Genetic consequences of packaging two RNA genomes in one retroviral particle: Pseudodiploidy and high rate of genetic recombination
(retrovirus replication/heterozygote)

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ABSTRACT Retroviruses contain two complete viral genomic RNAs in each virion. A system to study in a single round of replication the products of virions with two different genomic RNAs was established. A spleen necrosis virus-based splicing vector containing both the neomycin-resistance gene (neo) and the hygromycin B phosphotransferase gene (hygro) was used. Two frameshift mutants were derived from this vector such that the neo and the hygro genes were inactivated in separate vectors. Thus, each vector confers resistance to only one selection. The vectors with frameshift mutations were separately propagated and were pooled to infect DSDH helper cells. Doubly resistant cell clones were isolated, and viruses produced from these clones were used to infect D17 cells. This protocol allowed virions containing two different genomic RNAs (heterozygotes) to complete one round of retroviral replication. The molecular nature of progeny that conferred resistance to single or double selection and their ratio were determined. Our data demonstrate that each infectious heterozygous virion produces only one provirus. The rate of retroviral recombination is ~2% per kilobase per replication cycle. Recombinant proviruses are progeny of heterozygous virions.

The retroviral life cycle requires DNA molecules to be copied from viral RNA and to integrate into the host genome to form the provirus (1). However, a unique feature of retroviruses is that two RNA genomes are packaged in one virion (2–7). It has been suggested that one provirus is formed from the two copies of genomic RNA in one virion; that is, retroviruses are pseudodiploids (8, 9). Others have suggested that more than one copy of the provirus can be formed from one infectious event (10). Retroviruses have also been observed to undergo frequent genetic recombination (11–16). This frequent exchange of information was correlated with the presence of two different genomes in one virion—i.e., heterozygote formation (26). Is heterozygote formation a necessary condition for genetic recombination?

We established a system to study the products of a heterozygous virion in one round of retroviral replication. A spleen necrosis virus (SNV)-based vector, pJD216NeoHy (17), contains and expresses two resistant marker genes. Frameshift mutants were derived from pJD216NeoHy such that in each construct one of the resistant marker genes was mutated. Two constructs containing different mutated marker genes were introduced into helper cells (18) by means of coinfection. Viruses produced from these doubly infected cells were used to infect permissive D17 cells. Because neither these retroviral vectors nor D17 cells can provide the essential trans-acting viral proteins for viral replication, only one round of retroviral replication (from helper cell to D17 cell) occurs in this system. With this protocol, progeny derived from the viruses produced by the doubly infected cells can be studied. Our data demonstrate that each infectious virion produces only one provirus. Retroviral recombination requires the formation of heterozygotes. The rate of the recombination was 2% per kilobase.

MATERIALS AND METHODS

Definitions. One round of replication is defined as beginning with a provirus in one cell and ending with the formation of a provirus in another cell. Thus, the events in one round of replication include RNA transcription of the proviral DNA, assembly of the virus, entry of virus into a host cell, reverse transcription of the genome, and integration. Rate refers to the frequency of events occurring in one round of replication. Rate of recombination is calculated by comparing the titer of doubly resistant colonies (recombinant phenotype) with the lower titer of the two types of singly resistant colonies (parental phenotypes). The titers of the doubly resistant colonies were determined from the linear range of a series of 10-fold dilutions.

Plasmid Construction. pWH12, pWH13, and pWH14 were derived from pJD216NeoHy (17). pJD216NeoHy was partially digested with NcoI and the resulting recessed 3' ends were filled in by the Klenow fragment of Escherichia coli DNA polymerase I. These products were ligated and were used to transform competent E. coli cells. This approach resulted in two plasmids, pWH13 and pWH14. Each contained a 4-base pair (bp) insertion in either the neo (pWH13) or the hygro (pWH14) gene. Similarly, pWH12 was generated by a partial EcoRI digest, resulting in a 4-bp insertion within the hygro gene. Standard procedures were used to perform restriction enzyme digestions and fill-in reactions by Klenow enzyme (19).

Cells. A dog cell line, D17, which is permissive for SNV infection was used. Helper cell line DSDH was derived from D17 cells and contained the dehydrofolate reductase gene (pFR400) (20). Trans-acting viral proteins required for packaging were expressed by two separate vectors, pBR1 (gag-pol) and pFR102 (env), as in DSN cells (18).

