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The Nucleocapsid Domain Is Responsible for the Ability of Spleen Necrosis Virus (SNV) Gag Polyprotein To Package both SNV and Murine Leukemia Virus RNA

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Murine leukemia virus (MLV)-based vector RNA can be packaged and propagated by the proteins of spleen necrosis virus (SNV). We recently demonstrated that MLV proteins cannot support the replication of an SNV-based vector; RNA analysis revealed that MLV proteins cannot efficiently package SNV-based vector RNA. The domain in Gag responsible for the specificity of RNA packaging was identified using chimeric *gag-pol* expression constructs. A competitive packaging system was established by generating a cell line that expresses one viral vector RNA containing the MLV packaging signal (Ψ) and another viral vector RNA containing the SNV packaging signal (E). The chimeric *gag-pol* expression constructs were introduced into the cells, and vector titers as well as the efficiency of RNA packaging were examined. Our data confirm that Gag is solely responsible for the selection of viral RNAs. Furthermore, the nucleocapsid (NC) domain in the SNV Gag is responsible for its ability to interact with both SNV E and MLV Ψ. Replacement of the SNV NC with the MLV NC generated a chimeric Gag that could not package SNV RNA but retained its ability to package MLV RNA. A construct expressing SNV *gag*-MLV *pol* supported the replication of both MLV and SNV vectors, indicating that the *gag* and *pol* gene products from two different viruses can functionally cooperate to perform one cycle of retroviral replication. Viral titer data indicated that SNV *cis*-acting elements are not ideal substrates for MLV *pol* gene products since infectious viruses were generated at a lower efficiency. These results indicate that the nonreciprocal recognition between SNV and MLV extends beyond the Gag-RNA interaction and also includes interactions between Pol and other *cis*-acting elements.

Retroviral RNA encapsidation is achieved by interactions between Gag polyproteins and a packaging signal in the viral RNA. In the absence of *pol* and *env* gene products, Gag polyproteins select the viral RNA and form virus-like particles, indicating that Gag is the only polyprotein required for specific RNA packaging (1, 38, 44, 50, 55). After virus assembly and budding, Gag is processed into matrix (MA), capsid (CA), nucleocapsid (NC), and one or more other domains that vary among different viruses (7, 53). Experimental evidence indicates that NC plays a critical role in the RNA selection (11, 18–22, 24, 40, 41). With the exception of spumaviruses, all retroviruses encode an NC that contains one or two Cys-His boxes flanked by basic residues (7, 53). Mutations that alter the Cys-His box or basic residues result in a drastic reduction of RNA packaging (11, 18–22, 24, 40, 41). Although it is known that NC plays an important role in RNA packaging, it is unclear whether other domains in the Gag polyprotein such as MA and CA are also directly involved in RNA packaging. Although MA has a weaker affinity to RNA than NC (34, 35, 52), it was demonstrated that bovine leukemia virus MA binds specifically to the packaging signal and can enhance bovine leukemia virus RNA dimerization (26). This observation suggests that MA may cooperate with NC to achieve selective packaging of viral RNA (26). Additionally, CA may also play a role in RNA packaging, since deletion of a portion of CA caused a four-fold decrease in RNA packaging specificity of Rous sarcoma virus (RSV) (50).

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To determine whether replacement of NC with the NC derived from another virus is sufficient to alter the specificity of RNA packaging, various chimeric Gags were previously constructed and characterized. In the chimeras of RSV Gag containing murine leukemia virus (MLV) NC (14) and human immunodeficiency virus type 1 (HIV-1) Gag with MLV NC (2, 58), RNA analysis indicated that substituting the NC domain altered the specificity of RNA packaging. The RSV Gag with MLV NC chimeric polyprotein preferentially packaged MLV RNA, and the HIV-1 Gag with MLV NC chimeric polyprotein preferentially packaged MLV RNA. However, the packaging efficiencies were low, and no infectious virus was produced. Similarly, replacement of the HIV-2 NC with HIV-1 NC allowed the chimeric HIV-2 Gag polyprotein to package HIV-1 RNA, even though wild-type HIV-2 Gag cannot package HIV-1 RNA (28). Although the chimeric HIV-2 Gag with HIV-1 NC could package HIV-1 RNA, the packaging was enhanced when the HIV-1 p2 domain was also included, indicating another Gag domain(s) in addition to NC is also involved (28). These studies indicated that NC is, at least in part, responsible for RNA packaging specificity. In contrast, the chimeric HIV-1 Gag containing NC derived from mouse mammary tumor virus (MMTV) still preferentially packaged HIV-1 RNA (45). This observation indicated that replacement of the NC was not sufficient to alter the packaging specificity and that other Gag domains were involved.

