not and 3′ primer 1935OEN. The 3′ ends of the proviruses were amplified by reaction 2A or 2B by using 5′ primer NEO2007 and 3′ primers U5HMAB and U5ALC. The middle of the proviruses were amplified by reaction 3 or 4 by using primers NEO1115 and 3288YH or 3703U and U3360, respectively. The sequences of the primers used in these reactions are described in Materials and Methods.

only one functional drug resistance gene and can confer resistance to only one drug. WH13 and WH204 are otherwise identical constructs, except for six additional sets of restriction enzyme cleavage sites throughout the genome which result in a different restriction enzyme digestion pattern for each construct (Fig. 1). These differences do not affect vector virus replication. The conditions for the maintenance of cells, transfection, infection, and virus harvest were as described previously (9).

Mapping of the recombinant provirus by PCR and restriction enzyme mapping. Recombinant proviruses were mapped by polymerase chain reaction (PCR). DNA was prepared from clones resistant to both G418 and hygromycin and was used as substrates for PCR (7).

Proviral genomes were amplified with different sets of primers, and the amplified products were analyzed by restriction enzyme digestion (Fig. 1). The 5′ ends of the proviral genomes were amplified in two sets of reactions with a common 3′ primer and 5′ primers specific to the U3 markers derived from one of the parental viruses. The primers U3Sac and U3Not were specific to the polymorphic U3 regions of WH13 and WH204, respectively. One of these two primers and primer 1934OEN were used to amplify the 5′ end of the proviral genome (reactions 1A and 1B). In addition to amplifying the 5′ ends of the proviruses for mapping, these reactions also indicated the nature of the marker located in the 5′ U3 region.

The centers of the proviruses were amplified by either of two sets of reactions. The first set used 3703U and U3360 as primers (reaction 4) and amplified most of the genome (Fig. 1). The second set used NEO1115 and 3288YH as primers (reaction 3) and amplified most of the *neo* and *hyg* genes.

The 3′ ends of the proviruses were amplified by two sets of reactions with 3′ primers in the polymorphic U5 regions, U5HMAB and U5ALC, each specific to the U5 marker derived from one of the parental viruses. These two primers, together with primer NEO2007, amplified the 3′ ends of the proviruses (reactions 2A and 2B).

The sequences for the primers were as follows: U3Sac, TGGGAGGGAACTCTGGGGGA; U3Not, TGGGAGGG GCGGCCGCCTGGG; 1934OEN, ACACCCAGCCGGCCAC AGTCG; 3703U, TTCCCTCATTGGAGAGTAGCA; U3360, CACCCTGTAAGCTGTAAGCG; NEO1115, GGCGATAG CTAGACTGGGCGG; 3288YH, TGCCTCCGCTCGAAGT AGCGC; U5HMAB, CGGATTCAGTCCGGATCCCTG; U5 ALC, CGGATTCAGTCCGGATCGATC; and NEO2007, CC GCTTCCTCGTGCTTTACGG.

Virus irradiation and viral RNA isolation. Viruses harvested from different culture plates were pooled and divided into three portions to receive different doses of gamma radiation. The first portion (0 Gy) was not irradiated and was incubated at room temperature. The second and third portions were exposed to gamma radiation from a Cs-sourced Mark II irradiator. After the samples received 2,763 Gy of radiation, the second portion (designated the 3,000-Gy sample) was removed from the irradiator and was placed at room temperature with the 0-Gy sample. The third portion was returned to the irradiator and received 2,762 Gy of exposure, for a total of 5,525 Gy of radiation (designated the 6,000-Gy sample). After the irradiation was completed, all three portions were used either to infect target cells or to isolate viral RNA. Because the viruses originated from the same pool and were used to infect cells at the time, the differences observed between them resulted directly from the exposure to gamma radiation.

Viral RNA was isolated by the following procedure: viral pellets were collected after centrifugation (SW28 rotor, 25,000 rpm for 90 min in a Beckman ultracentrifuge) and were resuspended in TE buffer (10 mM Tris [pH 7.5] and 1 mM EDTA). Sodium dodecyl sulfate (SDS) and *Saccharomyces cerevisiae* tRNA were added to the samples to final concentrations of 0.1% and 200 μg/ml, respectively. Viral lysates were extracted with phenol and phenol-chloroform and were precipitated with ethanol. Viral RNAs were analyzed by the Northern (RNA) hybridization technique as previously described (12). The probe used in the Northern analysis was the *Bam*HI fragment from JD215NeoHy that contained all of *neo*, *hyg*, and the 3′ long terminal repeat (5).

