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Nonreciprocal Pseudotyping: Murine Leukemia Virus Proteins Cannot Efficiently Package Spleen Necrosis Virus-Based Vector RNA

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It has been documented that spleen necrosis virus (SNV) can package murine leukemia virus (MLV) RNA efficiently and propagate MLV vectors to the same titers as it propagates SNV-based vectors. Although the SNV packaging signal (E) and MLV packaging signal (Ψ) have little sequence homology, similar double-hairpin RNA structures were predicted and supported by experimental evidence. To test whether SNV RNA can be packaged by MLV proteins, we modified an SNV vector to be expressed in an MLV-based murine helper cell line. Surprisingly, we found that MLV proteins could not support the replication of SNV vectors. The decrease in titer was approximately 2,000- to 20,000-fold in one round of retroviral replication. RNA analysis revealed that SNV RNA was not efficiently packaged by MLV proteins. RNA hybridization of the cellular and viral RNAs indicated that SNV RNA was packaged at least 25-fold less efficiently than MLV RNA, which was the sensitivity limit of the hybridization assay. The contrast between the MLV and SNV packaging specificity is striking. SNV proteins can recognize both SNV E and MLV Ψ, but MLV can recognize only MLV Ψ. This is the first demonstration of two retroviruses with nonreciprocal packaging specificities.

Packaging of viral RNA is an essential process of retroviral assembly (13, 59, 60). Selection of the viral RNA during assembly is governed by the interactions between viral proteins and the packaging signal in viral RNA (13, 36, 37). Packaging signals have been identified in many retroviruses. In most if not all identified packaging signals, major portions of the signals are located in the 5′ untranslated region of the viral RNA between primer binding sites and *gag* (36, 37, 46). The Gag polyprotein has been shown to interact with the packaging signal in viral RNA and to select the RNA for packaging (4, 9, 10, 25, 26, 29, 30, 37, 43, 51).

"Pseudotyping" refers to viral particles that contain RNA from one virus and one or more proteins from another virus (7, 35, 50, 59, 60, 62, 68). In retroviral systems, pseudotyping is often observed with the RNA of a defective virus and the protein(s) of a closely related helper virus. Pseudotyping can also be observed with genetically distinct viruses. However, when distinct viruses are involved, the mixing is generally limited to the *env* gene products and not the *gag-pol* gene products (7, 59, 60, 62, 68). This limitation is probably due to the specific recognition between the Gag polyprotein and the RNA genome. The Gag polyprotein of one virus may not recognize the RNA of a different virus. There are, however, at least two examples in which the RNA of one retrovirus can be packaged and propagated entirely by the viral proteins of a different retrovirus. First, the proteins of the reticuloendotheliosis viruses (REV) can package the RNA of murine leukemia virus (MLV)-based vectors (17, 19, 32, 65, 67). Second, viral proteins of human immunodeficiency virus can package the RNA of simian immunodeficiency virus (49).

REV form a group of avian type C retroviruses, including isolates REV-A, spleen necrosis virus (SNV), duck infectious anemia virus, and chicken syncytial virus (44, 64). Although the natural hosts for these viruses are avian, these viruses are more similar to mammalian oncoviruses (12). Members of the REV group are classified as MLV-related viruses (12). The *gag-pol* region of REV is similar to those of MLV and gibbon ape leukemia virus (GaLV) by amino acid sequences, antigenicity of the *gag* gene products, and cation preference of the reverse transcriptase (2, 3, 32, 34, 53, 55, 56). It has been observed that the proteins of REV-A and SNV, two highly homologous members of the REV group, can efficiently package MLV-derived viral vectors. These pseudotyped viruses can reach titers similar to those of the SNV vectors (17, 19, 32, 65, 67). Secondary-structure and mutational analysis has demonstrated that even though the packaging signal of MLV (Ψ) and the packaging signal of SNV (E) lack sequence homology, they both contain a similar double-hairpin structure (33, 65). It was further demonstrated that the MLV double-hairpin structure could functionally replace the SNV double-hairpin structure; a vector with a chimeric packaging signal from MLV and SNV was efficiently packaged by SNV proteins (65).