The Gag polyproteins generally can package the RNA from the same or related viruses but cannot package the RNA of distantly related viruses. One of the exceptions is spleen necrosis virus (SNV), an avian virus that can efficiently package RNA from distantly related MLV (15). However, this recog-

TABLE 1. Primers used for construction of chimeric *gag-pol* expression plasmids

Primer	Sequence	Source(s) (nucleotide no.[a])
p1MCASNC	5′ GAGAGATGAGCAAGCTATTGCTCGCCCAGGAGAGTAGAGC 3′	pLGPS (2422) and pRD136 (2294)
2pSNCMPRO	5′ CCTGACCCTGACCTCCCTATTGTAATTCCTCTACCA 3′	pLGPS (2640) and pRD136 (2466)
p3MCA	5′ GACTGTCAGCAGCTGTTGGGGACTCTGCTG 3′	pLGPS (1812)
4pMCASNC	5′ GCTCTACTCTCCTGGGCGAGCAATAGCTTGCTCATCTCTC 3′	pLGPS (2441) and pRD136 (2302)
p5SNCMPRO	5′ TGGTAGAGGAATTACAATAGGGAGGTCAGGGTCAGG 3′	pLGPS (2624) and pRD136 (2447)
6pMRT	5′ GGGGGACTGGCAGGGTACCAGTATTCCCTG 3′	pLGPS (3263)
p7MCA	5′ CGGTGCGGGGCGATGATGGGC 3′	pLGPS (1885)
8pMRT	5′ GGTCCAACAGTCTCTGTATGTGGGG 3′	pLGPS (3234)
p1MLVU3	5′ CCGCCGCCCAGTCCTGCTCGC 3′	pLGPS (303)
2pMLSMA	5′ TTTCGATCCTGCCTGTCCCATATTTTCAGACAAATAC 3′	pLGPS (1010) and pRD136 (986)
p3MLSMA	5′ GTATTTGTCTGAAAATATGGGACAGGCAGGATCGAAGGGGC 3′	pLGPS (992) and pRD136 (967)
4pSCA	5′ CAGGTCCGATAAGCCTGATAG 3′	pRD136 (2072)
p3Sp18	5′ CCAGACTCTACTGTAATGACG 3′	pRD136 (1483)
p1SCAMNC	5′ AGAAGATGGCCAAAGTACTAGCCACTGTCGTTAGTGGACA 3′	pLGPS (2442) and pRD136 (2273)
2pMNCSPR	5′ GCGGGAGAACCCTGACGGCCCTAGTCATCTAGGGTCAGG 3′	pLGPS (2624) and pRD136 (2486)
4PSCAMNC	5′ TGTCCACTAACGACAGTGGCTAGTACTTTGGCCATCTTCT 3′	pLGPS (2461) and pRD136 (2293)
p5SNCMPR	5′ CCTGACCCTAGATGACTAGGGCCGTCAGGGTTCTCCCGC 3′	pLGPS (2606) and pRD136 (2466)
6pSRT	5′ TGTCCACTCGAATGCGAAGATC 3′	pRD136 (3345)
p7SCA	5′ ATGTTCCATTCACCACCTCGG 3′	pRD136 (1616)
8pSRT	5′ TATCTCGGCCCAGACTTTCGG 3′	pRD136 (2932)
MLVMA	5′ GTGGCGGCCGGGATGGGCCAGACTGTTACCAC 3′	pLGPS (1008)

[a] Nucleotide number of the first base (the most 5′ base) is shown. When a primer contains sequences from two plasmids, the nucleotide number of the first base derived from each of the plasmids is shown.

nition is nonreciprocal, and MLV proteins cannot package SNV vector RNA efficiently (5). We sought to utilize this system to explore which protein domain(s) in Gag is responsible for this nonreciprocal interaction. Chimeric MLV and SNV Gag or Gag-Pol polyproteins were generated by replacing either the NC domain or the entire Gag, and the packaging specificities of the chimeras were determined. We found that replacing the entire Gag altered the RNA packaging specificity, confirming previous observations that Gag is the only polyprotein involved in RNA selection (1, 38, 44, 50, 55). Furthermore, we found that replacement of the NC domain is sufficient to alter the RNA packaging specificity.

MATERIALS AND METHODS

Plasmid construction. Four chimeric *gag-pol* expression vectors were constructed by PCR and standard cloning techniques (51). MLV sequences were derived from plasmid pLGPS (43). SNV sequences were derived from plasmid pRD136 except that the SNV sequences in pSNVNC were derived from pVP1 (12, 39); the viral coding regions of pRD136 and pVP1 were derived from the same plasmid (pBR1) (12) and should contain identical sequences. Sequences of all the primers used for construction of the plasmids are listed in Table 1.

Plasmid pSNVNC was constructed as follows. SNV NC was amplified by PCR with primers p1MCASNC and 2pSNCMPRO (fragment 1); MLV CA was amplified by PCR with primers p3MCA and 4pMCASNC (fragment 2). DNA fragments 1 and 2 were joined together by PCR using overlap extension with primers 2pSNCMPRO and p3MCA (fragment 3). MLV *pol* was amplified with primers p5SNCMPRO and 6pMRT (fragment 4); fragments 3 and 4 were joined by PCR using primers p7MCA and 8pMRT. The final PCR product was digested with restriction enzymes *Xho*I and *Bcl*I; the resulting 1.3-kb DNA fragment was cloned into pLGPS to generate pSNVNC(old). A second plasmid, pSNVNC(new), was also constructed to verify results. Restriction enzyme mapping was performed to confirm the structures of the plasmids; the regions between *Xho*I and *Bcl*I sites were characterized by DNA sequencing. Sequencing analysis revealed the presence of a silent mutation in both plasmids (G to A, base 2207), which was not expected to affect these studies.