RESULTS

The effect of gamma radiation on the survival of the vector virus. To determine the effects of gamma radiation on the survival of the retroviral vectors, viruses were harvested from helper cell clones that contained both WH13 and WH204 proviruses. The viruses were separated into three samples and were then exposed to 0, 3,000, or 6,000 Gy of gamma radiation. The control samples (0-Gy exposure) were incubated at room temperature in parallel. The three viral samples were used to infect D17 target cells, which were then selected with G418, hygromycin, or G418 and hygromycin. Cells containing WH13 or WH204 proviruses conferred resistance to hygromycin or G418 selection, respectively. Cells containing recombinant proviruses with two functional genes conferred resistance to G418 and hygromycin selection. The number of resistant cell clones indicated

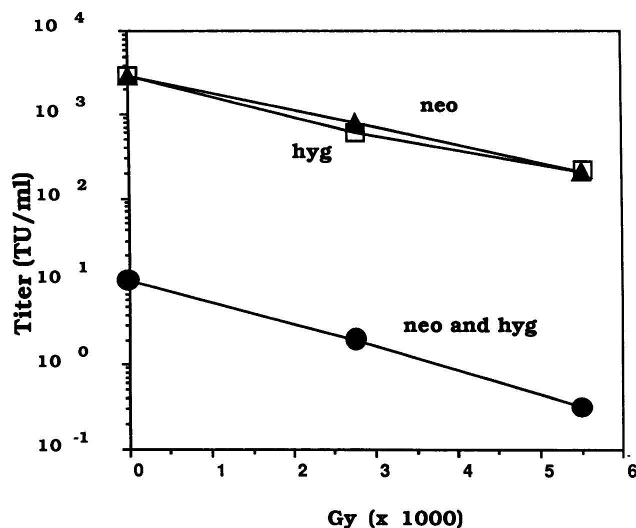

FIG. 2. The effect of gamma radiation on the survival of infectious vector virions. Symbols: □, titers in transforming units (TU) of the target colonies that were resistant to hygromycin selection; ▲, titers of the target colonies that were resistant to G418 selection; ●, titers of the target colonies that were resistant to hygromycin and G418 selection. These data are from an irradiation experiment using helper cell clone 9. In this experiment, the recombination rate was lower than average (see Table 1).

the transforming units of the vector virus (virus titers) (Fig. 2). In six independent experiments, we found that the virus titers were reduced to about 26% of control levels after 3,000 Gy of radiation and to about 6% of control levels after 6,000 Gy of radiation (Table 1). The reduction of the log of the viral titers was linear with the dose of radiation, suggesting one-hit inactivation and two to three lethal hits at 6,000 Gy. The hygromycin resistance titers decreased at the same rate as the G418 resistance titers, indicating that the two parental viruses (WH13 and WH204) responded similarly to radiation (Fig. 2 and data not shown). Furthermore, the double-resistance titer (G418 and hygromycin selection) decreased at the same rate as the two single-resistance titers (Fig. 2 and Table 1). Thus, irradiation did not significantly change the ratio of recombinant proviruses to the parental proviruses.

Molecular nature of the recombinant proviruses. To analyze the nature of the recombinants generated by irradiated virions, D17 cell clones that were both G418 and hygromycin resistant were isolated. DNA was prepared from these clones and was used as a substrate for PCR. The proviral genomes were amplified as three segments—the 5′ terminus, the center region, and the 3′ terminus—by using the strategy shown in Fig. 1. The primers used in these reactions are

TABLE 1. Effects of radiation on infectivity and recombination rate

Resistance[a]	Survival (%)[b] with radiation dose (Gly) of:		
	0	3,000	6,000
Single	100	26 ± 4	6 ± 4
Double	100[c]	17 ± 6	5 ± 2

[a] Data for single resistance were from six experiments. Data for double resistance (recombinants) were from four experiments.
[b] Percent survival ± standard deviation.
[c] The actual recombination rate was 1.6% ± 2.9%.

described in Materials and Methods. The amplified fragments were analyzed by restriction enzyme mapping, and the data were compiled to generate restriction enzyme cleavage maps for each provirus.

