RNAs from Genetically Distinct Retroviruses Can Copackage and Exchange Genetic Information In Vivo

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Sequence analysis suggests that ancient recombination events may have occurred between genetically distinct retroviruses. An experimental system was utilized to explore the genetic interaction between different viruses. Moloney murine sarcoma virus and spleen necrosis virus are type C retroviruses that belong to different subgenera. With vectors containing packaging signals from these two viruses, DNA proviruses containing genetic information from both RNAs can be generated. This is the first experimental evidence to indicate that RNA from different retroviruses can copackage and exchange genetic information.

Retroviruses package two copies of genomic RNA in one virion (9, 16, 31). During reverse transcription, genetic information from both RNAs can be used to generate a recombinant virus (9, 25, 43). Within a retrovirus population, recombination occurs frequently and increases genetic variation by allowing the distribution of various mutations (56). Genetic variation is beneficial to the virus population because it allows the survival of different variants in response to selective pressures from the environment.

Efficient recombination in retroviruses requires copackaging of the viral RNAs (25, 58). Interaction between the viral Gag polyprotein and the viral RNA is needed for the selective packaging of the viral RNA (2, 22, 40). The RNA of closely related viruses can often be recognized by the viral proteins and packaged efficiently. In addition, it has been shown that this recognition is retained in some genetically distinct but distantly related retroviruses such as murine leukemia virus (MLV) and spleen necrosis virus (SNV). MLV is a murine virus, and SNV is an avian virus; they belong to different subgenera (7). The primary sequences of the SNV and MLV packaging signals do not contain significant sequence homology, but they have similar double hairpin secondary structures (29, 60). Despite the lack of homology, MLV vector RNA can be packaged by SNV proteins even in the presence of competing SNV vector RNA (17, 60). This raises the question of whether SNV RNA and MLV RNA can be copackaged into a virion, and if so, whether a DNA copy containing genetic information from both RNAs can be generated.

Genetic interactions between different retroviruses have not been fully explored. A comparison of sequences suggests that ancient recombination events between different retroviruses may have occurred (8). SNV may be generated from such an event. The *gag-pol* region of SNV is related to that of MLV, whereas the *env* region of SNV is related to that of the Mason-Pfizer monkey virus. SNV and Mason-Pfizer monkey virus Env bind to the same receptor as demonstrated by interference studies (28, 30). However, recombination between different retroviruses has not been demonstrated experimentally.

In this report, we developed a system to explore the potential genetic interaction between SNV and MLV. Two retroviral vectors were used, one containing the packaging signal from SNV (E) and the other containing the packaging signal from Moloney murine sarcoma virus (Mo-MSV) (Ψ), which is derived from Moloney murine leukemia virus (Mo-MLV) (57). The vectors were designed so that neither could complete minus-strand DNA transfer efficiently when packaged separately. However, if RNAs from these two vectors were copackaged and both were used during reverse transcription, minus-strand DNA transfer could occur efficiently. This would result in the generation of a structurally unique provirus carrying genetic information from both parental RNAs.

Retroviral vectors used to study copackaging of RNA containing different packaging signals. Two retroviral vectors, pPY31 and pPS4, were used to study genetic interaction between Mo-MSV Ψ-containing RNA and SNV E-containing RNA (Fig. 1A). The plasmid constructs are referred to as pPY31 and pPS4, and the viruses derived from these plasmids are referred to as PY31 and PS4, respectively. Plasmid pPY31 has been described previously (61). Briefly, pPY31 contains an Mo-MSV-derived 5′ long terminal repeat (LTR), primer binding site (PBS), and packaging signal (Ψ) (1, 41). Mo-MSV Ψ contains homology to Mo-MLV Ψ (1, 48, 57). A hygromycin phosphotransferase B gene (*hygro*) (23) immediately follows the Mo-MSV sequences. The polypurine tract (PPT) and 3′ LTR that comprise the 3′ viral sequences of pPY31 are derived from SNV (14). Virus generated from the pPY31-transfected cells produced a 3,000-fold-lower titer of hygromycin-resistant target cells compared to a vector with two highly homologous LTRs (61). The lack of sequence similarity between the 5′ and 3′ R regions in the PY31 RNA forces the minus-strand DNA transfer to use short nucleotide homologies and alternative acceptor templates. All of the analyzed PY31 proviruses contain LTRs comprising truncated SNV U3 regions and Mo-MSV R-U5 regions (61). Most of the observed proviruses (85%) used a 6-nucleotide homology between the Mo-MSV R and the SNV U3 to mediate the minus-strand DNA transfer, resulting in proviruses with a 147-bp deletion in the SNV U3. This 147-bp deletion removes the TATA and the CATT elements (38, 52), which is likely to result in attenuated promoter activities of these proviruses.

Retroviral vector pPS4 contains an SNV-derived 5′ LTR, PBS, and packaging signal (E) (15). A puromycin *N*-acetyltransferase gene (*puro*) (12) is located immediately downstream of these sequences. The 3′ end of PS4 does not contain retroviral sequences but has a simian virus 40 termination signal (15).