Given the ability of REV and SNV proteins to support MLV vector replication, it is logical to question whether MLV proteins can also support SNV vector propagation. In this study, we performed the reciprocal experiment to examine the ability of MLV proteins to support the propagation of SNV vectors. We found that SNV vector RNAs were not packaged by the MLV helper cell lines; this indicates that the MLV proteins cannot recognize the SNV packaging signal.

MATERIALS AND METHODS

Construction of viral vectors. Retroviral vectors were constructed by standard cloning techniques (52). Plasmids of retroviral vectors are preceded by a p, whereas the viruses or proviruses generated from the plasmid are not. For example, pJS12 refers to the plasmid whereas JS12 refers to the virus or the provirus. pJS12 and pJS14 were both derived from plasmid pJD220SVHy (16). pJD220SVHy is an SNV-based vector containing an internal simian virus 40 (SV40) promoter expressing the hygromycin phosphotransferase B gene (*hygro*). The U3 promoter of the 3′ long terminal repeat (LTR) was removed by a 400-bp deletion without altering the 3′ attachment site. A full-length β-galactosidase gene (β-*gal*) was inserted upstream of the SV40 promoter to form pJS11. A 0.44-kb restriction enzyme fragment containing MLV U3 was inserted in the deleted U3 to form pJS12. This 0.44-kb fragment was derived from pME149 (21) and contains most of the MLV U3 sequences as well as 30 bp of R (57). A 0.4-kb

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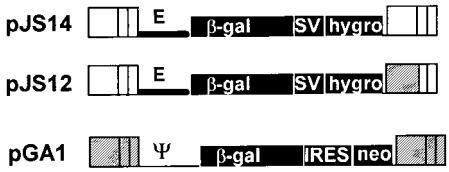

FIG. 1. Structures of retroviral vectors used to study pseudotyping. pJS12 and pJS14 are SNV-based retroviral vectors, whereas pGA1 is an MLV-based retroviral vector. SNV vectors pJS12 and pJS14 each contain β-*gal* expressed from the U3 promoter and *hygro* expressed from an internal SV40 promoter (SV). The 3′ LTR of pJS12 contains MLV U3 instead of SNV U3. pGA1, an MLV-based vector, also contains β-*gal* and a *neo* gene that is expressed by an encephalomyocarditis virus IRES. Open boxes represent SNV LTR sequences, and hatched boxes denote MLV LTR sequences. The SNV packaging signal (E) is designated by a thick line, whereas the MLV packaging signal (Ψ) is designated by a thin line.

DNA fragment containing SNV U3 was inserted in the same position in pJS11 to form pJS14. This 0.4-kb DNA fragment contained the DNA sequences between the *Sac*I and *Ava*I sites in the SNV U3 and replaced all of the deleted U3 sequences in pJD220SVHy.

Cells and virus propagation. D17 is a canine osteosarcoma cell line that is permissive to infection by SNV, REV-A, and MLV (48). C3A2 is derived from D17 and expresses REV-A *gag-pol* and *env* (61). REV-A and SNV are more than 90% homologous (19, 32), and REV-A proteins can package SNV-derived vectors with high efficiency (17, 19, 32, 65, 67). Both C3A2 cells and D17 cells were maintained in Dulbecco's modified Eagle's medium with 6% calf serum (HyClone laboratory). PG13 is a helper cell line that expresses MLV *gag-pol* and GaLV *env* (41). PA317 is an amphotropic MLV-based helper cell line (40). Both PG13 and PA317 are derived from NIH 3T3tk$^-$ cells and were maintained in Dulbecco's modified Eagle's medium with 10% calf serum. All cell lines were maintained in incubators at 37°C under 5% CO_2.

Plasmids were introduced into the helper cells by Polybrene-dimethyl sulfoxide transfection (31). Cell culture media were changed to fresh media 24 to 48 h before the virus was harvested. Virus-containing cell culture media were collected and subjected to centrifugation at 2,000 × *g* for 10 min to pellet the cells and cellular debris. Supernatants were isolated and used either to infect new cells or to isolate RNA. All the viruses used in infection were freshly harvested. Serial dilutions were made and used to infect target cells in the presence of Polybrene (50 μg/ml) for 4 h at 37°C.

