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Determinants of Moloney Murine Leukemia Virus Gag-Pol and Genomic RNA Proportions

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ABSTRACT

The Moloney murine leukemia virus (MoMLV) ribonucleoprotein complex is composed of an approximately 20:1 mixture of Gag and Gag-Pol polyproteins plus a single genomic RNA (gRNA) dimer. The mechanisms that regulate these proportions are unknown. Here, we examined whether virion proportions of Gag, Gag-Pol, and gRNA were determined by sampling (that is, if they reflected expression ratios or intracellular concentrations) or more specific recruitment. To this end, MoMLV Gag, Gag-Pol, and gRNA were expressed separately or together in various ratios. Varying the expression ratios of Gag and Gag-Pol revealed that Gag-Pol incorporation was stochastic and that the conserved 20:1 Gag/Gag-Pol ratio coincided with maximal particle production. When skewed expression ratios resulted in excess Gag-Pol, the released virions maintained the intracellular Gag/Gag-Pol ratios and the infectivity per virion was largely maintained, but virion production decreased sharply with high levels of Gag-Pol. The determinants of gRNA proportions were addressed by manipulating the amounts and contexts of functional nucleocapsid (NC) and the ratios of Gag to gRNA. The results showed that the NC domain of either Gag or Gag-Pol could provide gRNA packaging functions equally well. Unlike Gag-Pol, gRNA incorporation was saturable. An upper limit of gRNA incorporation was observed, and particle production was not disrupted by excess gRNA expression. These results indicate that the determinants of Gag/Gag-Pol proportions differ from those for Gag/gRNA. On the basis of the assumption that MoMLV evolved to produce virion components in optimal proportions, these data provide a means of estimating the proportion of unspliced MoMLV RNA that serves as genomic RNA.

IMPORTANCE

Viruses assemble their progeny from within the cells that they parasitize, where they must sort through a rich milieu of host proteins and nucleic acids to gather together their own building blocks, which are also proteins and nucleic acids. The research described here addresses whether or not the proportions of viral proteins and nucleic acids that are brought together to form a retroviral particle are determined by random sampling from the cell—and thus dictated by the components' availabilities within the cell—or if the amounts of each molecule are specified by the virus replication process. The results indicated that protein components of the murine retrovirus studied here are recruited by chance but that a specific counting mechanism defines the amount of nucleic acid incorporated into each progeny virion.

The cores of retroviruses, such as Moloney murine leukemia virus (MoMLV), can be considered ribonucleoprotein (RNP) complexes comprised of proteins and RNA, which are surrounded by a lipid envelope. The Gag and Gag-Pol polyproteins are the major and minor protein components of the MoMLV RNP, respectively, whereas the viral genomic RNA (gRNA) is the major RNA component. A budding MoMLV particle contains a few thousand Gag molecules, ~100 to 300 Gag-Pol molecules, and one gRNA dimer (1).

The MoMLV Gag polyprotein is encoded by the *gag* gene, which is located near the 5′ end of the viral genome, immediately upstream of *pol*. MoMLV Pol is not expressed from its own mRNA but instead is translated as a C-terminal extension of Gag when the *gag* stop codon is occasionally misread by a glutamine tRNA, resulting in readthrough into the *pol* reading frame (2; reviewed in reference 3). This generates a Gag-Pol fusion that is identical to Gag in its N-terminal sequences. An RNA pseudoknot structure (MoMLV-PK) is responsible for allowing translational readthrough of the MoMLV *gag* stop codon via termination suppression (reviewed in reference 4). Nuclear magnetic resonance-based structural studies demonstrate that, at physiological pH, the pseudoknot adopts a conformation permissive for stop codon readthrough in approximately 6% of MoMLV RNAs, whereas a nonpermissive conformation is observed in the remaining 94% (5). These ratios correlate well with the 20:1 Gag/Gag-Pol ratio reported for MLV (6).

The MoMLV Gag polyprotein directs virus assembly and gRNA packaging (reviewed in reference 7). The nucleocapsid (NC) domain of MoMLV Gag provides specificity in the protein-RNA interactions that are required for packaging via NC's conserved zinc knuckle motif (8). *In vitro* binding studies suggest that specific recruitment of a gRNA dimer requires high-affinity interactions with about 12 MoMLV NCs (9). Certain mutations within NC disrupt virus assembly, gRNA packaging, and infectivity (10–15). Because it shares sequences with Gag, the MoMLV Gag-Pol

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polyprotein also contains an NC domain. It is unknown if the RNA binding interactions of NC in the context of Gag-Pol differ from those in the context of Gag.

MoMLV gRNAs are packaged as dimers linked near their 5′ ends via a dimer linkage structure (DLS) that overlaps with the packaging signal (Ψ). gRNA dimerization and packaging are physically and functionally linked (reviewed in reference 16), and mutations or deletions in Ψ/DLS result in gRNA packaging defects (17–19). Genetic and biochemical studies suggest that the formation of MoMLV gRNA dimers occurs in the nucleus at or near sites of transcription (20–23). The formation of dimer pairs early in their biogenesis may explain the mechanism whereby MoMLV unspliced RNAs within host cells appear to segregate into nonequilibrating pools of gRNAs and mRNA (24–26).

MoMLV RNPs assemble from thousands of Gag molecules, each of which contains an NC domain with an intrinsic ability to form high-affinity interactions with gRNA. However, not every NC forms high-affinity interactions with a Ψ/DLS, and thus, there appears to be functional asymmetry among Gag molecules within the RNP. Presumably, MoMLV Gag molecules are separated into two distinct pools: Gag molecules that participate in high-affinity gRNA interactions and ones that do not (9, 15). Neither the counting mechanism that regulates the single-dimer packaging specificity of gRNA nor the mechanism that segregates Gag into two pools is known.

An additional asymmetry exists between Gag and Gag-Pol molecules. Each Gag contains a capsid (CA) domain with an intrinsic ability to interact with Gag-Pol, yet clearly not all Gag molecules interact with Gag-Pol molecules, as far fewer Gag-Pol molecules than Gag molecules are recruited per virion. The MoMLV Gag-Pol polyprotein is thought to be incorporated into assembling virions via CA-CA interactions between Gag and Gag-Pol, as is observed for HIV-1 (27, 28). That the counting mechanism that regulates the copy number of Gag-Pol is manifested by gene expression levels is suggested by the observation that the approximately 20:1 Gag/Gag-Pol copy number in retroviral RNPs mirrors the approximately 20:1 Gag/Gag-Pol expression ratio observed in cells (5, 29). In other words, this correlation suggests that MoMLV Gag-Pol incorporation into virions is stochastic and reflects sampling of the intracellular population of Gag and Gag-Pol.