The strategy to construct pSNVgag is described below. A portion of the untranslated region of MLV was amplified by PCR with primers p1MLVU3 and 2pMLSMA (fragment 1); SNV MA and p18 of SNV were amplified by PCR with primers p3MLSMA and 4pSCA (fragment 2). DNA fragments 1 and 2 were joined together by PCR with primers p1MLVU3 and 4pSCA (fragment 3). DNA fragment 3 was digested with the restriction enzymes *Sac*I and *Bam*HI and cloned into pUC19 to generate pMP3. SNV p18, CA, and NC were amplified by PCR with primers p3Sp18 and 2pSNCMPRO (fragment 4); MLV *pol* was amplified by PCR with primers p5SNCMPRO and 8pMRT (fragment 5). Fragments 4 and 5 were joined together by PCR using primers p3Sp18 and 8pMRT (fragment 6). DNA fragment 6 was digested with restriction enzymes *Bam*HI and *Bcl*I and cloned into the *Bam*HI site of pMP3 to generate pMP4. A 2-kb *Sac*I fragment from pMP4 containing SNV *gag* flanked by the MLV 5′ untranslated region and part of the MLV *pol* was cloned into pLGPS to generate pSNVgag. pSNVgag was analyzed by restriction enzyme digestions, and the PCR-amplified regions of the chimera were characterized by DNA sequencing. Silent mutations were found at these points in the plasmid: 932 (A to G), 1370 (T to C), 1640 (C to T), and 1942 (T to C). These mutations are not expected to affect these studies.

The strategy to construct pMLVNC is described below. MLV NC was amplified by PCR with primers p1SCAMNC and 2pMNCSPR (fragment 1); SNV CA was amplified by PCR with primers p3SP18 and 4pSCAMNC (fragment 2). DNA fragments 1 and 2 were joined together by PCR using primers 2pMNCSPR and p3SP18 (fragment 3). SNV *pol* was amplified with primers p5SNCMPR and 6pSRT (fragment 4). Fragments 3 and 4 were joined together by PCR using primers p7SCA and 8pSRT. This DNA was digested with the restriction enzymes *Bcl*I and *Apa*I, and the resulting 970-bp DNA fragment was subcloned into pRD136Apa$^-$ to generate pMLVNC. The construct pRD136Apa$^-$ is identical to pRD136 except that an *Apa*I site in the polylinker was destroyed. This plasmid was analyzed by restriction enzyme mapping and DNA sequencing of the region between *Bcl*I and *Apa*I sites; all of the sequences were as expected.

The strategy to construct pMLVgag is outlined below. The entire MLV *gag* was amplified by PCR with primers MLVMA and 2pMNCSPR. Primer MLVMA contains an *Eag*I site for cloning; the PCR product was digested with *Eag*I and *Pvu*I and cloned into pMLVNC that was digested with *Eag*I and partially digested with *Pvu*I to generate pMLVgag. The gross structure of pMLVgag was confirmed by restriction enzyme mapping; the 1.5-kb MLV *gag* amplified by PCR was further characterized by DNA sequencing. One silent mutation (C to T) was found at base 1040 in the plasmid, which is not expected to affect these studies.

DNA sequencing of plasmids. Chimeric constructs were sequenced by the method of Sanger et al. (51a). Double-stranded DNA was sequenced using the AutoRead kit (Pharmacia), using standard or quick-annealing methods as suggested by the manufacturer.

Cells, transfection, and infection. Cells were maintained in Dulbecco's modified Eagle's medium with 6% calf serum. DNA transfection was performed using the dimethyl sulfoxide-Polybrene method (27). Various *gag-pol* expression plasmids were introduced into E1 cells by cotransfection with pBSpac at a 10:1 ratio. Plasmid pBSpac conferred resistance to puromycin (9). For viral titer studies, transfected cells from each experimental group were plated at a density of 10^6 cells per 60-mm-diameter dish. Supernatant was harvested after 2 days, and cellular debris was removed by centrifugation. Viral infections were performed in the presence of Polybrene at 50 μg/ml (final concentration). The numbers of the drug-resistant cell colonies obtained were used to determine virus titers. Puromycin, hygromycin, and G418 selections were performed at a concentration of 175, 240, and 400 μg/ml, respectively.

E1 cell was derived from D17 cells, a dog osteosarcoma cell line that is permissive to both MLV and SNV infection (48). E1 cell line was constructed by first transfecting D17 cells with SV-A-MLV-env (31) plus pSVα3.6 (29) at a 10:1 ratio. Plasmid SV-A-MLV-env expressed the MLV *env* and plasmid pSVα3.6

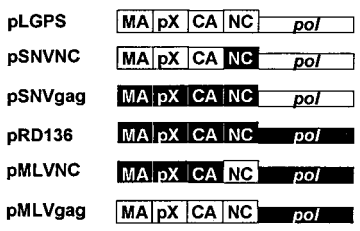

FIG. 1. Chimeric *gag-pol* expression constructs used to dissect the Gag determinants important in packaging specificity. MLV sequences are shown in white; SNV sequences are shown in black.

conferred resistance to ouabain. The resulting ouabain-resistant colonies were pooled (>900 colonies) and designated D17-Menv cells. The SNV-based retroviral vector JD215 (15) was transfected into C3A2 cells (54), and virus harvested was used to infect the D17-Menv cells. Clone D2 was selected from 10 G418-resistant (G418^r) cell clones analyzed. Vector JD215 contains a neomycin phosphotransferase gene (*neo*), which confers resistance to G418, a neomycin analog. MLV vector AR2 (57) was transfected into PA317 cells (42), and virus harvested was used to infect D2 cells; clone E1 was selected from 30 hygromycin-resistant (Hygro^r) cell clones analyzed. Vector AR2 contains the hygromycin phosphotransferase B gene (*hygro*), which confers resistance to hygromycin.