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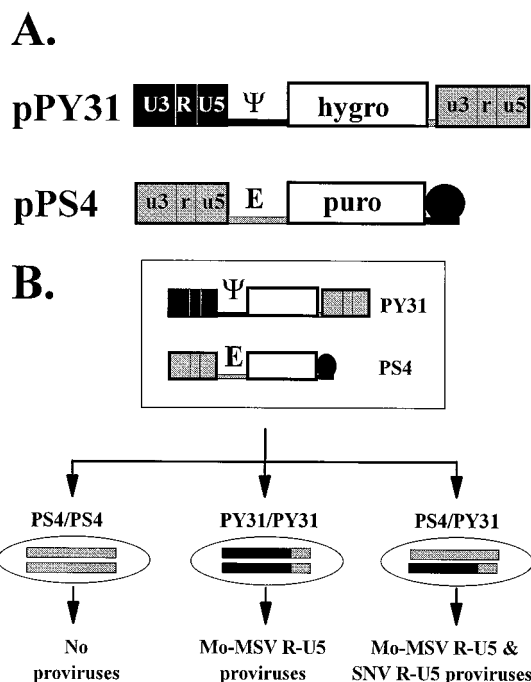
A.

B.

FIG. 1. Retroviral vectors used to study the genetic interaction of different viruses. (A) Structures of pPY31 and pPS4. Vector pPY31 contains Mo-MSV derived 5′ viral sequences including 5′ LTR and Ψ (black boxes and lines labelled by uppercase lettering), hygromycin phosphotransferase gene (hygro), and SNV-derived 3′ viral sequences including the PPT and 3′ LTR (shaded boxes labelled by lowercase lettering). Vector pPS4 contains SNV-derived 5′ viral sequences including 5′ LTR and E, puromycin *N*-acetyltransferase gene (puro), and a simian virus 40 termination sequence (black circle). (B) Viruses produced by helper cells containing both PY31 and PS4. The white box containing two viral constructs represents DSH134G helper cells containing PS4 and PY31. Three types of viruses (ovals) can be generated from the helper cells; viruses with two copies of PS4 RNA (shaded rectangles), with two copies of PY31 RNA (black rectangles with shaded ends), and with one copy each of PS4 and PY31 RNA are shown. Viruses with two copies of PS4 RNAs cannot generate any proviruses. Viruses with two copies of PY31 RNA can generate Mo-MSV R-U5-containing proviruses. Viruses with a copy each of PS4 and PY31 RNA can generate both Mo-MSV R-U5-containing proviruses and SNV R-U5-containing proviruses.

Minus-strand DNA synthesis can initiate in PS4 RNA because it contains a PBS. However, PS4 lacks the necessary 3′ viral sequences such as the PPT, attachment sites, and 3′ R to complete reverse transcription and allow efficient viral DNA integration.

Experimental protocol. An experimental protocol was designed to study the genetic interaction between PY31 and PS4. DSH134G is an SNV-based helper cell line (37). In order to generate cells expressing PS4 and PY31 RNA, DSH134G cells were first transfected with pPS4 by the dimethyl sulfoxide Polybrene method (27). After puromycin selection, the transfected DSH134G cell clones were pooled (>3,000 colonies). These cells were then transfected with pPY31 and placed on hygromycin selection. DSH134G cells that were both puromycin and hygromycin resistant were either isolated individually (clones) or pooled (pools), with an average pool size greater than 2,000 colonies. These cells are referred to as DSH134G-PS4-PY31 helper cells. To ensure that the helper cell clones and pools were derived from independent transfection events, four separate transfection experiments were performed; in addition, all helper cell clones were derived from different cell culture dishes. Both the clones and the pools of DSH134G-PS4-PY31 cells were plated with the same cell density. Viruses

were harvested 48 h later and used to infect target D17 cells (6, 50). The infected D17 cells were placed on hygromycin selection, and viral titers were determined. Virus titers were generated from six helper cell clones and three helper cell pools. The titers from the helper cell clones were 5×10^2 (E3), 2×10^4 (PP1), 5×10^4 (I3), 6×10^4 (GG1), and 10^5 CFU/ml (C3 and T1). The titers from the three helper cell pools were 10^5 (pool 1), 5×10^5 (pool 2), and 4×10^5 CFU/ml (pool 3).

Predicted proviral structures generated from virions containing PY31 RNA and PS4 RNA. DSH134G-PS4-PY31 cells can produce three types of virions: those with two copies of PS4 RNA (homozygotes), those with two copies of PY31 RNA (homozygotes), and those with one copy of PS4 RNA and one copy of PY31 RNA (heterozygotes) (Fig. 1B). Virions with two copies of PS4 RNA cannot produce proviruses because PS4 lacks the 3′ viral sequences. In contrast, virions containing two copies of PY31 RNA or virions containing one copy each of PY31 and PS4 RNA can produce proviruses.

As previously shown, virions containing two copies of PY31 RNA have dramatically reduced ability to generate proviruses (61); all the examined proviruses contain a hybrid LTR with a truncated SNV U3 and Mo-MSV R-U5. These will be referred to as Mo-MSV R-U5 proviruses.