Although Gag and Gag-Pol are expressed from a single species of mRNA at defined ratios during retroviral replication, the expression of these two polyproteins can be manipulated and/or separated experimentally. Using *pol* deletions, it has long been recognized that Pol is not necessary for MoMLV virus-like particle assembly and that expression of a constitutively fused version of MoMLV Gag-Pol prevents particle formation and proteolytic processing (30). Other previous studies have demonstrated complementation between Gag and Gag-Pol mutants (31), and several research groups have reported that altering the ratios of viral polyproteins has deleterious effects on particle formation for a broad range of retroviruses, including HIV-1, Rous sarcoma virus, and Mason-Pfizer monkey virus (32–35).

The goal of the present study was to test the hypothesis that the ratio of Gag to Gag-Pol and of Gag to gRNA in MoMLV particles is determined by intracellular expression ratios. In order to manipulate the ratios of Gag and Gag-Pol in the MoMLV RNP, an experimental complementation system was established in which intracellular expression of the two proteins was unlinked or proportions were altered by introducing mutations in the RNA pseudoknot that regulates Gag/Gag-Pol proportions. Similarly, altered expression ratios of gRNA to packaging-competent Gag were utilized to address the determinants of gRNA copy number within the MoMLV RNP.

MATERIALS AND METHODS

Cells and transfections. ET cells are 293T cells that constitutively express the murine ecotropic envelope (36). D17/pJET cells are canine osteosarcoma cells that constitutively express murine ecotropic receptor (37). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Gemini) for ET cells or 10% bovine serum (Invitrogen) for D17/pJET cells, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were grown at 37°C with 5% CO_2 in a humidified incubator. Transfections were carried out using polyethylenimine (PEI) (for data shown in Fig. 1, 2, 3B, and 5) or calcium phosphate precipitation (for experiments whose results are shown in Fig. 3A and 4), as described previously (36, 38). The molar ratios of the transfected plasmids are presented in the text and appropriate figures. For the experiments whose results are presented in Fig. 2, 1.7 pmol total MLV protein-expressing helper plasmid was used in each transfection, and the mass varied from 6.2 μg (for the construct that produces 100% Gag polyprotein [Gag^{only}]) to 12 μg (for the construct that produces 100% Gag-Pol polyprotein [$Gag\text{-}Pol^{only}$]). For the data shown in Fig. 3 and 4, 1.4 pmol total helper plasmid was used in each transfection. For the experiments whose results are shown in Fig. 5, the amount of pΔΨMLV helper was kept constant at 4.0 μg, and the following amounts of pMΨPuro were used: 0.07, 0.27, 1.09, 4.36, and 17.45 μg. The total number of moles of promoter per transfection of MoMLV long terminal repeats (LTRs) in the input DNA in each transfection was kept constant by the inclusion of the MoMLV solo LTR plasmid pM (described below).

Plasmids. The MoMLV helpers $p\Psi^{-}$MLV (pAM37-9) and pMΔΨMLV (pAO147-2) were derived from pNCA (39) and contain deletions in the packaging signal region from nucleotides (nt) 215 to 368 (36) and 215 to 568 (15), respectively. All other MoMLV helpers described here were derived from $p\Psi^{-}$MLV. $p\Psi^{-}MLV\text{-}Gag^{only}$ (pSD50-1) contains a deletion that removes *pol* and all but the last 100 nucleotides of *env*. $p\Psi^{-}MLV\text{-}Gag^{only}$ was engineered to contain a 4-nucleotide substitution (GGAG to ACGC) and a ClaI restriction site immediately downstream of the *gag* TAG stop codon. This ClaI site was used to fuse Gag directly to the ClaI site near the 3′ end of Env, generating a construct that lacked Pol and sequences required for stop codon readthrough via termination suppression. $p\Psi^{-}MLV\text{-}Gag\text{-}Pol^{only}$ (pSD45-1) is also a version of $p\Psi^{-}$MLV and was engineered to contain a single T-to-C substitution that replaces the *gag* stop codon with a glutamine codon and thus leads to constitutive Gag-Pol expression.

The W35G NC mutation (NC^{W35G}) was introduced into the MoMLV constructs $p\Psi^{-}MLV(NC^{W35G})$ (pSFJ346-1), $p\Psi^{-}MLV\text{-}Gag^{only}(NC^{W35G})$ (pSFJ22-1), and $p\Psi^{-}MLV\text{-}Gag\text{-}Pol^{only}(NC^{W35G})$ (pSFJ23-1), using a restriction fragment encompassing the W35G NC mutation of pGPP(W35G) (pEG479-1) (15). A similar strategy was employed to introduce W35G NC into $p\Psi^{-}MLV(NC^{W35G},PR^{D32S})$ (pSFJ370-1) and $p\Psi^{-}MLV\text{-}Gag\text{-}Pol^{only}(NC^{W35G},PR^{D32S})$ (pSFJ230-7). The D32S protease (PR) mutation (PR^{D32S}) was generated by site-directed mutagenesis and introduced into $p\Psi^{-}MLV(PR^{D32S})$ (pSFJ369-1) and $p\Psi^{-}MLV\text{-}Gag\text{-}Pol^{only}(PR^{D32S})$ (pSFJ229-7). The MoMLV-PK U38A (pk^{U38A}) and A39U (pk^{A39U}) mutations (5) were also introduced into $p\Psi^{-}MLV(pk^{U38A}, PR^{D32S})$ (pJBW124-2) and $p\Psi^{-}MLV(pk^{A39U}, PR^{D32S})$ (pSFJ576-1) by site-directed mutagenesis. All products of site-directed mutagenesis were confirmed by sequencing.

The MoMLV vector pMΨPuro (pAM86-5) contains a simian virus 40 (SV40)-driven puromycin resistance cassette (puroR) in the place of the viral genes and has been described previously (40). Deletion of Ψ and puroR from pMΨPuro produced the solo LTR vector pM (pSFJ591-1). This pM plasmid was generated by digesting pAM86-5 with AscI, which cuts once in each LTR, and circularizing. The riboprobe pD1040-2 is a

derivative of pBSII SK(+) (Stratagene) and contains a restriction fragment insert complementary to 330 bp (MscI to BsrBI) of the MoMLV Gag MA (41).

Virus and cell isolation. Tissue culture media were filtered at 48 h posttransfection for isolation of virus by sucrose cushion centrifugation (38). Viral pellets were either resuspended in TRIzol reagent (Invitrogen) for RNA isolation and subsequent RNase protection assay (RPA) analysis or resuspended in radioimmunoprecipitation assay (RIPA) lysis buffer (1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5) supplemented with 1× protease inhibitor cocktail tablet (Roche) and 1 mM phenylmethylsulfonyl fluoride to prepare samples for Western blotting. Cells were harvested at 48 h posttransfection either in RIPA lysis buffer to produce cell lysates for Western blotting as described previously (15) or in TRIzol reagent (Invitrogen) for RNA isolation and subsequent analysis by RPA. Equivalent volumes of recovery marker RNA (see below) were added to samples resuspended in TRIzol to normalize RNA recovery. Where applicable, virus was quantified in tissue culture supernatants using an exogenous reverse transcriptase (RT) assay (42) based on a previously described protocol (43).