To compare the viral titers of different vector constructs, viruses generated from each pool of the transfected cells were used to infect fresh helper cells. Pools of infected helper cells were generated; the pool size ranged between 1,500 and 6,000 colonies per pool. Helper cell pools containing different constructs were plated out at 5×10^6 cells per 100-mm dish. Viruses were harvested from these cells 36 to 48 h later and were used to infect D17 cells. Within each set of experiments, different viruses were generally harvested within the same hour.

RNA isolation and analysis. Cellular RNAs were isolated with the Trizol reagent (Gibco/BRL) as specified by the manufacturer. The integrity of the cellular RNA was examined by gel electrophoresis and by inspection of the ribosomal bands.

Viral RNAs were isolated by the following methods. Cells infected with vectors were plated at a density of 5×10^6 per 100-mm dish on the same day, and supernatants were collected 2 days later. An aliquot of the wild-type virus was added to serve as a control. Cell culture supernatants were subjected to low-speed centrifugation to remove cells and cellular debris. The supernatants were then centrifuged at 25,000 rpm for 90 min in an SW41 rotor. Viral pellets were resuspended in 50 mM Tris–1 mM EDTA (pH 7.5 to 8). A final concentration of 0.1% sodium dodecyl sulfate and 200 μg of tRNA per ml was added to the viruses, and the mixtures were extracted once with phenol, once with phenol-chloroform, and once with chloroform. A 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol were added to the RNAs. The RNA pellets were resuspended in 100 μl of diethylpyrocarbonate-treated water.

Slot blotting was performed with the convertible filtration manifold system (Gibco/BRL) under the conditions recommended by the manufacturer. Slot blots were hybridized with DNA fragments labeled with [α-^{32}P]dCTP by the random-priming method (22). All the probes used had a specific activity greater than 10^9 cpm/μg of DNA. The hybridization conditions were the same as previously described (27). The yield of the viral RNA was first standardized by the amount of wild-type SNV RNA. A 1.5-kb *Hin*dIII DNA fragment containing most of the REV-A *env* gene was used as a template for the random-priming reaction to generate a wild-type SNV-specific probe. A 3.8-kb DNA fragment containing β-*gal* was used to generate the probe to detect GA1, JS12, and JS14 RNAs. Quantitations were performed with a PhosphorImager (Molecular Dynamics) and ImageQuant software.

RESULTS

Retroviral vectors used to study viral RNA packaging. To determine whether SNV vector RNA can be pseudotyped by MLV proteins, a set of modified SNV vectors was constructed. It was necessary to modify the SNV vectors to use the currently available MLV-based murine helper cell lines, because the SNV U3 promoter is not transcriptionally active in murine cells (20). The structures of SNV-based vectors JS14 and JS12 are illustrated in Fig. 1. Both of these two vectors contain a complete 5′ LTR, E, β-*gal*, SV40 early promoter, and *hygro*. For both constructs, the viral U3 promoter drives the expression of β-*gal* whereas the internal SV40 promoter drives the expression of *hygro*. The 3′ LTRs of these two vectors contain different sequences. In pJS14, the U3 region of the 3′ LTR contains the SNV U3 promoter, whereas in pJS12 the SNV U3 region of the 3′ LTR was replaced by the MLV U3 promoter (Fig. 1). When pJS12 was transfected into helper cells, the full-length viral mRNA was expressed from the SNV U3 promoter located in the 5′ LTR. The full-length viral RNA contains the R-U5 from the 5′ LTR and the U3-R from the 3′ LTR. The 3′ U3 in pJS12 contains the MLV U3 sequences, which are used as a template for DNA synthesis during reverse transcription to generate proviruses containing MLV U3 in both LTRs (24). Therefore, in the progeny provirus resulting from infection, the full-length mRNA was expressed by the MLV U3.

