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Short communication

Reciprocal cross-packaging of primate lentiviral (HIV-1 and SIV) RNAs by heterologous non-lentiviral MPMV proteins

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ABSTRACT

Retroviral RNA packaging signal (ψ) allows the preferential packaging of genomic RNA into virus particles through its interaction with the nucleocapsid protein. The specificity of this interaction came into question when it was shown that primate retroviruses, such as HIV-1, could cross-package RNA from its simian cousin, SIV, and vice versa and that feline retrovirus, FIV could cross-package RNA from a distantly related primate retrovirus, MPMV. To study the generality of this phenomenon further, we determined whether there is a greater packaging restriction between the lentiviral class of retroviruses (HIV-1 and SIV) and a non-lentivirus, MPMV. Our results revealed that primate lentiviral RNAs can be cross-packaged by primate non-lentiviral particles reciprocally, but the cross-packaged RNAs could not be propagated by the heterologous particles. Packaging of RNA in the context of both retroviral vectors as well as non-retroviral RNA containing SIV, HIV, and MPMV packaging determinants by each others proteins further confirmed the specificity of cross-packaging conferred by the packaging sequences. These results reveal the promiscuous nature of retroviral packaging determinants and raise caution against their wide spread presence on retroviral vectors to be used for human gene therapy.

1. Introduction

Although retroviral genomic RNA represents less than 1% of the mRNA in the cytoplasm of an infected cell, this class of RNA is predominantly packaged in large amounts into the assembling virions (reviewed in D'Souza and Summers, 2005; Lever, 2007). Overall, the cis acting region responsible for retroviral RNA packaging is located at the 5′ end of the retroviral genome between the primer binding site (PBS) and Gag and is called the packaging signal (ψ); however, the precise requirements for packaging varies between different retroviruses (D'Souza and Summers, 2005; Lever, 2007). The specific incorporation of the retroviral genomic RNA into the virus particles is facilitated by the interaction between the cis acting packaging sequences and the nucleocapsid (NC) domain of Gag polyproteins in trans (D'Souza and Summers, 2005; Lever, 2007). Despite this interaction, several studies using a number of retroviruses have shown that RNA pseudotyping and/or cross-packaging (i.e. packaging of an RNA by heterologous virus particles) can take place among evolutionary related, yet molecularly different, retroviruses (reviewed in Al Dhaheri et al., 2009). Cross- and co-packaging among divergent retroviruses have been shown to result in the exchange of genetic information, which may lead to the generation of recombinant variants with new pathogenic potentials that may allow them to cause diseases, escape from the host's immune system, and acquire the ability to infect hosts they could not infect before (reviewed in An and Telesnitsky, 2002; Hu et al., 2003; Negroni and Buc, 2001).

Due to the increasing interest in using retroviral vectors in human gene therapy, pseudotyping among retroviruses has been extensively investigated recently and, at times, revealed noteworthy and unexpected findings (summarized in Table 1 and further reviewed in Al Dhaheri et al., 2009). For instance, co-packaging and exchange of genetic information between genetically distant retroviruses such as spleen necrosis virus (SNV) and murine leukemia virus (MLV) (Yin and Hu, 1997) as well as between human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (Motomura et al., 2008) have been reported. Furthermore, it has been shown that proteins of reticuloendotheliosis virus (REV) can package the RNA of a distantly related retrovirus, MLV (Embertson and Temin, 1987; Dougherty et al., 1989; Kewalramani et al., 1992; Yang and Temin, 1994; Yin and Hu, 1997). Similarly, among complex retroviruses, several studies revealed that lentiviruses (feline/human/simian immunodeficiency viruses, FIV/HIV-1/SIV) can reciprocally cross-package each others genomes (Browning et al., 2001; Rizvi and Panganiban, 1993; White et al., 1999). In addition, reciprocal cross-packaging between mouse mammary tumor virus (MMTV) and

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Table 1
Summary of retroviral RNA cross-packaging and propagation results reported in the literature.