Cells were grown in Temin-modified Eagle’s medium (30) with 6% calf serum at 37°C with 6% CO2. Helper cell clones containing the proviruses of interest were propagated in the presence of chicken anti-SNV antibody to prevent reinfection. G418 and hygromycin selection were done at 400 μg/ml and 80 μg/ml, respectively.

Transfection and Infection. Transfections were done by the dimethyl sulfoxide–Polybrene method (21). Viral infections

Abbreviations: All plasmids have a small p before their names; viruses derived from these plasmids do not; hygro and neo refer to the genes, whereas hygro' and neo' refer to the phenotypes of the cells; the superscript s refers to sensitive; SNV, spleen necrosis virus; mo, multiplicity of infection; nt, nucleotide(s).

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were done immediately after viral harvest. Virus collected from helper cells was centrifuged for 10 min at 3000 x g to remove cell debris. Ten-fold serial dilutions were made from each viral stock, and viral titers were determined by infecting 2 x 10^4 D17 cells per 60-mm dish.

**Southern Blot Analysis.** DNA purification, digestion, and hybridization were performed by standard techniques (19). DNA transfers were done with a vacuum blotter (LKB). All blots were hybridized with an internal Nco I-EcoRI fragment of pJD215Neo+X. pJD215Neo+X is identical to pJD216-NeoHy but without the splice-acceptor fragment (coordinates bp 5578-5799 in the SNV genome) (22). This fragment was chosen to avoid homology between the probe and the plasmids in the helper cell line [pBR1: nucleotides (nt) 676-6629, pPR102: nt 5653-7747]. The probe was labeled by the random-priming method (23).

**RESULTS**

**Vectors to Study the Progeny of Heterozygous Virions.** pJD216NeoHy is an SNV-based splicing vector that contains both neomycin (neo) and hygromycin B phosphotransferase (hygro) genes (Fig. 1). The neo gene is expressed from an unspliced message, whereas the hygro gene is expressed from a spliced message. To measure the relative neo, hygro, and neo plus hygro transforming units, the following experiment was performed. DSDh cells were infected with pJD216NeoHy from 2G helper cells (24) followed by selection with G418 plus hygromycin. Ten clones were isolated. Viruses were harvested from these clones and were assayed on D17 cells. Similar titers of neo, hygro, and neo plus hygro were obtained from these assays (data not shown).

pWH12, pWH13, and pWH14 differ from pJD216NeoHy in that each contains a 4-bp insertion in either the neo or the hygro gene, respectively. These insertions each destroyed one restriction enzyme site in the genome. The reversion rate of these frameshift mutants was <1 x 10^-7 (data not shown).

**Protocol to Characterize the Product of Heterozygotes in One Round of Retroviral Replication.** DSDh cells were transfected with pWH13 or pWH14. Appropriate selections were applied, and viruses were harvested from these transfected cells. Similar titers of WH13 and WH14 viruses were pooled and were used to infect fresh DSDh cells (Fig. 2). These cells were subjected to G418 plus hygromycin selection, and resistant colonies were cloned and propagated. Viruses were harvested from these doubly resistant clones and were assayed on D17 cells to determine the number of cells that were resistant to G418, hygromycin, and G418 plus hygromycin.

Ideally, this system measures events occurring in one cycle of viral replication. However, there are two possible events that may inadvertently allow more than one round of replication to occur: reinfection during expansion of the helper cell clones and the presence of replication-competent virus in the D17 assay cells. To ensure that the data truly reflect the events occurring during one round of replication, two precautions were taken. (i) Neutralizing anti-SNV antibody was present during the propagation of the helper cell clones to prevent reinfection. (ii) Supernatant media were harvested from each set of the assayed D17 cells and were tested for reverse transcriptase activity as well as the ability to transfer SNV genomes. None of the assay plates showed any detectable reverse transcriptase activity (data not shown). Fresh D17 cells were also inoculated with the supernatant media harvested from the D17 assay cells, and this measurement was used to measure any possible transfer of viral constructs. These supernatant media failed to transfer G418 or hygromycin resistance to D17 cells (data not shown). Therefore, a single round of retroviral replication was measured in this system.

**Comparison Between the Single and the Double Selection.** Eight doubly infected helper cell clones were assayed. The comparison of the titers for the different selections is given in Table 1. The ratio of the number of doubly resistant colonies to the lower of the two titers of the singly resistant colonies ranged from 2.6% to 1% (mean, 1.8%; SD, 0.6%).