MLV-based vector pGA1 was used as a positive control in these experiments (28); the structure of pGA1 is illustrated in Fig. 1. pGA1 contains MLV LTRs, Ψ, β-*gal*, an internal ribosomal entry site (IRES) from encephalomyocarditis virus (14), and the neomycin phosphotransferase gene (*neo*) (Fig. 1). Both β-*gal* and *neo* are expressed from the transcripts derived from 5′ U3; IRES allows translation of *neo.*

Experimental protocol. The experimental protocol used is illustrated in Fig. 2. Viral constructs pJS14 and pJS12 were separately transfected into a REV-A helper cell line, C3A2, which can package SNV vectors efficiently. Transfected C3A2 cells were placed under hygromycin selection, and hygromycin-resistant colonies were pooled. The MLV vector pGA1 was propagated in a similar manner, except that it was transfected into the MLV helper cell line PA317. PA317 was used to propagate GA1 because both GA1 and C3A2 contain *neo*; thus, the presence of GA1 in C3A2 cells cannot be directly selected.

JS14, JS12, and GA1 viruses were harvested from the transfected cells and used to separately infect PG13 helper cells.

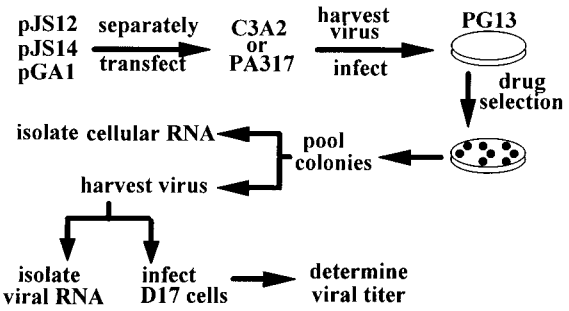

FIG. 2. Experimental protocol to study whether MLV proteins can pseudotype SNV vectors. Plasmid DNAs were used to separately transfect C3A2 (pJS12 and pJS14) or PA317 (pGA1) helper cells. JS12, JS14, and GA1 virus stocks were harvested separately from transfected helper cells and used to infect PG13 helper cells. Infected PG13 cells were selected with the appropriate drugs, pooled, and expanded. Viruses harvested from the pools were used for either RNA isolation or infection of D17 target cells to determine viral titers. DNA and RNA were also isolated from the infected PG13 cells for structural and expression analysis.

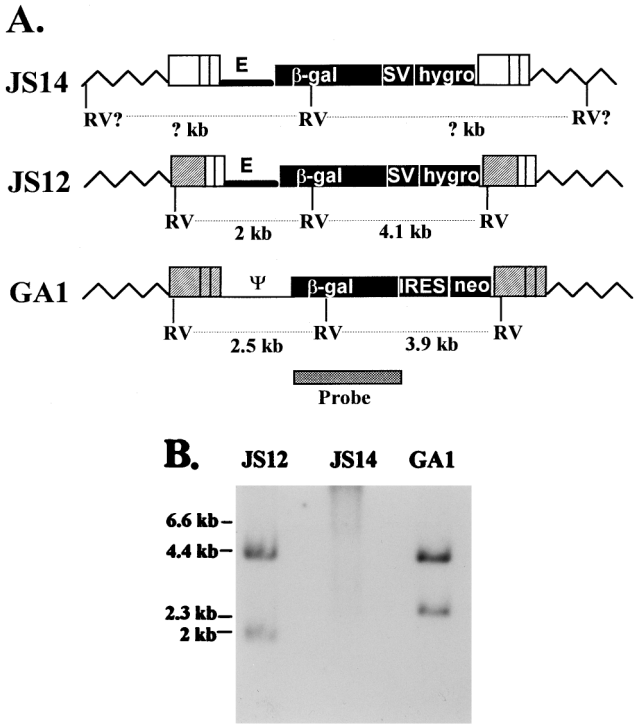

FIG. 3. (A) Predicted proviral structures of JS14, JS12, and GA-1. Zigzag lines, host DNA sequences; cross-hatched box labeled Probe, DNA fragment used to generate the radioactively labeled probe; RV, *Eco*RV restriction enzyme site. All the other abbreviations are identical to those in Fig. 1. (B) Southern analysis of genomic DNAs isolated from pools of PG13 cells infected with JS12, JS14, or GA1. The probe was a radioactively labeled β-*gal* DNA fragment (as shown in panel A). JS12 DNA produced 4.1- and 2-kb bands. JS14 DNA produced a smear because a pool of infected PG13 cells was used. GA1 containing genomic DNA produced 3.9- and 2.5-kb bands.