WH13 and WH204 differ in eight sets of markers (10). Thus, the sequences derived from each parent can be identified by analyzing the restriction enzyme cleavage maps of the recombinants. From this information, the recombinants were categorized as having either one internal template switch or more than one internal template switch during the elongation step of reverse transcription (Fig. 3).

The numbers of different kinds of recombinants generated after different doses of radiation are shown in Table 2. Of the 22 recombinants previously analyzed from virions without gamma irradiation, 10 underwent one internal template switch and 12 underwent more than one internal template switch (data from reference 10). Recombinants analyzed from virions exposed to 3,000 Gy of gamma radiation showed a different distribution: only 8 of 24 contained one internal template switch, whereas 16 of 24 contained more than one internal template switch. Recombinants analyzed from virions that were exposed to 6,000 Gy of radiation had a more striking distribution: none of the 19 recombinants analyzed contained only one internal template switch. Analysis of the recombinant proviruses recovered after exposure to increasing amounts of gamma radiation showed that the number of additional recombination events increased with increasing radiation dose, from an average of 0.7 to 1.4 per provirus.

Nature of minus-strand strong stop transfer. Two markers are located in the U3 and U5 regions of the two parental viruses and can be used to identify the nature of the minus-strand strong stop DNA transfer (18). By using recombinants generated from virions without irradiation, it was previously found that minus-strand strong stop transfer can be either intermolecular or intramolecular (10). Both types of minus-strand strong stop DNA transfer were also observed in recombinants generated from irradiated virions (Fig. 3 and Table 3).

A correlation between the type of minus-strand strong stop transfer and the number of internal template switches was previously found in recombinants generated from nonirradiated virions (10). Most recombinants that had undergone intermolecular minus-strand strong stop DNA transfer had one internal template switch, whereas most recombinants that had undergone intramolecular minus-strand strong stop DNA transfer had more than one internal template switch (Table 3).

Among the 24 recombinants generated from virions exposed to 3,000 Gy of radiation, 14 underwent intermolecular minus-strand strong stop DNA transfer and 10 underwent intramolecular minus-strand strong stop DNA transfer (Table 3). Among the 14 recombinants that underwent intermolecular minus-strand strong stop DNA transfer, 7 had one internal template switch and 7 had more than one internal template switch. Among the 10 recombinants that underwent intramolecular minus-strand strong stop DNA transfer, 1 had one internal template switch and 9 had more than one internal template switch.

Recombinants generated from virions exposed to 6,000 Gy of radiation all had more than one internal template switch, and they underwent both types of minus-strand strong stop transfer; 5 of 19 underwent intermolecular minus-strand strong stop DNA transfer, and 14 of 19 underwent intramolecular minus-strand strong stop DNA transfer.