To confirm the above observation, another virus WH12 (Fig. 1) was used with WH13 for coinfection. When viruses produced from WH12- and WH13-containing helper cell clones were assayed on D17 cells, similar results were obtained. Among 11 helper cell clones assayed, the ratio for doubly-to-singly resistant colonies ranged from 3.8% to 0.9% (mean, 2.2%; SD, 0.9%) (data not shown).

The Molecular Nature of the Doubly Resistant Colonies. Because each of the parental viruses (WH12, WH13, and WH14) confers only a single resistance, doubly resistant cells can be formed in two ways. The cells could acquire both parental proviruses or they could obtain a recombinant provirus that contained both functional resistant markers. These two possibilities can be easily distinguished by South-
ern hybridization analysis. Both WH13 and WH14 viruses contain a 4-base insertion that destroyed one of the two \textit{Nco I} restriction sites in the genome. When digested with \textit{BamHI} plus \textit{Nco I} and hybridized with an internal fragment, the WH13 provirus gives a 2-kb band, WH14 gives a 2.4-kb band, and the recombinant provirus that contains both functional selectable markers also has both \textit{Nco I} sites and thus produces a 1-kb fragment (Fig. 3A).

Eight doubly resistant helper cell clones were examined; all contained both parental bands but not the recombinant band (Fig. 3B, DSDh\#5 and data not shown). Fifteen doubly resistant D17 cell clones were isolated from low multiplicity of infection (moi) plates (=0.001 transforming units per cell for each parental virus) and were examined; all contained only the recombinant proviruses (Fig. 3B, D17\#5A-1, \#5B-1 and data not shown).

The copy number of the proviruses in these clones was also examined by digesting DNA with restriction enzymes that cleave only once in the provirus. Fifteen of 15 clones contained only one copy of the provirus (Fig. 3C and data not shown).

To look at a larger number of colonies, plates infected at a higher moi (=0.01 transforming units per cell for each parental virus) from each set of assays, which contained many doubly resistant colonies, were pooled. These pools contained 24–273 colonies. DNA from these pools was subjected to Southern analysis (Fig. 3B, D17\#5P and data not shown). Because both parental proviruses were required to confer resistance to double selection, the intensity of the recombinant band was compared with the weaker of the two parental bands. In D17\#5P, the intensity of the 2.4-kb band (parental provirus) was \(\times 10^4\) of the intensity of the 1-kb band (recombinant provirus). Thus, although some parental bands were observed in these pools, the major contributor to the double resistance in these cells were the recombinant proviruses. The presence of the parental viruses in the pools, but not in the clones of the target cells, is most likely the effect of increased double infection at a higher moi (see Discussion for details).

**Heterozygote Formation Is Required for Recombination.** To determine whether recombination occurred between two RNA genomes inside one viral particle or between genomes within different viral particles, we performed the following experiment. Similar titers of WH13 and WH14 viruses were propagated separately and were used to coinfect fresh D17 cells. G418 plus hygromycin selection was applied. Approximately 800 doubly resistant colonies were pooled from nine 100-mm plates. DNA samples were digested with \textit{BamHI} plus \textit{Nco I} and were subjected to Southern analysis (Fig. 3B, D17 coinfect). The 2.4-kb and 2-kb bands that represent the two parental proviruses were observed, but the 1-kb band representing the recombinant provirus was not observed.

### Table 1. Assay of WH13 and WH14 coinfect DSDh clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>G418 ((\times 10^4))</th>
<th>Hygro ((\times 10^4))</th>
<th>G418 + Hygro ((\times 10^4))</th>
<th>dbl/s, %</th>
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<tr>
<td>6</td>
<td>0.9</td>
<td>1.4</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>0.65</td>
<td>1.9</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>0.55</td>
<td>2</td>
<td>1.2</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>1.2</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
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<td>0.65</td>
<td>1</td>
<td>9.7</td>
<td>1.5</td>
</tr>
<tr>
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<td>0.65</td>
<td>1.1</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
<td>2.6</td>
<td>1</td>
<td>1.3</td>
</tr>
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<td>2</td>
<td>2.5</td>
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<td>1</td>
</tr>
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</table>

*Mean 1.8 ± 0.6

*Ratio of doubly resistant (dbl) titer versus the lower titer of the two parental titers.