RNA	Proteins																	
	HIV-1		HIV-2		SIV		FIV		MPMV		MMTV		MLV		SNV		MoMLV		REV	
	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep	Pack	Rep
HIV-1	Yes	Yes	No (1, 2)	No (1, 2)	Yes (3)	Yes (3)	Yes (4)	Yes (4)	Yes*	No*	Yes△ (5)	–	–	–	Yes (6)	Yes (6)	No (7)	–	–	–
HIV-2	Yes (1, 2)	Yes (1, 2)	Yes	Yes	Yes (2)	Yes (2)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SIV	Yes (2, 8)	Yes (2, 8)	No (2)	No (2)	Yes	Yes	Yes (4)	Yes (4)	Yes*	No*	–	–	–	–	–	–	–	–	–	–
FIV	Yes (4)	Yes (4)	–	–	Yes (4)	Yes (4)	Yes	Yes	No (4)	No (4)	–	–	–	–	–	–	–	–	–	–
MPMV	Yes*	No*	–	–	Yes*	No*	Yes (4)	No (4)	Yes	Yes	Yes (9)	No (9)	–	–	–	–	–	–	–	–
MMTV	Yes¶ (5)	–	–	–	–	–	–	–	Yes (9)	No (9)	Yes	Yes	–	–	–	–	No (10)	No (10)	–	–
MLV	–	–	–	–	–	–	–	–	–	–	–	–	Yes	Yes	Yes (11, 12)	Yes (11, 12)	–	–	Yes (11–13)	Yes (11–13)
SNV	No (6)	No (6)	–	–	–	–	–	–	–	–	–	–	No (14)	No (14)	Yes	Yes	–	–	–	–
MoMLV	Yes¶ (7)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Yes	Yes	–	–

HIV, SIV, FIV: human, simian, feline immunodeficiency virus, respectively; MPMV: Mason–Pfizer monkey virus; MMTV: mouse mammary tumor virus; SNV: spleen necrosis virus; MoMLV: Moloney murine leukemia virus; rev: reticuloendotheliosis virus; pack: RNA packaging; rep: replication indicating successful RNA propagation following RNA packaging; "–": indicates no available published reports in the literature regarding cross-packaging. Number(s) in parenthesis indicates the reference(s) cited: (1) Kaye and Lever (1998); (2) Strappe et al. (2005); (3) White et al. (1999); (4) Browning et al. (2001); (5) Poon et al. (1998); (6) Parveen et al. (2004); (7) Zhang and Barklis (1995); (8) Rizvi and Panganiban (1993); (9) Al Dhaheri et al. (2009); (10) Günzburg and Salmons (1986); (11) Embertson and Temin (1987); (12) Yang and Temin (1994); (13) Dougherty et al. (1989); (14) Certo et al. (1998).

* Represents data presented in the current study.

△ In the context of chimeric MMTV containing HIV-1 nucleocapsid (NC) in the background of MMTV genome.