Virions containing one copy each of PY31 and PS4 RNA can initiate minus-strand DNA synthesis on either strand of RNA, but only PY31 RNA has the necessary viral sequences at its 3′ end to complete reverse transcription. Minus-strand strong-stop DNA initiated from the PY31 RNA can transfer to the 3′ end of PY31 RNA (intramolecular transfer) with a reduced efficiency. The resulting proviruses have the same structure as those generated from virions with two copies of PY31 RNA (Mo-MSV R-U5 proviruses). Alternatively, the minus-strand strong-stop DNA initiated from PS4 can use the complementarity of the SNV R and transfer intermolecularly to the 3′ end of PY31 RNA. This is the most efficient means for the heterozygotic virions to complete reverse transcription. SNV and Mo-MSV contain identical PBS sequences; thus, plus-strand DNA transfer can occur efficiently after the intermolecular minus-strand DNA transfer. The resulting proviruses contain a complete SNV LTR and are referred to as SNV R-U5 proviruses.

The predicted structures of these two proviruses are shown in Fig. 2A. These two proviruses can be distinguished by restriction enzyme digestion and Southern hybridization analysis (54). An 800-bp *hygro* DNA fragment was used to generate probes by the random priming method for the Southern analysis (19). A *Not*I restriction enzyme site is present near the 5′ end of the SNV U3; thus, all proviruses will have the *Not*I sites in both the 5′ and 3′ LTRs. The size of this *Not*I-*Not*I fragment is 2,618 bp in SNV R-U5 proviruses and 2,478 bp in Mo-MSV R-U5 proviruses. In addition, a *Cla*I site is present in the SNV U5 but not in the Mo-MSV U5. Therefore, a *Not*I plus *Cla*I double digestion can also be used to confirm the origin of the R-U5 in these proviruses. *Cla*I sites are also present immediately downstream of *hygro* in both proviruses. Upon *Not*I plus *Cla*I double digestion, SNV R-U5 proviruses generate a 2,025-bp *Cla*I-*Cla*I fragment corresponding to the 5′ leader sequence and *hygro*. However, *Not*I plus *Cla*I digestion of Mo-MSV R-U5 proviruses results in a 2,418-bp *Not*I-*Cla*I fragment that includes most of the 5′ LTR, the leader sequence, and *hygro*.

Mo-MSV Ψ- and SNV E-containing RNAs can copackage within the same virion. Viruses from six clones and three pools of DSH134G-PS4-PY31 helper cells were used separately to infect target D17 cells. DNAs were isolated from infected, hygromycin-resistant D17 cell pools, and Southern hybridiza-

A.

Mo-MSV R-U5
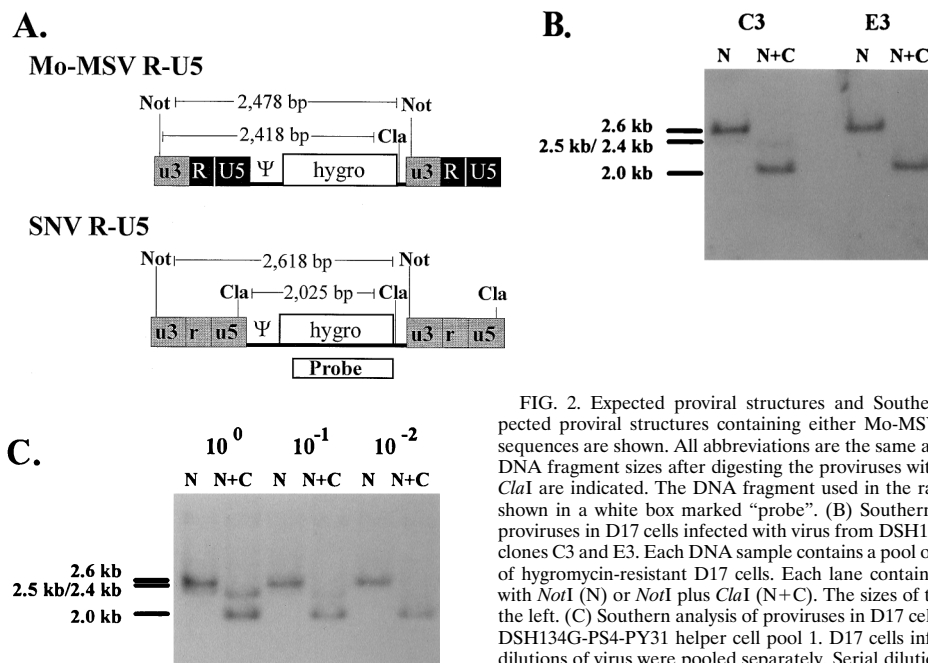

SNV R-U5

B.

C.