PG13 cells express MLV *gag-pol* and GaLV *env*. In each set of experiments, infections of PG13 with different viruses were performed on the same day. Infected PG13 cells were subjected to appropriate drug selections, and drug-resistant cells infected with each vector were pooled separately. Each pool represented a minimum of 1,000 colonies of infected cells. These pools of infected cells were plated at 5×10^6 cells per 100-mm dish. Two days after the cells were plated, viruses generated from each cell pool were harvested and used for either RNA isolation or infection of D17 cells to determine the viral titers. Cellular DNA and RNA were also isolated from these helper cell pools to analyze proviral structures and viral RNA expression.

PG13 is derived from a murine cell line, NIH 3T3tk$^-$, and expresses MLV *gag-pol* and GaLV *env*. The *gag* gene products select the packaged RNA; therefore, the interactions between MLV Gag polyproteins and RNA were analyzed in this system. Viruses propagated from PG13 contain the GaLV Env, and they cannot infect murine cells but can infect cells from other species such as D17 cells. This eliminates the possibility of reinfection during the propagation of the virus-containing helper cells and avoids possible amplification of differences in packaging efficiency between different vectors.

Proviral structures in infected PG13 helper cells. Genomic DNAs were isolated from pools of PG13 helper cells containing JS14, JS12, or GA1. The proviral structures of these vectors in helper cell pools were analyzed by restriction enzyme digestion and Southern hybridization. Viral DNA structures and restriction enzyme sites are illustrated in Fig. 3A. All three constructs contain an *Eco*RV site in β-*gal* (Fig. 3A). In addition, the MLV U3 contains two *Eco*RV restriction enzyme sites, whereas the SNV U3 does not contain any *Eco*RV sites. Therefore, if the JS12 proviruses in the PG13 cells contain the MLV U3, digestion with *Eco*RV should generate 4.1- and 2-kb DNA fragments that can be detected by Southern blotting with a probe derived from β-*gal* DNA. JS14 contains SNV U3; therefore, the only *Eco*RV site present in JS14 is located in β-*gal*. The sizes of the JS14 DNA fragments from *Eco*RV digestion vary depending on the locations of the enzyme sites in the flanking sequences in the cellular genome. Because retroviral integration is random, when DNA derived from a pool of JS14-infected cells is analyzed, a smear is expected. GA1 also contains the MLV U3; 3.9- and 2.5-kb bands are expected upon *Eco*RV digestion and hybridization with the β-*gal* probe (Fig. 3A). Southern analysis indicated that all of the proviral structures were as expected. An example of the Southern analysis is shown in Fig. 3B. These analyses demonstrate that during reverse transcription of JS12, the MLV U3 was duplicated (Fig. 3B).

The MLV helper cell line can propagate MLV vectors but not SNV vectors. Supernatants harvested from PG13 cells containing JS12, JS14, or GA1 proviruses were used to infect D17 cells. These infected D17 cells were placed under appropriate drug selections to determine the titers of these viruses. Data from three sets of experiments are shown in Table 1.

The MLV vector GA1 contains the MLV U3 promoter and MLV Ψ. Thus, as expected, the GA1 RNA was efficiently expressed and packaged in PG13 cells. GA1 was propagated efficiently in PG13 cells, with titers varying from 10^4 to 10^5 CFU/ml.

PG13 cells failed to produce infectious JS14 viruses. The SNV U3 promoter drives the transcription of the full-length JS14 RNA. Because SNV U3 promoter is not transcriptionally active in murine cells, it is expected that JS14 full-length RNA will not be expressed or packaged in PG13 cells to produce infectious viruses.

PG13 cells also failed to propagate the SNV E-containing JS12. After reverse transcription, JS12 contained MLV U3 in both LTRs (Fig. 3). Thus, full-length, E-containing JS12 RNA should be present in PG13 cells. However, the infection data indicated that infectious JS12 viruses were not produced by PG13. The defect in the virus production could be at the level of RNA expression, RNA packaging, or postassembly. To determine the cause of this defect, the cellular and viral RNAs of these vectors in PG13 cells were examined.