Western blotting. Cell and virus lysates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad), blocked, blotted, and detected using a LI-COR Odyssey system (LI-COR Biosciences) as previously described (15). The following primary antibodies were used: rat monoclonal antibody (MAb) against MoMLV p30 CA, which was derived from hybridoma supernatants (ATCC clone R187 from Bruce Chesebro), and mouse beta-actin MAb (AM4302; Ambion). Secondary antibodies included goat anti-rat IRDye 800CW and goat anti-mouse IRDye 680 (Li-Cor Biosciences).

RPA. The riboprobe was produced by linearizing pD1040-2 (41) (see above) with EcoRI, followed by *in vitro* transcription using T3 RNA polymerase (Promega) and [α-^{32}P]recombinant CTP (Perkin-Elmer). Recovery marker RNA was produced by linearizing pD1040-2 with BsrGI, followed by *in vitro* transcription with T7 RNA polymerase in the absence of radiolabeled nucleotides. The pD1040-2 riboprobe protects 291 nt of MΨPuro vector gRNA, 330 nt of wild-type (WT) MoMLV gRNA, and 185 nt of recovery marker. Hybridization of riboprobes with sample RNA, RNase digestion, and other RPA steps were carried out as described previously (9).

For the experiment whose results are shown in Fig. 3B, where protected products were not present in amounts sufficiently above the background to allow accurate quantification by a phosphorimager, values were estimated in reference to a standard curve. To achieve this, 2-fold serial dilutions of a radiolabeled riboprobe were separated on a denaturing polyacrylamide gel and imaged using a phosphorimager to generate the standard curve. Two different exposures of the experimental samples were also collected. The band intensities of the experimental samples were visually compared to images of the standard curve to estimate relative values among experimental samples.

Infections. For the single-cycle infectivity assay, virus produced in ET cells was sterile filtered and then used to infect D17/pJET target cells. Infection and puromycin selection conditions have been described previously (15). Infectious units are represented as the numbers of puromycin-resistant CFU per milliliter.

RESULTS

MoMLV particle production and incorporation of Gag-Pol. To test the hypothesis that the ratio of Gag to Gag-Pol in MoMLV particles is determined by intracellular expression ratios, it was necessary to establish an experimental system that allowed clear quantification of Gag and Gag-Pol. This was achieved by engineering derivatives of the MoMLV protein-expressing helper construct pΨ$^-$MLV (Fig. 1A) to include a protease (PR)-inactivating aspartic acid-to-serine (D32S) mutation. Use of PR^{D32S} prevented proteolytic processing of Gag and Gag-Pol, thereby allowing visualization of intact virion polyproteins by Western blotting. The PR^{D32S} Ψ-negative (Ψ$^-$) constructs provided all MoMLV proteins required for the assembly of virus-like particles containing the coexpressed MoMLV vectors; here pMΨPuro vector constructs were used (Fig. 1A).

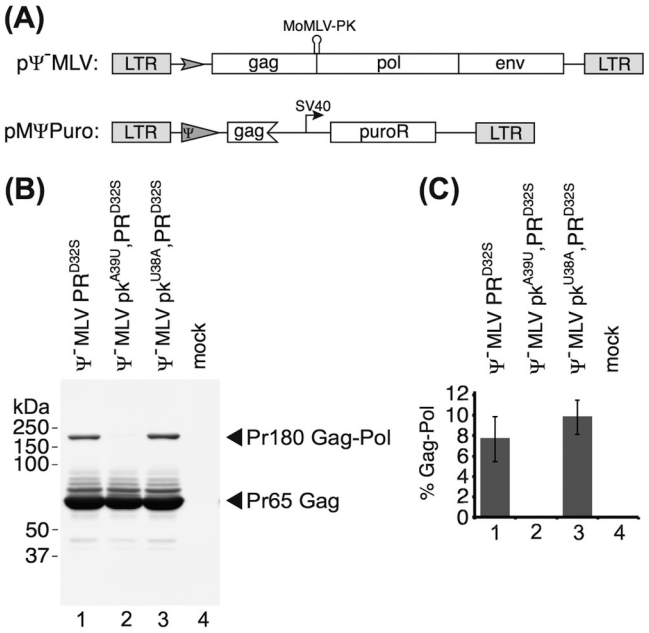

FIG 1 Quantification of Gag-Pol in MoMLV particles. (A) Diagram of MoMLV constructs used in the experiments whose results are presented here. The MoMLV constructs designated pΨ$^-$MLV contain Ψ deletions from nucleotides 215 to 368. The MoMLV vector pMΨPuro contains an SV40 promoter-driven puromycin resistance (puroR) cassette in place of the viral genes. (B) Western blot of virus lysates from ET cells transfected with the indicated MoMLV constructs. (C) Quantification of the percentage of Gag-Pol relative to the total amount of Gag (Pr65 Gag plus Pr180 Gag-Pol). Quantification results from three experimental repetitions. Note that data for cell-associated viral proteins are not provided because meaningful quantification of intracellular Gag-Pol proved unachievable with the reagents used here.

To confirm that Gag was not processed in virions in which PR was inactivated by the D32S mutation, 293T-derived ET cells (36) were cotransfected with pΨ$^-$MLV(PR^{D32S}) and pMΨPuro, and protein lysates of the resulting MoMLV particles were examined by Western blotting (Fig. 1B). The results confirmed the presence of full-length Pr65 Gag and Pr180 Gag-Pol and the absence of detectable proteolytic processing of these polyproteins (Fig. 1B, lane 1). Quantification of blots like the one shown in Fig. 1B revealed that on a molar basis, approximately 8% of total Gag (Pr65 Gag plus Pr180 Gag-Pol) was comprised of Pr180 Gag-Pol (Fig. 1C), assuming that Gag and Gag-Pol were detected equally. This correlates well with reported MoMLV *gag* stop codon readthrough rates (approximately 4 to 8%) (5, 29).