FIG. 2. Expected proviral structures and Southern analysis. (A) Two expected proviral structures containing either Mo-MSV- or SNV-derived R-U5 sequences are shown. All abbreviations are the same as those in Fig. 1. Expected DNA fragment sizes after digesting the proviruses with either *Not*I or *Not*I plus *Cla*I are indicated. The DNA fragment used in the random-priming reaction is shown in a white box marked "probe". (B) Southern hybridization analysis of proviruses in D17 cells infected with virus from DSH134G-PS4-PY31 helper cell clones C3 and E3. Each DNA sample contains a pool of more than 1,000 colonies of hygromycin-resistant D17 cells. Each lane contains 20 μg of DNA digested with *Not*I (N) or *Not*I plus *Cla*I (N+C). The sizes of the bands are indicated on the left. (C) Southern analysis of proviruses in D17 cells infected with virus from DSH134G-PS4-PY31 helper cell pool 1. D17 cells infected with different serial dilutions of virus were pooled separately. Serial dilutions used to infect cells are indicated above the figure. Each pool contains more than 500 colonies.

tions were performed. A representative Southern blot is shown in Fig. 2B. DNAs were isolated from D17 cells infected by virus harvested from helper cell clones E3 and C3; these DNAs are referred to as E3 and C3 DNA samples. In the E3 DNA sample, a 2.6-kb band or a 2.0-kb band was observed when DNA was digested with *Not*I or with *Not*I plus *Cla*I, respectively. The presence of these bands indicates that these hygromycin-resistant cells harbor the SNV R-U5 proviruses. Similar to the results for E3, Southern blot analysis of the C3 DNA sample revealed a 2.6-kb or a 2.0-kb band when the DNA was digested with *Not*I or *Not*I plus *Cla*I, respectively. However, the C3 DNA sample also revealed a faint 2.5-kb band or a 2.4-kb band upon *Not*I or *Not*I plus *Cla*I digestion, respectively. The presence of these bands indicated that in addition to the SNV R-U5 proviruses, these hygromycin-resistant cells contain Mo-MSV R-U5 proviruses. The intensities of these two additional bands were much weaker than those from the SNV R-U5 proviruses, indicating that the Mo-MSV R-U5 proviruses made up a small proportion of the integrated viral DNA.

Southern analysis revealed that SNV R-U5 proviruses were observed in both E3 and C3 DNA samples. Similar data were generated from the cells infected with viruses harvested from four additional helper cell clones (data not shown). This indicated that Mo-MSV Ψ-containing RNA and SNV E-containing RNA were copackaged and that genetic information from both RNAs was used to generate viral DNA. Mo-MSV R-U5 proviruses were also observed in the C3 sample but not in the E3 sample. It was observed that when the helper cells produced high viral titers, as did clone C3, the Mo-MSV R-U5 proviruses were present in the infected cell pools. In contrast, when the helper cell clones produced lower viral titers, as did clone E3, these proviruses were not present. SNV R-U5 proviruses have the full-length SNV promoter and can express *hygro*. In contrast, Mo-MSV R-U5 proviruses have truncated SNV U3 promoters and are impaired in their ability to express *hygro*. Thus, it is possible that Mo-MSV R-U5 proviruses could be observed only when the cells were infected at a high multiplicity of infection (MOI); under this condition they can be

carried along in cells that are coinfected with SNV R-U5 proviruses.

To test the effect of MOI on the generation of Mo-MSV R-U5 proviruses, hygromycin-resistant cells infected with different viral dilutions were pooled separately and Southern analysis was performed (Fig. 2C). Genomic DNA from cells infected with undiluted virus (10^0 in Fig. 2C; MOI was approximately 1) yielded 2.6-kb and 2.5-kb bands upon *Not*I digestion and 2.0-kb and 2.4-kb bands upon *Not*I plus *Cla*I double digestion. This indicates the presence of proviruses with both predicted structures. However, the intensity of the bands generated by the MSV R-U5 proviruses decreased as the viruses used to infect the D17 cells were diluted. In the DNA sample infected by the 10^{-2} virus dilutions (MOI was approximately 0.01), the 2.5-kb and the 2.4-kb bands were no longer detectable. In contrast, the bands that represented the SNV R-U5 proviruses could be easily detected in cells infected by all virus dilutions. This indicates that both the SNV R-U5 and the Mo-MSV R-U5 were used to generate proviruses. However, as the MOI of the virus decreases, the presence of the Mo-MSV R-U5 proviruses also decreases. Thus, the majority of Mo-MSV R-U5 proviruses are most likely carried along in cells coinfected with the SNV R-U5 proviruses. This also indicates that the formation of SNV R-U5 proviruses dictates the viral titers. Similar data was observed in two other independent experiments (data not shown).