Analysis of cellular and viral RNAs from PG13 cells. To analyze the expression of JS14, JS12, and GA1, total cellular RNAs were isolated from vector-infected PG13 cell pools. Equal amounts of cellular RNA from each sample were used to prepare fivefold serial dilutions; the first dilution of each sample contained 7 μg of total RNA. These RNAs were applied to nitrocellulose filters to generate slot blots. These RNA blots were hybridized with probes generated from DNA fragments containing β-*gal* to detect full-length, packaging signal-

TABLE 1. Virus titers produced by PG13 and C3A2 helper cells infected with JS12, JS14, or GA1

Expt	Titer (CFU/ml)				
	JS12-infected:		JS14-infected:		GA1-infected PG13
	PG13	C3A2	PG13	C3A2	
1	$<1 \times 10^0$	6×10^3	$<1 \times 10^0$	2×10^4	1×10^5
2	$<1 \times 10^0$	1×10^4	$<1 \times 10^0$	1×10^5	3×10^4
3	$<1 \times 10^0$	2×10^4	$<1 \times 10^0$	7×10^4	1×10^4

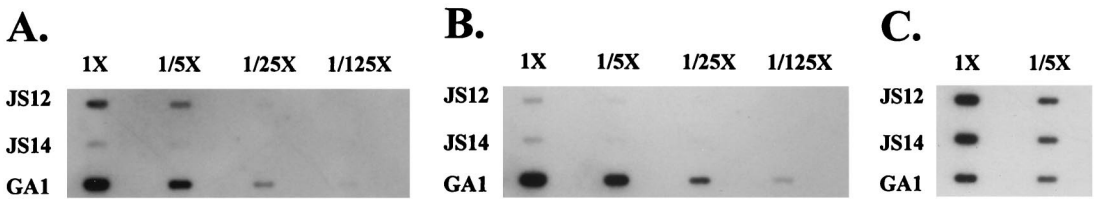

FIG. 4. RNA hybridization studies with cellular and viral RNA from infected PG13 cell pools. (A) RNA analysis of PG13 cellular RNA hybridized with the β-*gal* probe (Fig. 3A). The dilutions of RNA are shown above the blot. A 7-μg portion of total cellular RNA was used in the 1× dilution. (B) RNA analysis of viral RNA with the β-*gal* probe. (C) RNA analysis of spiked wild-type SNV RNA with the REV-A *env* probe. Wild-type SNV was added to the supernatant before the isolation of viral RNA to adjust for the possible loss of RNA during the purification procedures.

containing RNA. An example of the slot blots from cellular RNAs is shown in Fig. 4A.

Cellular RNA analysis indicated that both GA1 and JS12 RNA were expressed in PG13 cells. However, JS12 RNA is expressed at a fivefold-lower level than GA1 RNA. This may be caused by the interference of the internal SV40 promoter in JS12 (21). Expression of β-*gal* in JS14 is driven by the SNV U3 promoter. Slot blot analysis indicates that a very small amount of β-*gal*-containing RNA is present in JS14-infected PG13 cells, approximately 40-fold less than that of GA1 and 8-fold less than that of JS12.

Viral RNAs were isolated to examine the packaging of these vector RNAs in viral particles generated by PG13 (Fig. 4B and C). For each set of experiments, supernatants from pools of PG13 cells infected with JS12, JS14, or GA1 were collected. An aliquot of wild-type SNV was added to the supernatants at a 1:25 ratio to serve as an internal control to adjust for possible loss of RNAs during the isolation procedure. Viral RNAs were isolated, and fivefold dilutions were made; slot blots containing these RNAs were generated in duplicate. One set of the slot blots were hybridized with probe containing β-*gal* sequences. An example is shown in Fig. 4B. These blots demonstrated that GA1 was packaged efficiently, which is consistent with the infection data (Table 1). The sensitivity of our RNA analysis allows detection of up to a 125-fold dilution of the GA1 viral RNA. The signals from JS12 and JS14 RNAs were barely above background and were similar to those of the 125-fold dilution of GA1 viral RNA. The signals of JS12 and JS14 were similar to each other, although JS12 RNA was more abundant in the cellular RNA, indicating that the signal was either background in RNA detection or nonspecific packaging by the viral proteins. The other set of duplicated slot blots were hybridized to probes containing the SNV *env* sequences. An example is shown in Fig. 4C. These analyses showed that the differences observed in the viral RNA packaging (Fig. 4B) were not due to the loss of RNA during the purification procedure.