In order to manipulate the molar ratios of Gag and Gag-Pol in MoMLV particles, these polyproteins' expression ratios were altered by introducing mutations into the pseudoknot involved in recoding the stop codon between *gag* and *pol* (MoMLV-PK) (5). A mutant previously shown to be translational readthrough inactive (the A39U mutant, which contains an adenine-to-uracil change at position 39) and a second mutant that increases readthrough by approximately 30 to 80% (the U38A mutant) were used (5). Consistent with previous studies, MoMLV particles produced by

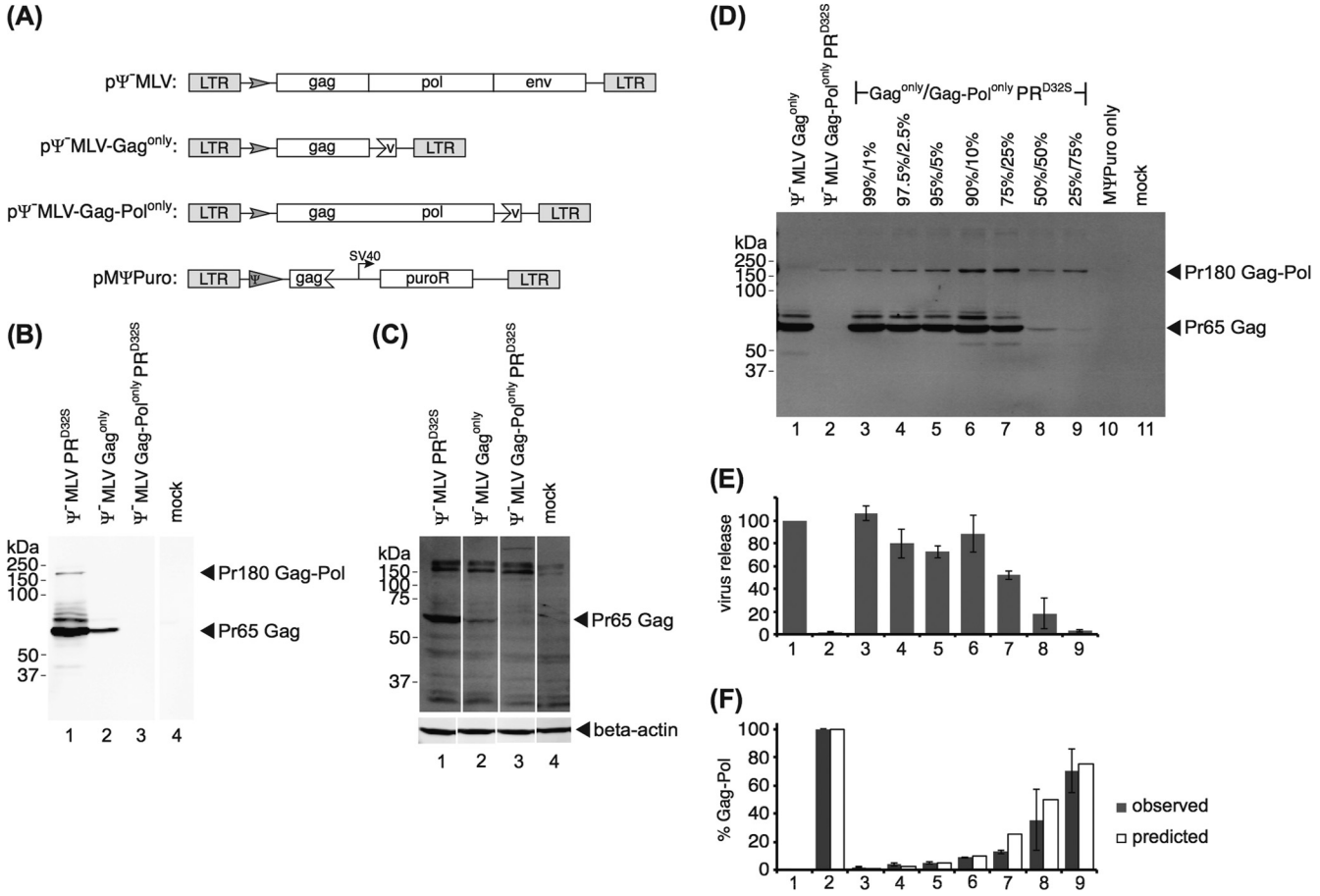

FIG 2 Incorporation of Gag-Pol into MoMLV particles. (A) Diagram of MoMLV constructs used in the experiments whose results are presented here. The MoMLV Gag^{only} constructs were derived from pΨ^{-}MLV and contain deletions of *pol* and *env*. The MoMLV Gag-Pol^{only} constructs were also derived from pΨ^{-}MLV but retained the *pol* open reading frame and contained a mutation that eliminates the *gag* stop codon. (B, C) Western blots of equal volumes of virus lysates (B) and equal volumes of cell lysates (C) of ET cells transfected with the indicated MoMLV constructs. (D) Western blot of equal volumes of virus lysates of ET cells either transfected with the indicated Gag^{only} or Gag-Pol^{only} constructs alone (lanes 1 and 2) or cotransfected with Gag^{only} and Gag-Pol^{only} constructs at the indicated ratios (lanes 3 through 9). (E) Quantification of MoMLV particle production shown in panel D presented as the total amount of Gag (Pr65 Gag plus Pr180 Gag-Pol) released into the medium relative to that in the sample with Gag^{only} alone, which was set to 100%. (F) Quantification of the percentage of Gag-Pol in MoMLV particles shown in panel D presented as the percentage of Gag-Pol relative to the total amount of Gag (Pr65 Gag plus Pr180 Gag-Pol). Black columns, quantification of Western blot bands (observed); white columns, the percentage of Gag-Pol predicted on the basis of input plasmid ratios (predicted). Data are based on those from experiments performed in duplicate.

pΨ^{-}MLV(pk^{A39U},PR^{D32S}) did not contain detectable levels of Pr180 Gag-Pol (Fig. 1B and C, lanes 2), and an approximately 25% increase in Pr180 Gag-Pol was detected in pΨ^{-}MLV(pk^{U38A,} PR^{D32S}) particles (Fig. 1B and C, lanes 3) (5). These results suggest that the amount of Gag-Pol incorporated into MoMLV virions is proportional to the readthrough-dependent protein expression amounts. Note, however, that although pseudoknot mutant samples consistently displayed higher Gag-Pol levels, the differences did not reach statistical significance.