With or without irradiation, most recombinants that un-

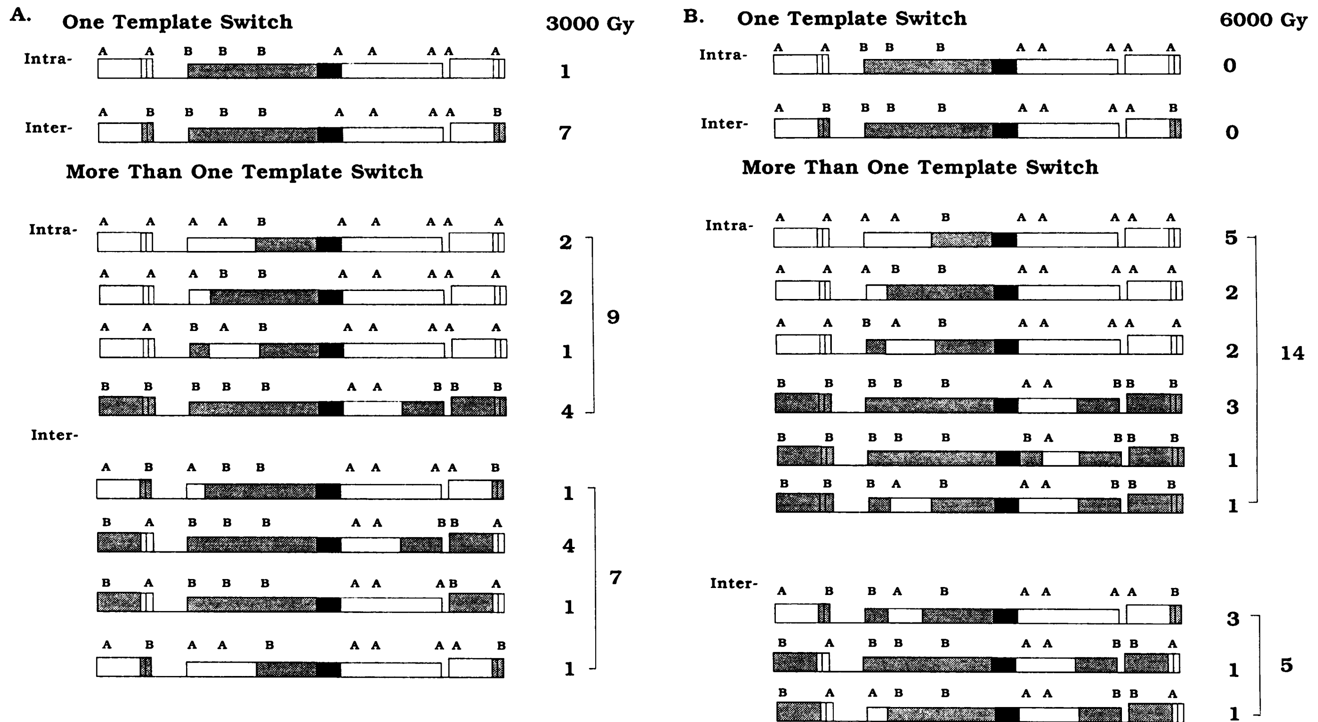

FIG. 3. Maps of recombinant proviruses generated from irradiated virions. (A) Categories of recombinant proviruses generated from virions exposed to 3,000 Gy. WH13-derived sequences are marked with restriction enzyme cleavage site designation A and are shown as white boxes, whereas WH204-derived sequences are marked with restriction enzyme cleavage site designation B and are shown as shaded boxes. The splice acceptor fragment is shown as black boxes. Intra- refers to recombinants with parental long terminal repeats that had undergone intrastrand minus-strand strong stop DNA transfer. Inter- refers to recombinants with recombinant long terminal repeats that had undergone interstrand minus-strand strong stop DNA transfer. The numbers to the right of the maps indicate the number of times a recombinant with that map was encountered. These recombinants were isolated from different petri dishes and thus represent independent recombination events. (B) Recombinant proviruses generated from virions exposed to 6,000 Gy.

derwent intramolecular minus-strand strong stop DNA transfer also had more than one internal template switch (this report and reference 10). However, the correlation between intermolecular minus-strand strong stop DNA transfer and one internal template switch was not observed with recombinants generated from irradiated virus. Instead, recombinants that had undergone intermolecular minus-strand strong stop DNA transfer and more than one internal template switch appeared with increasing radiation.

Analysis of the nature of irradiation damage. To analyze the nature of the damage in the virions, the virus-encoded reverse transcriptase activity and viral RNA were examined. Wild-type spleen necrosis virus was exposed to 0, 3,000, and 6,000 Gy of gamma radiation. Serial dilutions of these viral samples were made. A portion of these samples was used to infect chicken embryo fibroblasts, and another portion was used to assay directly for reverse transcriptase activity. The infectious titer of the spleen necrosis virus was determined by induction of cytopathic effect on chicken embryo fibroblasts and was found to decrease after irradiation at a rate similar to that of WH13 and WH204 (data not shown). Although the spleen necrosis virus titer decreased after irradiation, the reverse transcriptase activity remained at the same level (data not shown). These results indicated that the decrease in virus titer did not result from detectable damage to the reverse transcriptase activity.