Packaging efficiencies of Mo-MSV Ψ-containing and SNV E-containing RNAs by SNV proteins. The Southern analysis indicated that PY31 and PS4 can be copackaged in the helper cells. The relative levels at which PY31 and PS4 RNAs were packaged into virions by the SNV-based helper cells were determined (Fig. 3). One pool of DSH134G-PS4-PY31 helper cells and two helper cell clones (C3 and E3) were plated at equal densities; 48 h later, both cell-free viral RNAs and total cellular RNAs were harvested (26). Fivefold serial dilutions of viral RNA, cellular RNA, and a control plasmid DNA, pJS3, were prepared and applied onto a nylon membrane in duplicate by the convertible filtration manifold system (Gibco/
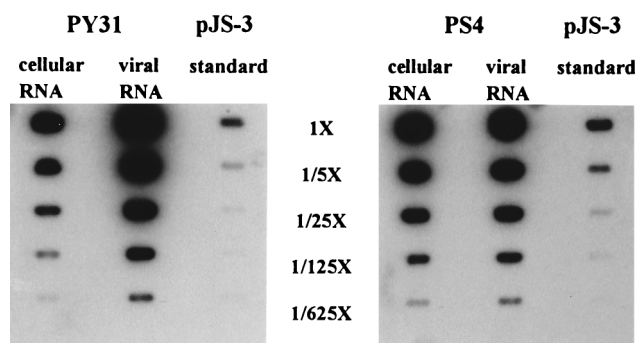
FIG. 3. Analysis of relative levels of PS4 and PY31 RNAs produced from DSH134G-PS4-PY31 cell clone C3. Total cellular and cell-free viral RNAs were isolated from DSH134G-PS4-PY31 cells. Fivefold serial dilutions of the RNAs were made, and slot blots were performed. ^{32}P-radiolabelled *hygro* and *puro* probes were used to hybridize to PY31 and PS4 RNAs, respectively. Plasmid pJS-3 contains both the *hygro* and *puro* genes and was used to normalize for the different efficiencies of the two probes.

BRL). The duplicate samples on the nylon membrane were separated, and each was hybridized to a probe generated from either a *hygro* DNA fragment or a *puro* DNA fragment. The intensities of the bands were quantified with a PhosphorImager (Molecular Dynamics). PY31 RNA hybridizes to probes containing the *hygro* DNA fragment, whereas PS4 RNA hybridizes to probes containing the *puro* DNA fragment. Both probes can hybridize to plasmid pJS3, which contains a copy of *puro* and a copy of *hygro*. Therefore, the difference in the specific activities of the two probes could be normalized by comparing their abilities to hybridize pJS3. The relative amounts of PY31 and PS4 RNAs could then be compared. A representative blot is shown in Fig. 3.

The ratio of PY31 RNA to PS4 RNA present in the total cellular RNA of the DSH134G-PS4-PY31 helper cell pool was 1.17 (Table 1). This demonstrates that the amounts of PS4 and PY31 RNAs from the pool of helper cells were relatively equal. In helper cell clones C3 and E3, the PY31/PS4 RNA ratios in the total cellular RNA preparations were 1.07 and 0.14, respectively. This variation in the amounts of PY31 and PS4 RNAs produced from helper cell clones is expected because of differences in the transfection efficiencies and in the expression of constructs integrated in different locations of the host genome. The ratio of PY31 RNA packaged into virus to PS4 RNA packaged varied from 0.71- to 10.42-fold. After the cellular RNA expression level was standardized, PY31 RNA was found to package into viruses 5.1 to 9.5 times more efficiently than PS4 RNA (Table 1). This confirmed that the reduced efficiency of the generation of Mo-MSV R-U5 proviruses was not due to the packaging of the PY31 RNA but resulted from the inefficient minus-strand DNA transfer and the deletion of the U3 promoter.

The RNA analysis indicated that SNV proteins preferentially packaged PY31 (Mo-MSV Ψ-containing) RNA over PS4 (SNV E-containing) RNA. Previously, it was observed that SNV proteins preferentially packaged competing SNV RNA over MLV RNA (17). Several possibilities could explain this difference. First, we used the Mo-MSV Ψ, while the researchers of previous work used the Mo-MLV Ψ. Other studies have demonstrated that Mo-MSV Ψ may be more efficient than Mo-MLV Ψ in directing the encapsidation of RNA despite the high degree of homology between the two sequences (1, 48). Second, the SNV E-containing vector, PS4, used in these experiments lacked SNV viral sequences at its 3′ end (SNV PPT, 3′ LTR). This may have affected the encapsidation of SNV

E-containing RNAs by SNV proteins. Sequences at the 3′ end of the human immunodeficiency virus type 1 (HIV-1) genome (49) and the Rous sarcoma virus genome (53) have been implicated in viral RNA packaging and provide some precedent for this possibility. Finally, the relative expression of the E- or Ψ-containing vectors in the helper cells was not determined in the previous experiments (17); this may have affected the virus titers and the conclusion.

Different retroviral species can interact genetically. During reverse transcription, template switching events occur frequently between copackaged RNAs (9, 24, 43). This includes minus-strand DNA transfer as well as recombination events. This is the first report to demonstrate that RNAs with encapsidation sequences derived from genetically distinct retroviruses can be copackaged in a virion. Furthermore, both viral RNAs were used to generate a DNA; this demonstrates that different viruses can interact genetically. However, the efficiency of this interaction cannot be clearly defined in these experiments. PY31 replicates with a 3,000-fold-reduced efficiency compared to a vector with two highly homologous LTRs (wild-type vector) (61). Southern analysis from all DNA samples demonstrated that there were more SNV R-U5 proviruses than Mo-MSV R-U5 proviruses. Therefore, the lower limit for generating SNV R-U5 proviruses must be at least 0.03% (1/3,000) of a wild-type vector. The higher limit of these events is more difficult to define. When a lower MOI was used for infection, the resulting hygromycin-resistant cells harbored mostly SNV R-U5 proviruses. Since virus titers were determined by counting hygromycin-resistant colonies at lower MOIs, the formation of the SNV R-U5 proviruses dictates the virus titer. The titers of the DSH134G-PS4-PY31 helper cells were between 5×10^2 and 1×10^5 CFU/ml. Thus, some of the titers are comparable to the published titers of the DSH134G helper cells with SNV vectors (2×10^5 to 5×10^5 CFU/ml) (37). This indicates that the copackaging of RNA from different viruses and the usage of both RNAs to generate DNA can be quite efficient. More experimental data will be needed to further define the efficiency of this interaction.