Thus, to permit systematic variation of Gag and Gag-Pol expression ratios, *gag* and *gag-pol* were genetically separated, and genes for each polyprotein were placed on separate expression constructs (Fig. 2A). Consistent with the findings of previous studies (6), transfection of ET cells with pΨ^{-}MLV-Gag^{only} (Fig. 2A), which produces only the Gag polyprotein, resulted in moderately efficient particle production (Fig. 2B, lane 2). Specifically, virus production remained approximately 20% as high for cells transfected with pΨ^{-}MLV-Gag^{only} as for cells transfected with pΨ^{-}MLV(PR^{D32S}) (Fig. 2B, lanes 1 and 2). Cell-associated proteins were compared by Western blot analysis (Fig. 2C), and the analysis revealed an approximately 5-fold decrease in cell-associated Gag for cells transfected with pΨ^{-}MLV-Gag^{only} relative to that for cells transfected with pΨ^{-}MLV(PR^{D32S}) (Fig. 2C, lanes 1 and 2). This suggests that differences in Gag protein levels in cells largely explained the observed decrease in pΨ^{-}MLV-Gag^{only} production. Although other possibilities for this 5-fold decrease in virion production cannot be ruled out, some, such as the potential that cellular Gag may be destabilized by the absence of Gag-Pol, appear to be unlikely because the coexpression of Gag^{only} and Gag-Pol^{only} did not increase virus release (see below).

Also consistent with the findings of previous studies (30), transfection of ET cells with pΨ^{-}MLV-Gag-Pol^{only}(PR^{D32S}) (Fig. 2A), which produces only the Gag-Pol polyprotein, resulted in drastic reductions in particle production, with the amount of Pr180 Gag-Pol released from cells expressing this construct being only ~1% as much as the amount of Pr65 Gag released from

pΨ⁻MLV-Gag^{only}-transfected cells (Fig. 2B, lanes 2 and 3, and D, lanes 1 and 2).

pΨ⁻MLV-Gag^{only} and pΨ⁻MLV-Gag-Pol^{only}(PR^{D32S}) were then transfected into ET cells, either alone or in combination at various molar ratios, and particle production was examined by Western blotting (Fig. 2D). The protein ratios in virions, predicted on the basis of the molar ratios of cotransfected protein expression plasmids, are indicated at the top of Fig. 2D. The results demonstrated that coexpression of Gag and Gag-Pol supported efficient particle production across a wide range of expression ratios (Fig. 2D, lanes 3 through 7; quantification results are presented in Fig. 2E). However, when Gag-Pol represented 50% or greater of the total amount of coexpressed viral polyprotein, particle release decreased approximately 5- to 30-fold relative to that by cells expressing Gag/Gag-Pol at the 20:1 wild-type-like ratios (Fig. 2D and E, lanes 8 and 9).

Note that as the amount of input Gag-Pol expression plasmid increased from 1% to 75% of the total amount of cotransfected MoMLV expression plasmids, the proportion of Gag-Pol in the released particles correspondingly increased (Fig. 2D, lanes 3 through 9). No examination of the density, morphology, or other analyses of the released particles that would illuminate the extent to which the resulting particles resembled authentic virions was performed. Although intracellular Gag-Pol was not observable with the reagents used here (the low-mobility bands present in all lanes of Fig. 2C are presumed to be background associated with the antibody preparation), quantification of the Gag-Pol in the extracellular samples (Fig. 2F) revealed that the proportion of Gag-Pol released from cells (observed) closely matched the input transfected plasmid ratios (predicted) across all coexpression ratios, even those where particle production was decreased more than 10-fold. Taken together, these results suggest that the incorporation of Gag-Pol into MoMLV particles is stochastic across a broad range of protein coexpression ratios.

Impact of altered MoMLV Gag/Gag-Pol ratios on single-cycle infectivity. To assess the impact of altered MoMLV Gag/Gag-Pol polyprotein ratios on virus infectivity, Gag and Gag-Pol constructs were transfected into ET cells either alone or in combination with each other at various molar ratios, and proviral titers for the resulting particles were determined. Here, the Gag-Pol construct pΨ⁻MLV-Gag-Pol^{only} (Fig. 2A), which contains a functional PR, was utilized so that single-cycle infectivity could be measured, and pMΨPuro was included to provide a vector with a selectable marker. Virions produced in this manner were used to infect fresh target cells, and provirus titers, represented as the number of puromycin-resistant CFU per milliliter, were determined (Fig. 3A). The results showed that the titers remained relatively unchanged for virus containing 1% to 25% Gag-Pol (Fig. 3A, lanes 3 through 7). In contrast, the titers of virus containing 50% Gag-Pol or greater (Fig. 3A, lanes 8 and 9) were decreased approximately 100-fold or more relative to those of virus with 25% or less Gag-Pol. These approximately 100-fold decreases in numbers of CFU per ml observed for virions with high Gag-Pol contents were similar to the decreases in virus release that were measured in experiments using the PR^{D32S} forms of the same Gag/Gag-Pol ratios described above (Fig. 2D and E). Virions produced from the Gag^{only} and from the Gag-Pol^{only} helper constructs alone yielded no or very low proviral titers that were considered background (Fig. 3A, lanes 1 and 2). Thus, together, these results suggest that, in contrast to reports for HIV (44), the MoMLV infectivity per virion remained relatively unchanged by at least a moderate excess of Gag-Pol.