In the infected PG13 cells, GA1 RNA is approximately fivefold more abundant than JS12. However, the viral RNA analysis indicated at least a 125-fold difference in the viral RNA signal. Thus, GA1 RNA is packaged at least 25-fold better than the SNV E-containing JS12 RNA. This is in sharp contrast to our observations that SNV proteins can package MLV Ψ-containing vector RNA with the same efficiency as they can package the SNV E-containing RNA (67).

The REV helper cell line propagates SNV vectors JS12 and JS14 efficiently. It was possible that MLV Gag proteins could not package JS12 RNA because JS12 contained defective packaging signals and not because MLV Gag cannot recognize SNV E. To eliminate this possibility, the efficiency of JS12 propagation in the REV-A helper cell line, C3A2, was examined. A protocol similar to the one in Fig. 2 was used, except that C3A2 was used instead of PG13. JS12 and JS14 viruses were harvested from transfected C3A2 cells and used to infect fresh C3A2 cells. Infected C3A2 cells were pooled, and viruses were harvested from these cells to infect D17 cells to determine virus titers. Viral RNAs and cellular RNAs were also isolated and subjected to hybridization analysis. Infection data indicated that JS12 and JS14 could be propagated efficiently in C3A2 cells (Table 1). Both of these vectors achieved titers similar to the titers generated by typical SNV-based vectors in C3A2 cells. In all experiments, JS12 consistently produced a 3- to 10-fold-lower titer than JS14. Examples of cellular RNA and viral RNA analysis are shown in Fig. 5. Cellular RNA analysis revealed that JS12 is expressed at a fivefold-lower efficiency than JS14 (Fig. 5A), most probably because these two vectors were expressed from different promoters. The same fivefold difference is reflected in the packaged viral RNA (Fig. 5B). Thus, JS12 RNA is packaged just as efficiently as the JS14 RNA in C3A2 cells. Taken together, these data indicate that JS12 can be packaged and propagated efficiently in C3A2 cells. Thus, the lack of JS12 RNA in PG13-produced virions is due to the failure of MLV Gag proteins to recognize SNV E.

In summary, the MLV helper cell line does not support the propagation of SNV vectors, although MLV vectors can be propagated by SNV-based or REV-A-based helper cell lines very efficiently. In parallel experiments, the MLV vector was propagated at 10^4 to 10^5 CFU/ml; however, no SNV vector titer was detected. Considering that SNV vector RNA was expressed at a 5-fold-lower level, the decrease in titer is approximately 2,000- to 20,000-fold. RNA analysis revealed

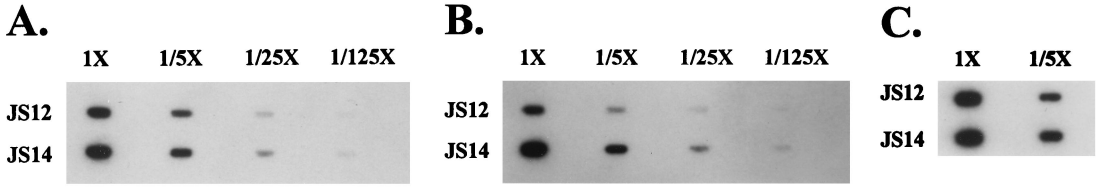

FIG. 5. RNA hybridization studies with cellular and viral RNA from infected C3A2 cells. (A) RNA analysis of C3A2 cellular RNA hybridized with the β-*gal* probe. The dilutions are shown above the blot. A 7-μg portion of total cellular RNA was used in the 1× dilution. (B) RNA analysis of viral RNA with the β-*gal* probe. (C) RNA analysis of spiked wild-type SNV viral RNA with the REV-A *env* probe.

that SNV E-containing RNA is packaged at least 25-fold less efficiently than is MLV RNA. The lack of recognition between MLV Gag polyproteins and SNV E-containing RNA plays a major role in the inability of the MLV helper cell line to propagate SNV vectors that can be expressed in murine cells.