RNA isolation and analysis. Cellular RNA was isolated using Trizol reagent (Gibco/BRL) according to the manufacturer's instructions. The integrity of the cellular RNA was verified by gel electrophoresis and inspection of the ribosomal bands. Cell-free virion RNA was also isolated. E1 cells transfected with different plasmids were plated at equal densities (5×10^6 cells per 100-mm-diameter dish), and virus supernatants were harvested 2 days later. Cellular debris was cleared by low-speed centrifugation, and the viral supernatants were centrifuged at 25,000 4 rpm for 90 min in a Sorvall SW41 rotor. Viral pellets were resuspended in 50 mM Tris–1 mM EDTA (pH 7.5 to 8) and lysed with 0.1% sodium dodecyl sulfate (SDS) in the presence of 200 μg of tRNA per ml. The mixtures were extracted with phenol and chloroform and precipitated by ethanol, and the RNA pellets were resuspended in 100 μl of diethyl pyrocarbonate-treated water.

Fivefold serial dilutions were prepared from the RNA samples, and slot blots were generated using the convertible filtration manifold system (Gibco/BRL) according to the conditions recommended by the manufacturer. A 1.3-kb DNA fragment containing *neo* and a 0.8-kb DNA fragment containing *hygro* were used for random priming reactions (16) to generate probes that specifically hybridized to JD215 and AR2 vector RNA, respectively (specific activity, $>10^9$ cpm/μg of DNA). A plasmid containing both *hygro* and *neo*, pWH11 (25), was used as a standard on the blots probed with *hygro* and *neo* to normalize the two probes. Slot blots were quantified using a PhosphorImager and the ImageQuant program (Molecular Dynamics).

Western analysis. Western blotting analyses of the cell-free viruses were performed using standard procedures (51) and ECL (enhanced chemiluminescence) Western blotting detection reagents (Amersham Life Science). Protein samples were separated on SDS-polyacrylamide (10 or 16%) gels for the analysis of MLV CA or MLV NC, respectively, and transferred to Biotrace polyvinylidene fluoride 0.45-μm-pore-size membranes (Gelman Sciences). A rat monoclonal antibody against MLV CA was prepared from R187 cells obtained from American Type Culture Collection (6), and goat anti-rat immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (Southern Biotechnology Associates Inc.) was used as a secondary antibody. A rabbit antibody against MLV NC was a generous gift from Alan Rein. A sheep anti-rabbit IgG antibody conjugated with horseradish peroxidase (Boehringer Mannheim) was used as a secondary antibody.

RESULTS

Chimermic *gag-pol* expression constructs used to examine the role of the NC domain in RNA packaging. After virus assembly and budding, Gag polyproteins from MLV and SNV are processed into MA, a protein of unknown function (pX), CA, and NC (Fig. 1). Four chimeric *gag-pol* expression constructs were generated to determine whether the NC domains of Gag are important to the nonreciprocal packaging of MLV and SNV. The structures of these constructs as well as the parental plasmids are shown in Fig. 1. The chimeric constructs were derived from the MLV *gag-pol* expression construct pLGPS (43) and the SNV *gag-pol* expression construct pRD136 (39). The pSNVNC chimera contains MLV *gag-pol* with SNV NC replacing MLV NC. The pSNVgag chimera contains SNV *gag* and MLV *pol*. The pMLVNC chimera contains SNV *gag-pol* with MLV NC, and the pMLVgag chimera contains MLV *gag* and SNV *pol*. PCR and standard cloning techniques were used for the generation of these expression constructs, which created precise junctions between domains and maintained all of the reading frames. All of the regions that were subjected to PCR amplification were characterized by DNA sequencing to ensure that none of the chimeric constructs contained any inadvertent mutations that changed the encoded amino acids.

Cell culture system used to determine efficiency of viral RNA packaging by chimeric *gag-pol* vectors. A cell line, E1, was generated to analyze packaging specificities of the chimeric *gag-pol* expression constructs. To construct the E1 cell line, D17 cells were transfected with a plasmid, SV-A-MLV-env, that expresses amphotropic MLV *env* (31); then the SNV-based vector JD215 was introduced by infection, and finally the MLV-based vector AR2 was introduced by infection. MLV Env can interact with both MLV Gag/Gag-Pol and SNV Gag/Gag-Pol to form infectious viruses (12). The vector JD215 contains *neo* and all of the *cis*-acting elements from SNV, which include the packaging signal (E) (15). The vector AR2 contains *hygro* and all of the *cis*-acting elements from MLV, which include the packaging signal (Ψ) (57). The presence of both SNV vector RNA and MLV vector RNA in E1 cells allows this system to measure the packaging specificities of the Gag/Gag-Pol polyproteins in a competitive manner.

Viral titers generated by the *gag-pol* expression constructs in E1 cells. The *gag-pol* expression constructs pLGPS, pSNVNC (old), pSNVNC(new), pSNVgag, pRD136, pMLVNC, and pMLVgag were separately introduced into E1 cells by transfection. Because the *gag-pol* expression constructs lack selectable markers, they were cotransfected with plasmid pBSpac at a 10:1 ratio. Plasmid pBSpac encodes the puromycin acetyltransferase gene and confers resistance to puromycin (9). Puromycin-resistant colonies obtained after drug selection were pooled from plates transfected with each *gag-pol* expression construct or with only pBSpac, each pool containing more than 500 colonies. Cells containing the different constructs were plated at the same density, and viruses were harvested and used to infect D17 target cells. The infected D17 cells were placed on either hygromycin or G418 selection. Cells infected with the MLV vector AR2 were Hygro^r, whereas cells infected with the SNV vector JD215 were G418^r. The numbers of Hygro^r or G418^r colonies obtained were used to determine the titers of AR2 or JD215, respectively.