In this report, we demonstrate that genetic interaction between different retroviruses can occur after two viruses infect the same cells and their RNAs are copackaged. Abundant literature indicates that genetically distinct retroviruses can infect the same host cells. Many individuals infected with HIV-1 are also infected with other retroviruses such as HIV-2 (18, 21, 44, 47) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and -2) (4, 33, 42). Furthermore, it has been demonstrated that HIV-1 can infect the same host cells as HIV-2 (34) and HTLV-1 (13, 32). It has been reported that HIV-1 proteins can package the RNA of simian immunodeficiency virus, which is similar to HIV-2 (51). However, it is not clear whether copackaging of the two RNAs can occur.

TABLE 1. Packaging efficiency of PY31 RNA and PS4 RNA by SNV proteins[a]

DSH134G-PS4-PY31 helper cell	Ratio		
	PY31 viral RNA/PS4 viral RNA	PY31 cellular RNA/PS4 cellular RNA	Viral RNA/cellular RNA
Pool	7.75	1.17	6.6
Clone C3	10.42	1.07	9.5
Clone E3	0.71	0.14	5.1

[a] RNA was analyzed by slot blot hybridization and quantitated by a PhosphorImager.

Elements important for dimerization overlap the encapsidation sequence of some retroviruses (5, 10, 11, 46). It has been suggested that retroviral RNAs dimerize prior to encapsidation (20, 35, 39). The formation of heterodimers consisting of RNA from different retroviruses has been previously demonstrated in vitro. For example, HIV-1 RNA can form a heterodimer with either Mo-MLV RNA or Rous sarcoma virus RNA (36). In this report, we demonstrate that minus-strand DNA transfer is possible between Ψ- and E-containing RNAs, which suggests that the two RNAs can form heterodimers in vivo. If other species of retroviral RNAs can also form heterodimers in vivo, then the exchange of genetic information between different retroviruses may be feasible. These events may occur infrequently, and most of the resulting recombinant viruses may not be viable. However, if a chimeric virus with distinct properties is generated, it could have unknown pathogenic potential. Therefore, copackaging of heterologous RNAs from different retroviral species may have a significant role in the evolution of retroviruses in nature.

This work may also have public safety implications for xenotransplantation. Currently, organs and bone marrow from nonhuman primates or other animals are used or being considered for transplantation into humans (3, 45, 59). These xenotransplants can serve as reservoirs for the introduction of foreign retroviruses into human hosts (55). Once in the human host, these viruses may interact with human retroviruses or endogenous retrovirus-like elements to generate recombinant viruses with unknown properties. This underscores the importance of understanding the potential interactions between unrelated retroviruses.