![Fig. 3. Molecular nature of the doubly resistant cells.](image-url)

(A) Parental proviruses and recombinant proviruses can be distinguished by Southern blotting analysis. A \[^{32}P\] labeled internal \textit{Nco I–EcoRl} fragment lacking the splice-acceptor sequences was used to hybridize with the Southern blots (cross-hatched boxes). Upon a \textit{BamHI} plus \textit{Nco I} double digestion, parental proviruses like WH13 give a 2-kb fragment, parental proviruses like WH14 give a 2.4-kb fragment, and the recombinant proviruses containing both functional markers give a 1-kb fragment. Open boxes, long terminal repeats; shadowed box, neo; hatched box, hygro; A and E, Asp-718 and EcoRl enzyme recognition site, respectively. (B) The first set of samples, DSDh\#5 (lanes 1 and 2), are DNA isolated from a doubly resistant helper cell clone, DSDh\#5. The second set, D17\#5P (lanes 3 and 4), are DNA isolated from 49 doubly resistant D17 clones resulting from infection with virus harvested from DSDh\#5. D17\#5A-1 and \#5B-1 are samples isolated from doubly resistant D17 clones resulting from infection with virus harvested from DSDh\#5. The fifth set, D17 coinfect, is from a pool of \(\approx 800\) doubly resistant colonies resulting from infection with pooled WH13 and WH14 viruses. B, \textit{BamHI} digestion; B+N, \textit{BamHI} plus \textit{Nco I} digest. (C) DNAs of clones D17\#5A-1 and \#5B-1 were digested with enzymes that cleaved the proviral genome once. Neither of these recognition sites is within the region of the fragment used for hybridization. A and E, Asp-718 and EcoRl digestions, respectively. Numbers at right represent kb.

This result contrasts with the doubly resistant colonies generated from heterozygote-containing viral stocks. Although similar moi values were used to generate these pools (Fig. 3B, D17\#5P and D17 coinfect), recombinant proviruses were only observed after infecting D17 cells with heterozygote-containing viral stocks. However, it is possible for DNA...
recombination to occur between the two proviruses and generate a recombinant provirus in the DSDh helper cell clones; however, the frequency of these events is $10^{-4}$ (25), four orders of magnitude lower than the retroviral recombination events that we have observed. Further, Southern analysis of the DSDh helper cell clones did not reveal any evidence of this DNA recombination.

**DISCUSSION**

We have described a system to study the genetic consequences of packaging two retroviral RNAs in one virion. Two aspects of their behavior were studied: (i) How many proviruses are generated from infection with one virion? (ii) What are the requirements and the rate of retroviral recombination?

The system that we used allows us to measure events occurring during one round of retroviral replication. The two constructs that we used were extremely homologous to each other. Within a viral genome of 3628 nt, there were eight nucleotide differences. These different nucleotides were in the coding region of the selectable markers and should not affect the cis-acting sequences required for packaging. The extreme homology eliminates any potential discrimination for packaging as a result of nucleotide sequence differences. Therefore, random packaging can be assumed. Another advantage of the system is that both the parents and the recombinants can be assayed simultaneously. This property allowed us to compare numbers of cells that contain parental proviruses with cells that contain recombinant proviruses.

All of the 15 doubly resistant clones we examined acquired only one provirus. This result demonstrated that even though two RNA genomes are packaged in each retroviral virion, only one provirus is produced from each infectious event.

It was proposed that packaging two different genomes in one virion is required to observe genetic recombination (26). To test this hypothesis, we compared the progeny of heterozygote-containing viral stocks and stocks that do not contain heterozygotes. When D17 cells were infected with viruses harvested from cells that contained both parental proviruses to allow the formation of heterozygotes, recombinant proviruses were observed at a rate of ~2% (Table 1). When D17 cells were infected with a mixture of the two separately propagated parental viruses, no recombinant proviruses could be detected. The only difference between these two experiments was the presence of the heterozygotes. In addition to analyzing pools of cells, we also examined individual cell clones in various experiments. All 15 D17 cell clones generated from infection with heterozygote-containing viral stocks contained recombinant proviruses. However, when the doubly resistant DSDh clones were generated by infection with two separately propagated parental proviruses, all of them contained only the parental viruses (Fig. 3B, DSDh#5 and data not shown). These results indicate that recombinant proviruses are the progeny of heterozygous virions. Dually resistant cells generated during coinfection result not from recombination but from double infection.