Viral titers generated from five independent sets of transfections and infections are shown in Table 2. As expected in all five sets of experiments, the SNV *gag-pol* expression construct pRD136 supported efficient replication of both SNV vector JD215 and MLV vector AR2. The SNV vector titers (G418^r) varied from 4.7×10^2 to 8×10^3 CFU/ml, with a mean of 2.6×10^3 CFU/ml. Similarly, the MLV vector (Hygro^r) titers varied from 7.7×10^2 to 6.5×10^3 CFU/ml, with a mean of 2.6×10^3 CFU/ml. In all five sets of experiments, the MLV *gag-pol* expression construct pLGPS supported efficient replication of MLV vector AR2. The MLV vector titers ranged from 3.9×10^4 to 1.6×10^5 CFU/ml, with a mean of 10^5 CFU/ml. In contrast, pLGPS did not support efficient replication of SNV vector JD215, with virus titers that ranged from <1 to 20 CFU/ml, with a mean of 6.6 CFU/ml. This result was in agreement with our previous observation that the SNV proteins can support both SNV and MLV vector replication whereas the MLV proteins can support only MLV vector replication (5).

The chimeric construct pSNVgag that expressed SNV *gag* and MLV *pol* supported the replication of both the MLV and

TABLE 2. MLV (Hygro^r) and SNV (G418^r) vector titers generated by E1 cells transfected with *gag-pol* expression constructs

Expt	Titer[a] (CFU/ml)					
	pRDI36 (SNV)		pLGPS (MLV)		pSNVgag	
	MLV vector (10^3)	SNV vector (10^3)	MLV vector (10^5)	SNV vector (10^0)	MLV vector (10^3)	SNV vector (10^1)
1	6.5	8	1.6	20	10	8.3
2	2.6	1	1.2	8	6.5	1
3	1.3	3	1.2	3	2.9	2.1
4	1.9	0.7	0.85	<1	8.8	1.8
5	0.77	0.47	0.39	2	2.8	1.2
Mean	2.6	2.6	1	6.6	6.2	2.9

[a] pMLVNC, pMLVgag, pSNVNC(old), pSNVNC(new), and pBSpac, MLV and SNV vector titers were <1 CFU/ml in all experiments.

the SNV vector (Table 2). The MLV vector titers (Hygro^r) ranged from 2.8×10^3 to 1.0×10^4 CFU/ml, with a mean of 6.2×10^3 CFU/ml. The SNV vector (G418^r) titers ranged from 1.0×10^1 to 8.3×10^1 CFU/ml, with a mean of 2.9×10^1 CFU/ml. These results demonstrated that a chimeric *gag-pol* expression construct derived from distinct viruses could support viral replication. In contrast to pSNVgag, however, none of the other chimeric *gag-pol* expression constructs, specifically pSNVNC(old), pSNVNC(new), pMLVgag, and pMLVNC, supported the replication of MLV or SNV vectors. Both the MLV and the SNV vector titers generated from all these chimeras were less than 1 CFU/ml in all experiments (Table 2). As expected, cells transfected with only pBSpac did not express *gag-pol* and therefore did not generate infectious viral particles.

Direct determination of packaging specificities of chimeric polyproteins by analysis of cellular and viral RNA. To directly examine the specificities of RNA packaging by these *gag-pol* expression constructs, cellular and cell-free viral RNA analyses were performed. Total cellular RNAs were isolated from cells transfected with pBSpac plus various *gag-pol* expression constructs or with pBSpac alone. Viruses were harvested from these transfected cells and cell-free viral RNAs were isolated. Fivefold serial dilutions were performed with both cellular and viral RNAs, and the samples were applied to duplicate slot blots, which were hybridized to probes generated from either a 0.8-kb DNA fragment containing *hygro* or a 1.3-kb DNA fragment containing *neo*. A set of representative slot blots is shown in Fig. 2. The probe generated from *hygro* DNA fragment hybridized to the RNA of MLV vector AR2, whereas the probe generated from *neo* DNA fragment hybridized to the RNA of SNV vector JD215. Plasmid pWH11, which contains a copy of *hygro* and a copy of *neo*, was also applied to both blots as a control to standardize the signals obtained from the two probes. The RNA packaging specificities of different *gag-pol* expression constructs were determined by comparison of blots hybridized with *hygro* and *neo* probes.

Analysis of cellular RNAs isolated from cells transfected with various *gag-pol* expression constructs indicated that AR2 was expressed at a two- to fivefold-higher level than JD215 in all experiments (Fig. 2). As expected, there was little variation