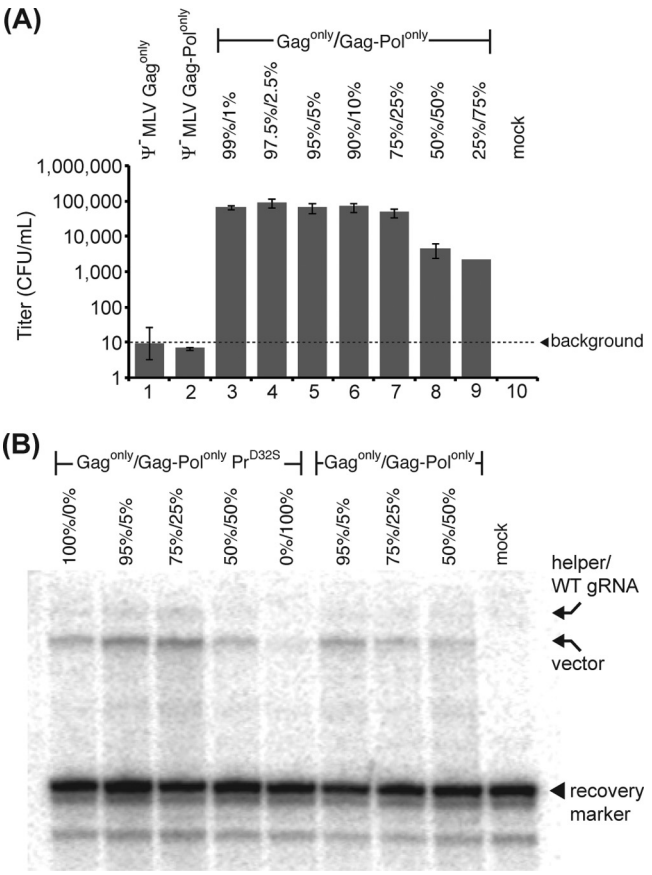

FIG 3 Impact of MoMLV Gag/Gag-Pol ratio on single-cycle infectivity. (A) Titer of virus produced from ET cells transfected with the indicated Gag^{only} or Gag-Pol^{only} constructs (lanes 1 and 2) or cotransfected with the Gag^{only} and Gag-Pol^{only} constructs at the indicated ratios (lanes 3 through 9). Error bars indicate the standard errors of the means for experiments replicated at least twice in all lanes with the exception of lane 9, which reflects the results of a single experiment. The MoMLV vector pMΨPuro was included in each transfection to serve as gRNA. Note that virions produced from the Gag^{only} helper constructs yielded very low proviral titers in some but not all experimental replications (lanes 1 and 10), and thus, this titer was considered the background titer. (B) Production of virus-like particles, addressed by assessing RNA packaging. ET cells were transfected with the indicated constructs and molar ratios of plasmids. The amount of RNA packaging per ml was quantified using RNase protection assays of samples to which recovery marker RNA (for sample normalization) was added, as described in Materials and Methods. The MoMLV vector pMΨPuro was included in each transfection to serve as gRNA. The results of one of two experimental replicates are shown. Note that because virion production is reduced about 5-fold under protein complementation conditions, product levels were too low to allow robust quantification, and thus, gRNA level estimation was performed using a standard curve, as described in Materials and Methods.

Studies with HIV-1 have suggested that the yields of PR-positive (PR^{+}) and PR-negative virions differ from one another and that overexpression of Gag-Pol results in high levels of proteolytic processing and decreased virion production (45, 46). If particle release were vastly altered for PR^{+} MoMLV particles, the results presented in Fig. 3A would require reinterpretation. Thus, to compare particle release, the amount of gRNA per ml in virus-containing medium for PR^{+} and PR^{D32S} MoMLVs was addressed

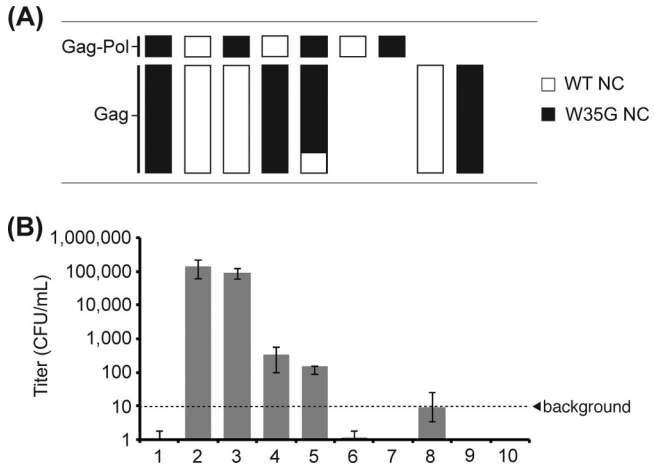

FIG 4 gRNA packaging function of the MoMLV Gag-Pol polyprotein and the titer of virus produced from ET cells cotransfected with the NC Gag^{only} and/or $Gag\text{-}Pol^{only}$ constructs at WT-like 95% Gag/5% Gag-Pol ratios. (A) The WT NC (white bars) or W35G NC (black bars) constructs used. Note that for column 5 samples, ET cells were transfected with a combination of 5% NC^{WT} Gag^{only}/90% NC^{W35G} Gag^{only}/5% NC^{W35G} $Gag\text{-}Pol^{only}$. (B) Titers are presented as numbers of CFU/ml, and error bars indicate the standard errors of the means. Reported data are from experimental triplicates. As noted in the legend to Fig. 3A, products with Gag^{only} were observed inconsistently, and thus, this value is considered the background.

(Fig. 3B). In contrast to what has been reported for HIV-1, these results suggested that the release of PR^{+} virions was only slightly reduced (about 2-fold) relative to that of PR^{D32S} virions, suggesting that MoMLV and HIV-1 differ in this regard.

gRNA packaging function of the Gag-Pol polyprotein. Our previous work has shown that packaging of a full complement of MoMLV gRNA requires that only a small fraction of the total Gag molecules in each particle provide packaging-competent NC (15). Because so little packaging-competent NC is necessary and because NC is present both in Gag and in Gag-Pol, it seemed possible that only one of these two polyproteins might function in gRNA packaging. Here, to test whether or not either Gag or Gag-Pol could serve as the source of packaging-competent NC, an NC mutation known to decrease packaging 20-fold, W35G, was introduced into the Gag^{only} and $Gag\text{-}Pol^{only}$ expression constructs (Fig. 2A) (15). The W35G mutation leads to significant reductions in MoMLV gRNA packaging but only nominal defects in other assembly functions of NC when virus is produced by transient transfection in human cells (10, 11, 15). Here, functional PR constructs and a single-cycle infectivity assay were used to examine gRNA packaging indirectly.

To determine if NC's packaging function could be provided only in the context of one polyprotein or the other, Gag^{only} and $Gag\text{-}Pol^{only}$ expression constructs containing W35G and/or WT NC (NC^{WT}) were cotransfected into ET cells at 20:1 molar ratios to establish WT-like Gag/Gag-Pol levels. The cotransfection partners are represented schematically in Fig. 4A, with the smaller rectangles at the top specifying the nature of the $Gag\text{-}Pol^{only}$ construct used in each cotransfection and the larger rectangles specifying the Gag^{only} alleles used. The MoMLV vector pMΨPuro was included in the transfections so that RNA packaging could be monitored by determining the number of puromycin-resistant CFU per milliliter in infected target cells.

The titer of particles containing W35G NC in both Gag and Gag-Pol was reduced to background levels (<1 CFU/ml) (Fig. 4B, lane 1), whereas the titer of virus containing NC^{WT} Gag and NC^{WT} Gag-Pol (Fig. 4B, lane 2) was relatively high (1×10^5 CFU/ml). Virus containing NC^{WT} Gag/NC^{W35G} Gag-Pol generated a titer similar to that generated by virus containing NC^{WT} Gag/NC^{WT} Gag-Pol (Fig. 4B, lane 3), demonstrating that Pol is recruited well and is fully functional in the context of W35G.