From theoretical calculations, it is expected that when two viral stocks are mixed and used for infection at moi values of 0.1, 0.01, and 0.001 transforming units per cell, 10%, 1%, and 0.1% of the infected cells will be doubly infected, respectively. The proportion of doubly infected cells declines as the moi is reduced. We tested the frequency of double infection at different moi by coinfecting D17 cells with separately propagated WH13 and WH14 viral stocks. Our data from six experiments generally conform to the results expected from theoretical calculations (R. Wisniewski, W.-S.H., and H.M.T., unpublished observation).

This phenomenon can also be seen in our Southern analysis. All of the doubly resistant D17 clones contained recombinant proviruses only. However, when doubly resistant colonies from plates infected with 10-fold higher moi were pooled and were examined, minor parental fragments were detected (Fig. 3B, D17t#5 and data not shown). These parental proviruses were probably the result of double infection. To avoid misleading calculations, the titer of doubly resistant colonies was determined in the linear range of the titrations.

In this system, with two markers that are 1 kb apart, 2% recombination was detected. This rate is in contrast with the previous reports in which 10-40% of recombinants were detected (10, 12). The main difference between the systems is that we measured recombination in one round of replication, whereas the previous reports studied the frequencies of recombination during multiple rounds of replication.

We encountered the problem of reinfection in another set of experiments similar to the ones we have described in this paper. In these experiments, helper cell clones were propagated without the presence of antibody. Reinfecion occurred despite superinfection interference. As a result, when 11 clones were assayed, the percentage of recombination varied greatly, ranging from 0.3 to 13% (mean, 4.6%; SD, 4.5%) (data not shown). With prolonged propagation of these helper clones, recombinant bands could be detected in Southern blot hybridization (data not shown). However, when helper cell clones were propagated in the presence of antibody, the recombination rate of different clones varied little, and no recombinant band could be detected in the Southern analysis of the helper cell clones (Fig. 3B, DSDh#5 and data not shown).

Heterozygous virions would result in four phenotypes of proviruses: parental type like WH113 (neo' hygro'), parental type like WH14 (neo' hygro'), a recombinant that contains two functional genes (neo' hygro'), and a recombinant that contains two mutant genes (neo' hygro'). It is thought that recombination occurs at similar frequencies in all regions of the viral genome (15, 16). Assuming this hypothesis is correct and that there is no gradient of recombination in the retroviral genome, the frequency of recombination is directly proportional to the distance between the two markers. In an ideal population where the two parental viruses are expressed equally and packaging is random, 50% of the virions would be heterozygous. The ratio of the distance between the two mutations (1029 nt) and the viral RNA length (3628 nt) indicates that 28% of the recombination events occurred between the two mutations. Because only half of the recombinants possess a neo' hygro' phenotype, only 14% of the recombination events are detectable. If recombination occurs once in each heterozygous virion, 7% of the total progeny will be neo' hygro' recombinants (14% × 50%), and the frequency of each of the parental phenotypes will be 43%. After a single selection, the parental phenotype that is resistant to this selection plus the doubly resistant recombinant phenotype will be measured (43% + 7%), therefore, a frequency of 7/50 or 14% of recombination will be observed.

The observed recombination rate in our system is 2%. Hence, we estimate that for a viral population containing a 3.6-kb genome, one of seven viruses experiences at least one recombination event. Viral populations with larger genomes probably have more recombination per virion. For a wild-type virus (10 kb), one of three viruses would experience at least one recombination event.

In summary, we found that although two RNA genomes are packaged in one retrovirus virion, only one provirus is produced per infectious particle. Having two RNA genomes in the same particle facilitates the exchange of the genetic information because heterozygotes are required for frequent recombination. In this system, with the 3.6-kb length of RNA and the 1-kb distance between the two mutations, ~2% recombination was observed per cycle.
Currently, there are two hypotheses regarding the mechanism of retroviral recombination, the copy-choice model (27) and the strand-displacement model (28). One prediction of the copy-choice model is that only one DNA molecule will result from one virion, whereas the strand-displacement model predicts two copies of DNA molecules will be produced. Although we have concluded that one infectious virion produces only one provirus, we cannot rule out the possibility that two unintegrated viral DNA molecules are generally produced, but that only one of the molecules successfully integrates to form a provirus as a result of a low integration efficiency (9, 29). Therefore, the mechanism of recombination cannot be elucidated from these data, and other approaches to distinguish between these two hypotheses regarding the mechanism of retroviral recombination need to be used.

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