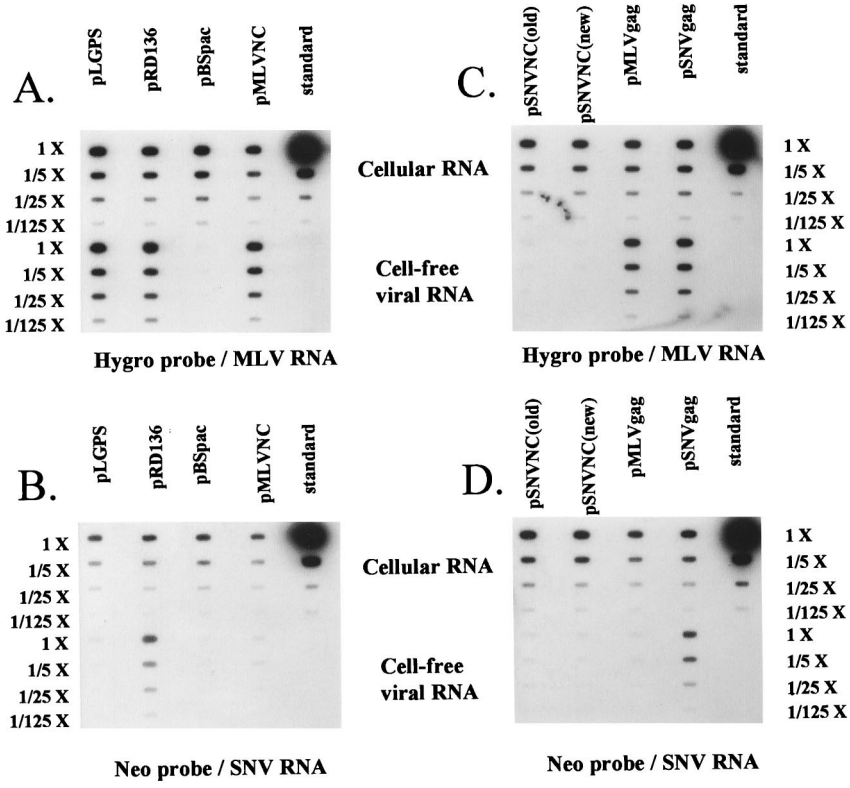

FIG. 2. Slot blot hybridization analyses of cellular RNA and cell-free viral RNA generated from transfected E1 cells. In each blot, cellular RNAs are on the top half and the cell-free viral RNAs are on the bottom half. Fivefold serial dilutions of each RNA sample were applied to the slot blot, and the dilutions are indicated at the sides. (A and C) Blots hybridized with the *hygro* probe, which detected MLV vector AR2 RNA; (B and D) blots hybridized with the *neo* probe, which detected SNV vector JD215 RNA. Standard, plasmid pWH11 DNA containing a copy of *neo* and a copy of *hygro*.

in the level of expression of AR2 and JD215 RNAs between E1 cells transfected with various *gag-pol* expression constructs (Fig. 2). The uniform levels of expression of the AR2 and JD215 RNAs in cells transfected with various *gag-pol* expression constructs ensured that any differences observed in the analysis of cell-free viral RNAs was due to the packaging specificities of the viral proteins. Furthermore, analyses of the cell-free supernatant from cells transfected with only pBSpac revealed that in the absence of *gag-pol* expression, no significant cell-free viral RNAs were produced (Fig. 2A and B). This result indicated that any cell-free viral RNA detected in cells transfected with *gag-pol* expression constructs most likely represented RNA packaging by the polyproteins generated by the transfected constructs.

Cell-free viral RNA analyses indicated that in cells transfected with pLGPS, MLV vector AR2 RNA was efficiently packaged but the SNV vector JD215 RNA was not packaged at a significant level (Fig. 2A and B). RNA of MLV vector AR2 was easily detected after a 125-fold dilution. In contrast, the signal from undiluted JD215 RNA was not significantly higher than the background (Fig. 2A and B). Considering the difference in AR2 and JD215 expression observed in analysis of the cellular RNA, MLV Gag/Gag-Pol packaged AR2 RNA at least 25-fold more efficiently than the JD215 RNA. This result was consistent with our previous observation that MLV polyproteins cannot package SNV RNA efficiently (5).

Analysis of cell-free viral RNAs derived from cells transfected with pRD136, which expressed wild-type SNV *gag-pol*, indicated that both AR2 and JD215 RNAs were efficiently packaged (Fig. 2A and B). After adjusting the results for the levels of expression of the two vectors as determined from analysis of cellular RNAs, we found that SNV Gag/Gag-Pol packaged AR2 RNA and JD215 RNA at equal efficiencies. This result was consistent with previous observations that SNV proteins can efficiently package both SNV and MLV vector RNA (15).

Cell-free viral RNAs derived from cells transfected with the pMLVNC chimera indicated that the chimeric Gag/Gag-Pol polyproteins containing the MLV NC domain could package MLV vector AR2 RNA at levels comparable to those for cells transfected with pRD136 (Fig. 2A). However, the same Gag/Gag-Pol polyproteins could not efficiently package SNV vector JD215 RNA and exhibited at least a 25-fold decrease in comparison to cells transfected with pRD136 (Fig. 2B). Since the only difference between the Gag/Gag-Pol polyproteins produced by pRD136 and pMLVNC was the NC domain, it was concluded that changing the NC domain of SNV to the MLV NC domain led to a minimum 25-fold decrease in the ability of the chimeric Gag/Gag-Pol to interact with JD215 RNA. In contrast, the chimeric pMLVNC construct packaged AR2 RNA as efficiently as pRD136, indicating that its ability to interact with AR2 RNA was not affected. Therefore, replacing the NC domain altered the packaging specificity of the Gag/Gag-Pol polyprotein.