The observation that virions composed of packaging-competent Gag and packaging-defective Gag-Pol encapsidated nearly WT levels of gRNA demonstrated that Gag can provide packaging functions during MoMLV assembly but did not rule out the possibility that Gag-Pol may also be capable of mediating packaging. To address this, experiments were performed in which packaging-competent NC was present only in Gag-Pol in which the Gag polyprotein harbored NC^{W35G} (Fig. 4B, lane 4). The titer of these NC^{W35G} Gag/NC^{WT} Gag-Pol particles was approximately 2.5 log units lower than that of NC^{WT} Gag/NC^{WT} Gag-Pol virus. However, NC^{W35G} Gag/NC^{WT} Gag-Pol particles contained only ~5% as much functional NC as NC^{WT} Gag/NC^{W35G} Gag-Pol virions. Retroviral NCs are known to contribute to postassembly functions, such as reverse transcription, as well as to packaging (47), and NC^{W35G} is known to generate virions with lower single-cycle infectivity assay titers that would be predicted by their packaging defects (15). Thus, additional experiments were performed to allow a direct comparison of the polyproteins' packaging capabilities by establishing conditions where a constant amount of packaging-competent NC (5% of the total) was provided either by Gag or by Gag-Pol. When the packaging-competent NC was present in Gag and represented 5% of total Gag (with the remainder of the Gag in this experiment harboring W35G NC), the titer was indistinguishable from that of NC^{W35G} Gag/NC^{WT} Gag-Pol virus (Fig. 4B; compare lanes 4 and 5). These results demonstrate that a packaging-competent NC can support virus replication equally well when provided in the context of either the Gag or Gag-Pol polyprotein and suggest that NC in the context of Gag, Gag-Pol, or a combination of the two can provide gRNA packaging functions for MoMLV.

Limits of MoMLV gRNA packaging. The experiments described above demonstrated that Gag-Pol incorporation into MoMLV particles is principally stochastic, with defects in virion release occurring when Gag-Pol is expressed in significant excess. In order to test if the quantity of gRNA packaged by MoMLV is also determined by intracellular expression ratios, gRNA/*gag* mRNA ratios were manipulated by experimental separation and coexpression at various ratios. Specifically, MoMLV particles were produced by cotransfecting ET cells with constant amounts of the Ψ^- helper construct pΔΨMLV with or without the MoMLV vector pMΨPuro, whose amount was varied over a 256-fold range (Fig. 5A). Particle production was quantified (Fig. 5B) and revealed that no decreases in virion production were observed, even with the largest amounts of cotransfected MoMLV vector.

Next, vector RNA expression in cells and packaging into particles were quantified by an RNase protection assay (Fig. 5C). At coexpression ratios where the amount of Ψ^- helper was in molar excess of the amount of vector, packaging increased roughly in proportion to vector expression levels (Fig. 5C, lanes 2 to 4 and 10 to 12). Vector expression ratios in cells continued to increase in proportion to the amount of transfected DNA, as cotransfected vector levels exceeded those of the helper, but packaging levels plateaued (Fig. 5C, lanes 5 and 6 and lanes 13 and 14). When

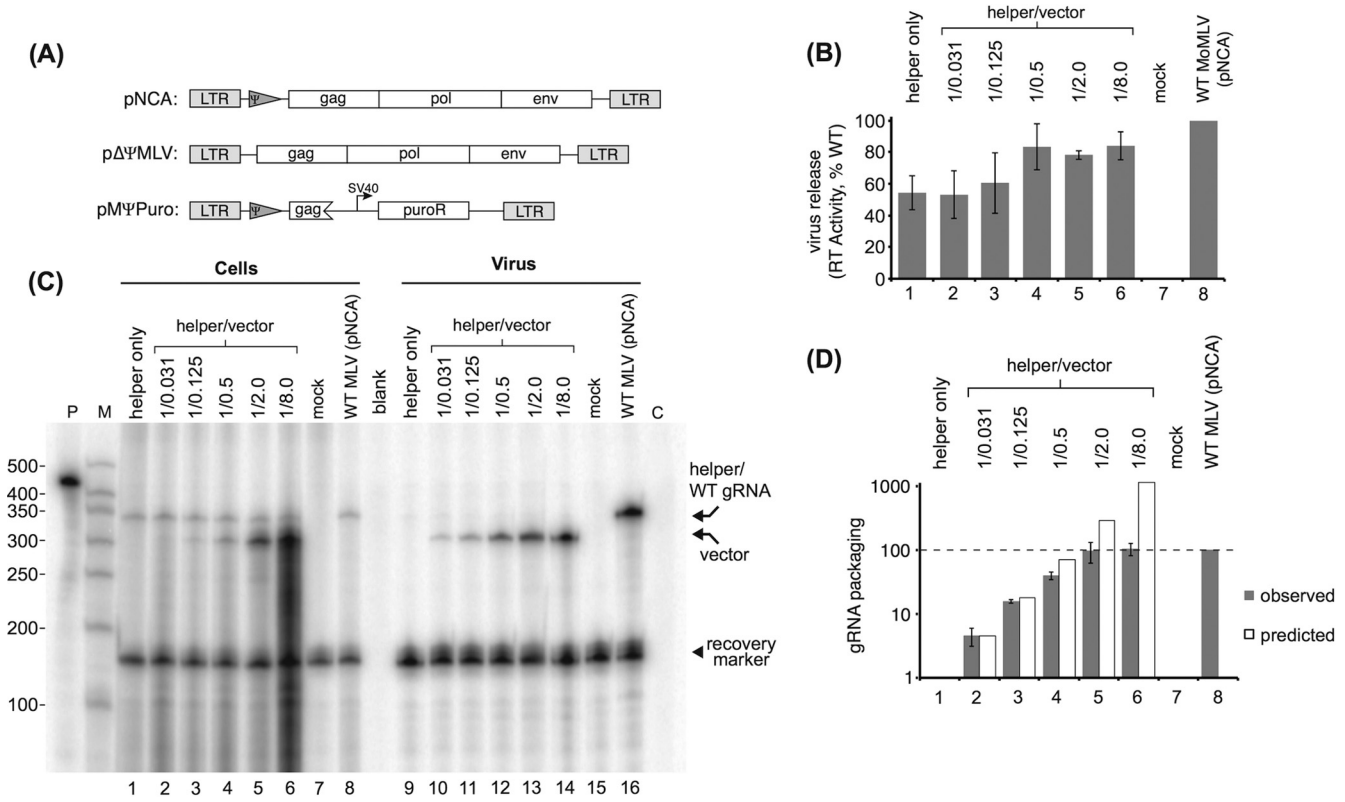

FIG 5 Limits of MoMLV gRNA packaging. (A) Diagram of MoMLV constructs used in the experiments described here. pNCA is the parental WT MoMLV clone from which all other constructs were derived. pMΔΨMLV contains a Ψ deletion from nucleotides 215 to 568. (B) MoMLV particle release from cells cotransfected with the MoMLV construct pΔΨMLV and increasing concentrations of the MoMLV vector pMΨPuro was determined by determination of the RT activity of medium harvested from cells and normalized to that for WT MoMLV, which was set to 100%. Data shown are from two independent repetitions. (C) RPA of RNA harvested from virus and cells transfected as described in the legend to panel B and probed with the pD1040-2 riboprobe. Lane P, probe; lane M, marker; lane C, control. Numbers on the left are in kilodaltons. (D) Quantification of MoMLV RNA packaging shown in panel C normalized to the particle release shown in panel B and normalized to the amount of WT MoMLV, which was set to 100. Gray columns, measured values for RNA packaging (observed) from panel C; white columns, predicted packaging levels based on the quantity of input vector plasmid (predicted). The input molar ratio of helper and vector is indicated above each panel, with the amount of helper set to 1. See Materials and Methods for further experimental details.