TABLE 2. Effect of gamma irradiation on the average number of internal template switches in the resulting recombinants

Radiation dose (Gy)	No. of recombinants with following no. of internal template switches:			
	1	2	3	4
0[a]	10	9	3	0
3,000	8	15	1	0
6,000	0	12	6	1

[a] Data from reference 10.

The effect of irradiation on viral RNA was examined by Northern analysis. Virus was harvested, aliquoted, and irradiated as before. The samples were then subjected to ultracentrifugation, and the resulting pellets were resuspended, extracted with phenol-chloroform, and precipitated with ethanol. In each experiment, isolation of RNA from the three samples was performed in parallel. RNA isolated from the same amount of supernatant medium was taken from each sample and was subjected to Northern analysis (Fig. 4). Northern analysis indicated that after irradiation, the amount of full-length RNA recovered decreased. This phenomenon was consistent over three sets of experiments. On the average, relative to unirradiated virions, about 50% of the RNA was isolated from the 3,000-Gy virions and 25% was isolated from the 6,000-Gy virions, as determined with a Betascope 603 Blot Analyzer. (The relative infectivities were 26 and 6%.) No discrete species of smaller RNA was seen. In some of the Northern analyses, an additional band at about

TABLE 3. Types of minus-strand strong stop DNA transfer and number of internal template switches in recombinants

Minus-strand strong stop DNA transfer	No. of recombinants at radiation dose (Gy) of:					
	0[a]		3,000		6,000	
	One switch	More than one switch	One switch	More than one switch	One switch	More than one switch
Intrastrand	3	10	1	9	0	14
Interstrand	7	1	7	7	0	5

[a] Data from reference 10. In the 0-Gy population, one provirus could not be scored for the type of minus-strand strong stop DNA transfer.

7 to 7.5 kb was observed. It is likely that this band was dimer RNA.

To test whether this decrease in RNA recovery was the result of cross-linking between viral RNA and protein, lysates of viruses were treated with proteinase K prior to phenol-chloroform extraction. Northern analysis indicated that after proteinase K treatment, more RNA was recovered from irradiated virions (data not shown). This result suggests that protein-RNA cross-linking contributed to the loss of RNA recovered after irradiation. This result also suggested that the damage to nucleic acids was partly a result of the cross-linking of RNA with protein.

DISCUSSION

We wanted to use breaks in viral RNA to test the mechanisms of retroviral recombination. In our previous study using vectors with multiple markers, the rate of selected recombination was 2%; approximately half of the recombinants analyzed after one round of retroviral replication contained one internal template switch, and the other half contained more than one internal template switch (10). On the basis of the current recombination models, each recombination event during minus-strand DNA synthesis generates one internal template switch in the recombinant, whereas most displacement/assimilation events during plus-strand DNA synthesis generate a recombinant with two internal template switches. Thus, recombinants with one internal template switch are more likely to be generated by minus-strand recombination.

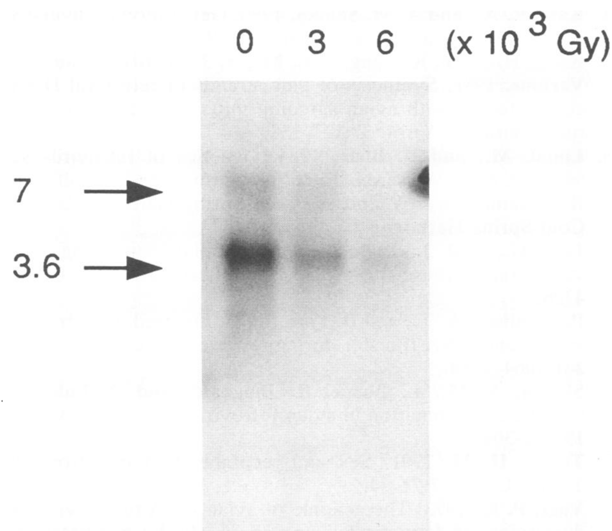

FIG. 4. Northern analysis of viral RNA from virions exposed to different doses of gamma radiation. The doses of gamma radiation to which the viruses were exposed are indicated on the top of the Northern blot. The sizes of the bands (in kilobases) are indicated. The 3.6-kb band is the full-length vector viral RNA. These viral RNAs were isolated in parallel and were not treated with proteinase K prior to phenol extraction. The procedures for RNA isolation, Northern analysis, and the probes used are described in Materials and Methods.