Analysis of cell-free viral RNA derived from cells transfected with pSNVNC(old) and pSNVNC(new) indicated that neither AR2 nor JD215 RNA was packaged even though both RNAs were expressed in the cells (Fig. 2C and D). This observation suggested that these chimeric Gag/Gag-Pol polyproteins were not functional and could not interact with either MLV or SNV vector RNA. The chimeric pSNVNC(old) and pSNVNC(new) constructs were identical in sequence but were independently generated at different times to confirm reproducibility of the results. During the generation of the chimeric constructs, PCR amplified regions were characterized by DNA sequencing. It was possible that pSNVNC(old) contained a mutation located outside the sequenced region and caused the loss of function of the polyprotein. To rule out this possibility, pSNVNC(new) was generated to confirm data obtained by pSNVNC(old).

Analysis of cell-free viral RNA derived from cells transfected with pMLVgag indicated that MLV vector AR2 RNA was efficiently packaged but the SNV vector JD215 RNA was not efficiently packaged (Fig. 2C and D). In contrast, cells transfected with pSNVgag packaged AR2 RNA and JD215 RNA with similar efficiencies (Fig. 2C and D). The packaging specificities of the Gag/Gag-Pol polyproteins produced from pMLVgag (MLV *gag* and SNV *pol*) and pSNVgag (SNV *gag* and MLV *pol*) were similar to those of pLGPS (MLV *gag-pol*) and pRD136 (SNV *gag-pol*), respectively. These data indicated that replacement of the entire *gag* regions altered the specificity of RNA packaging and the identity of *pol* did not affect packaging specificity. This result was consistent with the previous observation that the Gag polyprotein alone determines the packaging specificity (1, 38, 44, 50, 55).

Characterization of viral proteins generated from the chimeric *gag-pol* expression constructs. Analysis of cell-free viral RNA derived from cells transfected with the chimeric construct pSNVgag indicated that viral RNA was efficiently packaged by these Gag/Gag-Pol polyproteins. In addition, the chimeric pSNVgag supported virus replication, indicating that the chimeric SNV Gag-MLV Pol polyproteins were incorporated into viral particles, were correctly processed, and performed all of the other functions needed for successful viral replication.

The other three chimeric expression vectors, pSNVNC, pMLVNC, and pMLVgag, were not able to support the replication of the viral vectors, indicating that at least one of the processes during viral replication was impaired. RNA analyses revealed that both pSNVNC constructs could not package vector RNAs, which explained, at least in part, why this chimeric construct failed to generate infectious viruses. RNA analyses also revealed that the polyproteins generated from pMLVNC and pMLVgag efficiently packaged viral RNA but failed to produce infectious viral particles. Therefore, defects in viral replication other than RNA packaging abolished the ability of these constructs to generate infectious viruses.

Western blotting analyses of cell-free viruses were performed to examine the processing of Gag polyproteins in various chimeric constructs. E1 cells transfected with different constructs were plated at the same density; viruses were harvested 3 days later, concentrated by ultracentrifugation, and used to characterize the viral proteins. A representative Western blot using antibody against MLV CA is shown in Fig. 3. The viral particles produced by cells transfected with pLGPS, pSNVNC(old), pSNVNC(new), or pMLVgag are expected to generate an MLV CA if the Gag polyprotein is efficiently processed. As expected, processed MLV CA was detected using a monoclonal antibody against MLV CA in viruses produced by cells transfected with pLGPS. However, little or no processed MLV CA was detected in samples generated from cells transfected with the chimeric construct pSNVNC(old), pSNVNC(new), or pMLVgag (Fig. 3).

Western blotting analyses using an antibody against MLV NC were also performed to examine viruses generated from cells transfected with pLGPS, pMLVgag, and pMLVNC, which all contained the MLV NC domain (Fig. 1). Processed MLV NC protein was not observed in samples generated from cells transfected with pMLVgag or pMLVNC but was observed in samples generated from pLGPS (data not shown). The lack of processed MLV NC in samples transfected with pMLVgag was consistent with the absence of processed MLV CA in the same samples. Together, these data indicated that the Gag

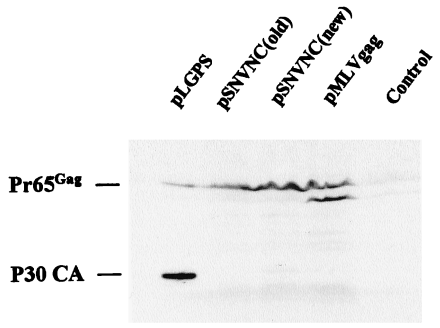

FIG. 3. Western analysis of the cell-free virion proteins, using antibody against the MLV CA. A rat monoclonal antibody against MLV CA generated from R187 cells (6) was used as a primary antibody, and a goat anti-rat IgG antibody conjugated with horseradish peroxidase was used as a secondary antibody. Medium with serum was loaded as a control. The unprocessed Gag ($Pr65^{Gag}$) and processed MLV CA (P30) are indicated.

polyproteins produced by pSNVNC(old), pSNVNC(new), pMLVgag, and pMLVNC were not processed at a detectable level. These data explain, at least in part, the inability of polyproteins generated by pMLVgag and pMLVNC to support viral replication despite the fact that they both packaged viral RNA efficiently. The processing of viral proteins derived from pSNVgag was not examined because antibodies against the SNV Gag domains were not available. However, it is expected that the polyproteins generated from pSNVgag were processed correctly because this construct supported the replication of viral vectors.