DISCUSSION

The results of this study demonstrate striking differences between the MLV and SNV packaging specificities. SNV proteins can recognize both SNV E and MLV Ψ, but MLV proteins can recognize only MLV Ψ. This demonstrates a difference in packaging specificity between the two viruses; MLV proteins are more selective in RNA packaging, and SNV proteins are more relaxed. Why should viruses have different packaging specificities? One possibility is that the selection pressures placed on these two viruses are different. Analyses of various murine cells indicate that numerous MLV-like endogenous elements are present in the murine genome (6, 11). For example, VL-30 RNA can be packaged by MLV proteins (25, 39, 45, 54). Thus, MLV may have evolved a higher specificity to suppress the packaging of endogenous MLV-like elements present in the murine genome. In contrast, REV and SNV are known only as exogenous viruses. The avian genome may not contain endogenous SNV-like elements to force the selection pressure on a stringent RNA packaging selection. Therefore, the difference between these two viruses may reflect the genetic environment where the viruses propagate.

Alternatively, SNV proteins may not have a general property of relaxed packaging but may specifically recognize the MLV Ψ. It is thought that REV, the group of avian viruses that includes SNV, is derived from mammalian oncoviruses (32, 34). The packaging signal and the coding region may have evolved at different rates. The *gag* gene codes for the polyprotein that contains the signal to go to the cell membrane, select the packaged RNA, and interact with Env protein (63). Any changes that drastically decrease any of the above-described functions will confer a selective disadvantage. Thus, changes that result in the termination of the open reading frame, alteration of the polyprotein structure, or protein folding will be negatively selected. On the other hand, the functional unit of a packaging signal is RNA. Changes on most bases may be tolerated as long as the overall structures are maintained. Thus, it is possible that the SNV packaging signal diverged at a higher speed than *gag-pol*. If so, Gag-Pol of SNV would be expected to maintain its recognition of the MLV Ψ, but the SNV E could have diverged to the point that it can no longer be recognized by MLV Gag-Pol.

The difference in the packaging specificities of MLV and SNV provides a unique system to dissect the recognition between Gag polyprotein and RNA. It is thought that the nucleocapsid (NC) portion of the *gag* gene products plays an important role in packaging specificity (1, 15, 18, 25, 26, 38, 39, 47). In several viral systems, chimeric Gag proteins had been constructed to contain NC derived from a different virus (5, 18, 51, 69). The packaging specificity of these chimeric Gag proteins was altered. We are currently examining the factors that are responsible for the differences in packaging specificity between these two viruses.

The difference in the virus titers between JS12 and GA1 is 2,000- to 20,000-fold. The detected RNA packaging difference is 25-fold. It is possible that the difference in RNA packaging is greater; however, other factors may contribute to the difference as well. For example, the SNV genome may not be a suitable substrate for MLV reverse transcriptase or integrase. The lack of RNA packaging makes it difficult to further dissect the efficiency of these processes. These two viruses contain the same primer binding sites and use the same tRNA primer; thus, the initiation of reverse transcription is unlikely to be affected.

The efficient propagation of MLV vectors in SNV-based helper cell lines clearly indicated that MLV attachment sites (*att*) can be effectively used by SNV integrase. However, it is not clear whether SNV *att* sites are suitable substrates for MLV integrase. The *att* sites of the two viruses only contain the same four terminal bases that are known to be critical for most retroviral integrases, including those of MLV and SNV (13). It is known that MLV integrase can tolerate multiple mutations in the *att* sites (42); DNA containing SNV 3′ *att* and MLV 5′ *att* can be used as a substrate by MLV integrase (67), although with unknown efficiency. As a result, the role of integration efficiency in the decrease of viral titer is unclear.

Altered U3 vectors were used in this study. It has been previously demonstrated that by altering the U3 enhancers, the expression of the viral vector can be manipulated (23, 58). In the JS12 vector described in this report, the entire U3 was altered. Similar strategies can be used to generate retroviral vectors with different regulation of expression for gene therapy. While this work was in progress, it was demonstrated that a similar strategy could be used to generate Tat-inducible MLV vectors (8). Therefore, this strategy is likely to be useful for the generation of cell-type-specific vectors.

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