REFERENCES

1. **Adam, M. A., and A. D. Miller.** 1988. Identification of a signal in a murine retrovirus that is sufficient for packaging of nonretroviral RNA into virions. J. Virol. **62:**3802–3806.
2. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64:**1920–1926.
3. **Bach, F. H., S. C. Robson, H. Winkler, C. Ferran, K. M. Stuhlmeier, C. J. Wrighton, and W. W. Hancock.** 1995. Barriers to xenotransplantation. Nat. Med. **1:**869–873.
4. **Bartholomew, C., W. Blattner, and F. Cleghorn.** 1987. Progression to AIDS in homosexual men co-infected with HIV-1 and HTLV-1 in Trinidad. Lancet **ii:**1469.
5. **Bieth, E., C. Gabus, and J.-L. Darlix.** 1990. A study of the dimer formation of Rous sarcoma virus RNA and of its effect on viral protein synthesis in vitro. Nucleic Acids Res. **18:**119–127.
6. **Bowman, E. H., V. K. Pathak, and W.-S. Hu.** 1996. Efficient initiation and strand transfer of polypurine tract-primed plus-strand DNA prevent strand transfer of internally initiated plus-strand DNAs. J. Virol. **70:**1687–1694.
7. **Coffin, J. M.** 1992. Structure and classification of retroviruses, p. 19–49. *In* J. A. Levy (ed.), The retroviridae, vol. 1. Plenum Press, New York, N.Y.
8. **Coffin, J. M.** 1993. Reverse transcription and evolution, p. 445–479. *In* A. M. Skalka and S. P. Goff (ed.), Reverse transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
9. **Coffin, J. M.** 1996. Retroviridae: the viruses and their replication, p. 1767–1848. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, vol. 3. Raven Press, New York, N.Y.
10. **Darlix, J.-L., C. Gabus, and B. Allain.** 1992. Analytical study of avian reticuloendotheliosis virus dimeric RNA generated in vivo and in vitro. J. Virol. **66:**7245–7252.
11. **Darlix, J.-L., C. Gabus, M.-T. Nugeyre, F. Clavel, and F. Barre-Sinoussi.** 1990. Cis elements and trans-acting factors involved in the RNA dimerization of the human immunodeficiency virus HIV-1. J. Mol. Biol. **216:**689–699.
12. **de la Luna, S., I. Soria, D. Pulido, J. Ortin, and A. Jimenez.** 1988. Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. Gene **62:**121–126.
13. **de Rossi, A., G. Franchini, A. Aldovini, A. del Mistro, L. Chieco-Bianchi, R. C. Gallo, and F. Wong-Staal.** 1986. Differential response to the cytopathic effects of human T-cell lymphotropic virus type III (HTLV-III) superinfection in T4+ (helper) and T8+ (suppressor) T-cell clones transformed by HTLV-I. Proc. Natl. Acad. Sci. USA **83:**4297–4301.
14. **Dougherty, J. P., and H. M. Temin.** 1986. High mutation rate of a spleen necrosis virus-based retroviral vector. Mol. Cell. Biol. **6:**4387–4395.
15. **Dougherty, J. P., and H. M. Temin.** 1987. A promoterless retroviral vector indicates that there are sequences in U3 required for 3′ RNA processing. Proc. Natl. Acad. Sci. USA **84:**1197–1201.
16. **Duesberg, P. H.** 1968. Physical properties of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. USA **60:**1511–1518.
17. **Embreston, J. E., and H. M. Temin.** 1987. Lack of competition results in efficient packaging of heterologous murine retroviral RNAs and reticuloendotheliosis virus encapsidation-minus RNAs by the reticuloendotheliosis virus helper cell line. J. Virol. **61:**2675–2683.
18. **Evans, L. A., J. Moreau, K. Odehouri, D. Seto, G. Thompson-Honnebier, H. Legg, A. Barboza, C. Cheng-Mayer, and J. A. Levy.** 1988. Simultaneous isolation of HIV-1 and HIV-2 from an AIDS patient. Lancet **iii:**1389–1391.
19. **Feinberg, A. P., and B. Volgelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specificity. Anal. Biochem. **132:**6–13.
20. **Fu, W., R. J. Gorelick, and A. Rein.** 1994. Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions. J. Virol. **68:**5013–5018.
21. **George, J. R., C. Y. Ou, B. Parekh, K. Brattegaard, V. Brown, E. Boateng, and K. M. De Cock.** 1992. Prevalence of HIV-1 and HIV-2 mixed infections in Cote d'Ivoire. Lancet **340:**337–339.
22. **Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein.** 1988. Point mutants of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zinc finger-like" protein sequence. Proc. Natl. Acad. Sci. USA **85:**8420–8424.
23. **Gritz, L., and J. Davies.** 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression. Gene **25:**179–188.
24. **Hu, W.-S., and H. M. Temin.** 1990. Retroviral recombination and reverse transcription. Science **250:**1227–1233.
25. **Hu, W.-S., and H. M. Temin.** 1990. Genetic consequences of packaging two RNA genomes in one retroviral particle: psuedodiploidy and high rate of genetic recombination. Proc. Natl. Acad. Sci. USA **87:**1156–1560.
26. **Hu, W.-S., and H. M. Temin.** 1992. Effect of gamma radiation on retroviral recombination. J. Virol. **66:**4457–4463.
27. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4:**1172–1174.
28. **Kewalramani, V. N., A. T. Panganiban, and M. Emerman.** 1992. Spleen necrosis virus, an avian immunosuppressive retrovirus, shares a receptor with the type D simian retroviruses. J. Virol. **66:**3026–3031.
29. **Konings, D. A. M., M. A. Nash, J. V. Maizel, and R. B. Arlinghaus.** 1992. Novel GACG-hairpin pair motif in the 5′ untranslated region of type C retroviruses related to murine leukemia virus. J. Virol. **66:**632–640.
30. **Koo, H.-M., J. Gu, A. Varela-Echavarria, Y. Ron, and J. P. Dougherty.** 1992. Reticuloendotheliosis type C and primate type D oncoretroviruses are members of the same receptor interference group. J. Virol. **66:**3448–3454.
31. **Kung, H.-J., J. M. Bailey, N. Davidson, M. O. Nicolson, and R. M. McAllister.** 1975. Structure, subunit composition, and molecular weight of RD-114 RNA. J. Virol. **16:**397–411.
32. **Landau, N. R., K. A. Page, and D. R. Littman.** 1991. Psuedotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J. Virol. **65:**162–169.
33. **Lee, H., P. Swanson, V. S. Shorty, J. A. Zack, J. D. Rosenblatt, and I. S. Chen.** 1989. High rate of HTLV-II infection in seropositive I. V. drug abusers in New Orleans. Science **244:**471–475.
34. **Le Guern, M., and J. A. Levy.** 1992. Human immunodeficiency virus (HIV) type 1 can superinfect HIV-2-infected cells: pseudotype virions produced with expanded cellular host range. Proc. Natl. Acad. Sci. USA **89:**363–367.
35. **Levin, J. G., P. M. Grimley, J. M. Ramseur, and I. K. Berezesky.** 1974. Deficiency of 60 to 70S RNA in murine leukemia virus particles assembled in cells treated with actinomycin D. J. Virol. **14:**152–161.
36. **Marquet, R., F. B. Baudin, C. Gabus, J.-L. Darlix, M. Mougel, C. Ehresmann, and B. Ehresmann.** 1991. Dimerization of human immunodeficiency virus (type 1) RNA: stimulation by cations and possible mechanism. Nucleic Acids Res. **19:**2349–2357.
37. **Martinez, I., and R. Dornburg.** 1995. Improved retroviral packaging lines derived from spleen necrosis virus. Virology **208:**234–241.
38. **McKnight, S. L.** 1992. CCAAT/enhancer binding protein, p. 771–795. *In* S. L. McKnight (ed.), Transcriptional regulation. Cold Spring Harbor Lab-