DISCUSSION

The NC domain is responsible for nonreciprocal RNA packaging of MLV and SNV. Several chimeras of Gag containing NC from different viruses have been previously tested, and mixed results were obtained (2, 14, 28, 45, 58). For example, chimeric HIV-1 Gag with MLV NC preferentially packaged MLV RNA (2, 58), but chimeric HIV-1 Gag with MMTV NC still preferentially packaged HIV-1 RNA (45). These differences were hypothesized to be caused by the number of Cys-His boxes in the NC domain (45). The NC domains of HIV-1 and MMTV both contain two Cys-His boxes, whereas MLV NC contains only one Cys-His box. It was postulated that because the NC of HIV-1 and MMTV contained the same number of Cys-His boxes, substitution of the NC did not alter the RNA packaging specificity (45). It was also hypothesized that NC domains that contain the same number of Cys-His boxes are likely to be effectively substituted for one another and that the determinant for RNA packaging specificity is located elsewhere in Gag (45).

The results of our studies provide insights into the mechanisms used by retroviruses to achieve RNA packaging specificity. The chimeric pMLVNC construct, which expressed SNV Gag containing MLV NC, could efficiently package MLV vector RNA but could not package SNV vector RNA. This result indicates that the NC domain of the Gag polyprotein confers the specificity of RNA packaging. This result also indicates that the SNV NC and MLV NC are not functionally interchangeable even though both NC domains contain one Cys-His box (Fig. 4).

HIV-1 and HIV-2 provide another example of nonreciprocal packaging that is similar to the SNV and MLV system used in these studies (28). HIV-1 can package both HIV-1 and HIV-2 RNAs, whereas HIV-2 can package only HIV-2 RNA. Substituting the NC domain also changed the packaging specificity even though both HIV-1 and HIV-2 NC domains contained two Cys-His boxes. The results of our studies are in agreement with these studies and indicate that the NC domains of both oncoviruses and lentiviruses are likely to be the determinants that confer RNA packaging specificity. NC domains containing the same number of Cys-His boxes cannot be effectively substituted for one another without changing the packaging specificity.

Interactions between processed Gag products, Pol products, and *cis*-acting elements. We observed that the chimeric pSNVgag expression construct supported the replication of both MLV and SNV vectors. This is the first *gag-pol* chimera that has been shown to support viral replication. Several studies have indicated that the NC protein plays an important role in both reverse transcription and integration during the viral life cycle (3, 4, 8, 10, 11, 13, 17, 21–23, 30, 32, 33, 36, 37, 46, 47, 49, 56). The fact that pSNVgag supports viral replication indicates for the first time that the SNV NC protein can functionally cooperate with MLV reverse transcriptase (RT) and MLV integrase (IN) in vivo to complete reverse transcription and integration. Furthermore, the MLV vector titers ($Hygro^r$) generated by pSNVgag are equivalent to the vector titers generated by pRD136 (Table 2), indicating that the functional cooperation between these heterologous viral proteins was efficient.

Polyproteins generated by pSNVgag packaged both SNV and MLV vector RNAs efficiently. Interestingly, the pSNVgag chimera supported the replication of both SNV and MLV, but the titer of the SNV vector was approximately 2 orders of magnitude lower than the titer of the MLV vector in all five sets of independent experiments (Table 2). The difference between SNV and MLV virus titers indicates that the MLV *pol* gene products use SNV *cis*-acting elements less efficiently than the MLV *cis*-acting elements. In contrast, SNV RT and IN can efficiently use MLV *cis*-acting elements, since the SNV *gag-pol* expression construct pRD136 can propagate both SNV and MLV vectors with equal efficiency. This indicates that the nonreciprocal recognition between SNV and MLV extend beyond the interaction between Gag polyproteins and packaging signals. The interactions between the *pol* gene products and the *cis*-acting elements of these two viruses are also nonreciprocal. It should be noted that SNV and MLV RTs use the same tRNA primer but different polypurine tracts and the SNV and MLV INs use different attachment sites. Therefore, the MLV RT and/or IN may use the SNV *cis*-acting sequences with reduced efficiencies.

```
MLV
ATVVSGQKQDR-----QGGERRRSQLDRDQCAYCKEKGHWAKDCPKKPRGPRGPRPQTSLLT-LDD
*   *  * **       * * *  * *  ******* *** * ***   *   *     *   *
*   *    *        *   *  *   ******* *** * ***   *   *     *   *
LA-QES--RAERGSKKTPPG-KGRPPLGKNQCAYCKEEGHWKKNCPKLVSGAA-PV----LVEELQ
SNV
```

FIG. 4. Alignment of MLV NC and SNV NC amino acid sequences. Amino acids constituting the Cys-His boxes are shown in bold.

In cells expressing pRD136, pSNVgag, and pLGPS, the SNV vector titers were approximately the same as, 2 orders of magnitude lower than, and 4 orders of magnitude lower than the MLV vector titers, respectively (Fig. 1 and Table 2). As discussed above, the efficiencies with which MLV and SNV RTs and INs use the SNV *cis*-acting elements most likely resulted in a reduced SNV titer with pSNVgag in comparison to pRD136. The SNV vector titer difference of 2 orders of magnitude between pSNVgag and pLGPS probably reflects the difference in the efficiency of SNV RNA packaging by the SNV and MLV Gag polyproteins. The observed reduction in the SNV titer is in agreement with the RNA analysis that SNV Gag packages SNV vector RNA at least 25-fold more efficiently than the MLV Gag.

In summary, a competitive RNA packaging system was established in this study to test the functionality and the packaging specificity of the chimeric Gag polyproteins. Using the nonreciprocal RNA packaging between MLV and SNV, it is possible to measure the gain and the loss of functions of these chimeric proteins. Further analysis of *cis*- and *trans*-acting elements important for nonreciprocal RNA packaging are under way.

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