normalized for particle production (Fig. 5B), the amount of gRNA packaged per particle increased approximately 10-fold as the amount of cotransfected vector plasmid increased 16-fold (from a helper/vector plasmid molar ratio of 1:0.031 to one of 1:0.5; Fig. 5D, lanes 2 to 4) but increased significantly less than predicted at a helper/vector ratio of 1:2 (Fig. 5D, lane 5). Further increasing the helper/vector ratio to 1:8 did not increase the amount of gRNA packaged per particle, suggesting that the amount of gRNA packaging had reached saturation (Fig. 5D, lane 6).

RNA packaging in MoMLV particles produced by transfection of ET cells with the WT MoMLV proviral plasmid pNCA (Fig. 5A) was compared to packaging in Ψ^- particles containing saturating levels of pMΨPuro (Fig. 5C and D). The observation that the plateau level of MoMLV vector packaging was indistinguishable from the amount of gRNA packaged per WT MoMLV particle (Fig. 5D, lanes 6 and 8) suggests that the upper limit of vector gRNA packaging observed in Fig. 5 closely matches the amount of gRNA packaged per virion by WT MoMLV.

DISCUSSION

The goal of the present study was to determine whether or not Gag/Gag-Pol ratios and the numbers of gRNA molecules per MoMLV particle are dictated solely by intracellular expression ratios. Here, the proportion of total Gag present in virions that was Gag-Pol was determined to be approximately 8% under native Gag/Gag-Pol expression conditions, a value that matches reasonably well with the values reported previously (5, 29). When Gag/Gag-Pol ratios were manipulated, either by mutating residues within the pseudoknot that regulates Gag/Gag-Pol proportions or by expressing Gag and Gag-Pol from separate constructs, the molar proportion of Gag-Pol in virions consistently matched intracellular expression ratios. This suggests that the incorporation of Gag-Pol into virions is principally a stochastic process.

When MoMLV Gag and Gag-Pol were coexpressed at equimolar ratios and when Gag-Pol represented the majority polyprotein, marked defects in particle production were observed. These observations of production defects in the presence of excess Gag-Pol are consistent with previous findings for both MLV and HIV-1 (44, 48). It has been suggested for HIV-1 that when Gag-Pol is overrepresented in cells, assembly may be disrupted due to recruitment of monomeric RNAs (44), improper membrane curvature, or the inability of assembling virions to accommodate the larger Gag-Pol molecule in excess (49). Previous reports of aberrant virion morphology for HIV-1 when Gag-Pol is overrepresented in cells are also consistent with this notion (48).

Interestingly, the results presented here suggest that MoMLV virions with a moderate excess of Gag-Pol retain infectivity and, thus, that MLV and HIV may differ in this regard. Both release and infectivity are decreased for HIV particles containing excess Gag-Pol, and these decreases are at least partially due to premature PR activation (45, 46). This activation may result from dimerization of HIV-1 reverse transcriptase (RT), which, when it occurs in the context of Gag-Pol, can activate PR and have negative effects on budding and maturation (50). In contrast, MoMLV RT is monomeric, at least in solution (51), and thus, MoMLV RT likely cannot contribute to Gag-Pol–Gag-Pol dimerization and premature PR activation to the extent that HIV-1 RT can.

Additional experiments performed here revealed that either the Gag or the Gag-Pol polyprotein can provide the interactions with gRNA necessary for packaging. Specifically, proviral titers were indistinguishable when the molar levels of packaging-competent NC were kept constant, regardless of whether the functional NC was provided in the context of Gag or Gag-Pol. This finding mirrors previous observations with the *Saccharomyces cerevisiae* yeast retrotransposon Ty3, which showed that the zinc knuckle motif of NC can support retrotransposition when provided in the context of either Gag or Gag-Pol (52).

In contrast to observations with Gag and Gag-Pol, gRNAs were not recruited in strict proportion to their intracellular expression levels. Instead, when Gag expression was constant and gRNA expression in cells increased, the amount of gRNA per particle increased proportionately until an upper limit was reached, beyond which a 10-fold further increase in vector expression neither increased the amount packaged per virion nor decreased the level of virus production. These observations of saturable gRNA packaging and no impairment of virion release in the presence of excess gRNA support the hypothesis that recruitment of the conserved copy number of gRNAs per MoMLV particle—presumably a single dimer—triggers an assembly transition that prevents the inclusion of excess gRNA (53, 54).

The notion that MoMLV virions assemble when a single Ψ/DLS has been recruited is consistent with previous work demonstrating that MoMLV can assemble well with genomes three times the normal length (41). Also consistent with this notion are the results of recent studies with Pol-minus HIV-1 virus-like particles generated using Rev-independent expression. These results suggest that the encapsidation of two copies of gRNA is regulated not by RNA mass but instead by the specific recognition of a single dimer of RNAs (54).

The findings from the gRNA overexpression experiments described here can be used to estimate the proportions of MoMLV unspliced RNAs within cells that will serve as gRNAs versus those that will function as mRNAs. Both genetic and biochemical studies of MoMLV gRNA dimer formation (20–23) and inhibitor-based studies that suggest that MoMLV gRNAs and mRNAs differ in half-lives (24–26) suggest that the unspliced MoMLV RNAs within host cells segregate into nonequilibrating pools: one that provides gRNAs and the other that consists of viral mRNA. In using long-established approaches to genetically separate gRNA from mRNA by using Ψ-positive (Ψ^+) and Ψ^- derivatives of MoMLV, the experiments described here allowed us to experimentally manipulate the ratios of these intracellular pools. Analysis of intracellular RNA proportions showed that saturable packaging levels were achieved when the mRNA (Ψ^- helper) pool was roughly half the gRNA (Ψ^+ vector) pool and that these ratios were indistinguishable from those observed for WT MLV (pNCA) (Fig. 5). Previous packaging competition experiments have suggested that the Ψ^+ vectors used here and WT MLV RNAs are packaged equally well (9). Although caveats remain, if MoMLV has evolved to ensure that replication occurs under conditions of optimal gRNA/mRNA ratios, then these findings suggest that roughly twice as much of the total unspliced MoMLV RNA in a cell resides in the gRNA pool as in the mRNA pool.

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