oratory Press, Cold Spring Harbor, N.Y.

39. **Méric, C., and S. P. Goff.** 1989. Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. **63:**1558–1568.

40. **Meric, C., and P.-F. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60:**450–459.

41. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **5:**431–437.

42. **Page, J. B., S. Lai, D. D. Chitwood, N. G. Klimas, P. C. Smith, and M. A. Fletcher.** 1990. HTLV-I/II seropositivity and death from AIDS among HIV-1 seropositive intravenous drug users. Lancet **334:**1664–1667.

43. **Panganiban, A. T., and D. Fiore.** 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. Science **241:**1064–1069.

44. **Peeters, M., C.-Y. Gershy-Damet, K. Fransen, K. Koffi, M. Coulibaly, E. Delaporte, P. Piot, and G. van der Groen.** 1992. Virological and polymerase chain reaction studies of HIV-1/HIV-2 dual infection in Cote d'Ivoire. Lancet **340:**339–340.

45. **Platt, J. L., G. M. Vercellotti, A. P. Dalmasso, A. J. Matas, R. M. Bolman, J. S. Najarian, and F. H. Bach.** 1990. Transplantation of discordant xenografts: a review of progress. Immunol. Today **11:**450–456.

46. **Prats, A.-C., C. Roy, P. Wang, M. Erard, V. Housset, C. Gabus, C. Paoletti, and J.-L. Darlix.** 1990. *cis* elements and *trans*-acting factors involved in dimer formation of murine leukemia virus RNA. J. Virol. **64:**774–783.

47. **Rayfield, M., K. De Cock, W. Heyward, L. Goldstein, J. Krebs, S. Kwok, S. Lee, J. McCormick, J. M. Moreau, K. Odehouri, G. Schochetman, J. Sninsky, and C.-Y. Ou.** 1988. Mixed human immunodeficiency virus (HIV) infection in an individual: demonstration of both HIV type 1 and type 2 proviral sequences by using polymerase chain reaction. J. Infect. Dis. **158:**1170–1176.

48. **Rein, A.** 1994. Retroviral RNA packaging: a review. Arch. Virol. **9:**513–522.

49. **Richardson, J. H., L. A. Child, and A. M. L. Lever.** 1993. Packaging of human immunodeficiency virus type 1 RNA requires *cis*-acting sequences outside the 5′ leader region. J. Virol. **67:**3997–4005.

50. **Riggs, J. L., R. M. McAllister, and E. H. Lennette.** 1974. Immunofluorescent studies of RD-114 virus replication in cell culture. J. Gen. Virol. **25:**21–29.

51. **Rizvi, T. A., and A. T. Panganiban.** 1993. Simian immunodeficiency virus RNA is efficiently encapsidated by human immunodeficiency virus type 1 particles. J. Virol. **67:**2681–2688.

52. **Saltzman, A. G., and R. Weinman.** 1989. Promoter specificity and modulation of RNA polymerase II transcription. FASEB J. **3:**1723–1733.

53. **Sorge, J., W. Ricci, and S. H. Hughes.** 1983. *cis*-acting RNA packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. J. Virol. **48:**667–675.

54. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.

55. **Stoye, J. P., and J. M. Coffin.** 1995. The dangers of xenotransplantation. Nat. Med. **1:**100.

56. **Temin, H. M.** 1991. Sex and recombination in retroviruses. Trends Genet. **7:**71–74.

57. **Van Beveren, C., J. M. Coffin, and S. Hughes.** 1985. Nucleotide sequences complemented with functional and structural analysis, p. 567–1148. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

58. **Weiss, R. A., W. S. Mason, and P. K. Vogt.** 1973. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses. Virology **52:**535–552.

59. **White, D., and J. Wallwork.** 1993. Xenografting: probability, possibility, or pipe dream? Lancet **342:**879–880.

60. **Yang, S., and H. M. Temin.** 1994. A double hairpin structure is necessary for the efficient encapsidation of spleen necrosis virus retroviral RNA. EMBO J. **13:**713–726.

61. **Yin, P. D., V. K. Pathak, A. E. Rowan, R. J. Teufel II, and W.-S. Hu.** 1997. Utilization of nonhomologous minus-strand DNA transfer to generate recombinant retroviruses. J. Virol. **71:**2487–2494.