Recombinants with more than one internal template switch can be generated from two or more recombination events during minus-strand DNA synthesis or from plus-strand recombination. Recombinants with more than one internal template switch occurred more frequently than expected from the overall rate of recombination (10). Thus, if recombination events are independent, recombinants with more than one internal template switch are more likely to be generated by plus-strand recombination.

An additional finding from the previous work (10) was that recombinants that underwent intramolecular minus-strand strong stop DNA transfer usually contained more than one internal template switch, whereas recombinants that underwent intermolecular minus-strand strong stop DNA transfer usually contained one internal template switch. To explain these data, we proposed that retroviral recombination occurs through both the minus-strand and the plus-strand recombination pathways (two-pathway hypothesis), each pathway generating genotypically different recombinants. Therefore, under this hypothesis, the fact that double recombinants occurred more frequently than expected is a reflection of the two recombination mechanisms retroviruses use.

On the basis of the current models of retroviral recombination, breaking viral RNA should promote minus-strand recombination, decrease or not affect plus-strand recombination, and shift plus-strand recombination towards the 3′ end of the genome. To gain further understanding of retroviral recombination as well as to test the two-pathway hypothesis, particularly the correlation of intrastrand minus-strand strong stop DNA transfer and more than one recombination event, we attempted in this study to alter the pathway of retroviral recombination by breaking virion RNA. We chose gamma radiation because its effect is directly related to the size of the molecule; the larger the molecule, the more likely it is to be damaged by gamma irradiation (3). However, although we found that the amount of recoverable RNA decreased after irradiation, no specific smaller RNAs were seen. Gamma radiation-induced damage to nucleic acid is complex (22). Our results indicated that viral RNA was damaged not primarily by breaks but by protein-RNA cross-linking. Since breaks were not the major effect of the gamma radiation, inactivation of viral infectivity did not result from breaks. It is likely that reverse transcriptase cannot use the gamma radiation-damaged portion of viral RNA as a template for DNA synthesis, resulting in inactivation of the virus.

TABLE 4. Distribution of additional recombination events[a]

Radiation dose (Gy)	No. of recombinants located:		% Located 3′
	5′	3′	
0[b]	12	4	25
3,000	8	9	53
6,000	20	7	26

[a] The location of each additional recombination event was determined from the maps in Fig. 3.
[b] Data from reference 10.

In spite of this finding, we still found an effect on recombination. Virions exposed to 3,000 and 6,000 Gy of gamma radiation were used to infect D17 cells. The ratio of parental proviruses to recombinant proviruses did not change significantly compared with proviruses that resulted from the nonirradiated control virions even at 6% survival (Table 1), in agreement with the hypothesis that inactivation did not result from breaks. However, the number of recombinants with only one internal template switch decreased with irradiation, and as a result, the average number of additional internal template switch events in the recombinants increased from 0.7 to 1.4 at 6,000 Gy (Table 2).

The disappearance of the recombinants with one internal template switch, which primarily had interstrand minus-strand strong stop DNA switches, supports a two-pathway hypothesis. We propose that recombinants with only one internal template switch were formed by a copy-choice mechanism. The mechanism responsible for the recombinants with multiple internal template switches could then be strand displacement/assimilation or forced copy choice. Breaks in viral RNA distinguish these two mechanisms: with increasing numbers of breaks, the distribution of recombination events should shift 3′ for strand displacement/assimilation and be unchanged for forced copy choice.

The location of the additional recombinants was determined (Table 4). Although at 3,000 Gy there was a shift 3′, the 6,000-Gy population had only 25% of the additional recombination events 3′, just as the unirradiated population did (10). Thus, these data do not support a plus-strand mechanism for the additional recombination induced by irradiation. The results are consistent with a forced copy-choice mechanism, which proposes that the number of internal template switches increases with the number of breaks in the viral RNA. This prediction of the copy-choice model is confirmed for the first time by these experiments. Other experiments (6, 17) and our previous experiments (10) also indicate that recombination can occur during minus-strand DNA synthesis. However, one can still propose that, in addition to copy-choice and forced copy-choice mechanisms, plus-strand recombination exists (24).

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