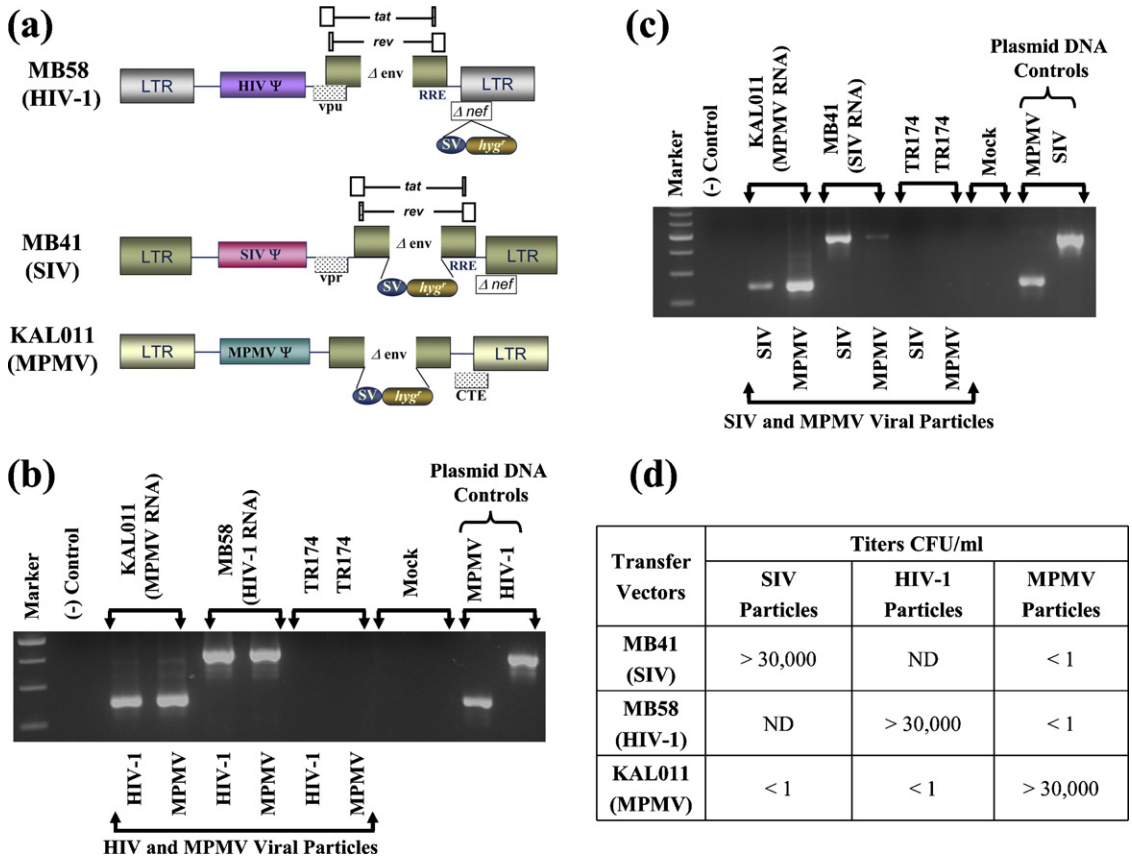
¶ In the context of chimeric viruses containing HIV-1 NC in the background of the respective parental viruses.

Mason–Pfizer monkey virus (MPMV) has been reported recently (Al Dhaheri et al., 2009). Non-reciprocal packaging has also been observed in both lentiviruses (HIV-1 and HIV-2) (Kaye and Lever, 1998; Strappe et al., 2005) as well as simple retroviruses (SNV, MLV, REV; Embertson and Temin, 1987; Yang and Temin, 1994; Certo et al., 1998). Moreover, it has been shown that a complex retrovirus (HIV-1) RNA could be cross-packaged by a simple retrovirus, SNV, proteins but not vice versa (Parveen et al., 2004). On the contrary, it has also been observed that proteins from a complex retrovirus such as FIV are efficient in packaging RNA from a simple retrovirus such as MPMV but MPMV proteins are unable to package FIV RNA (Browning et al., 2001). To study the generality of the cross-packaging phenomenon among divergent retroviruses further, we investigated whether there is a greater cross-packaging restriction(s) between the lentiviruses such as HIV-1 and SIV and a primate non-lentivirus such as MPMV.

2. HIV-1 and SIV RNAs can be cross-packaged by MPMV proteins

In order to study whether HIV-1 and SIV RNAs can be cross-packaged by a simple retrovirus (MPMV), we took advantage of the earlier established trans complementation assays for HIV-1, SIV, and MPMV (Naldini et al., 1996; White et al., 1999; Browning et al., 2001). HIV-1 (MB58) and SIV (MB41) transfer vectors (Fig. 1a) containing all the cis-acting sequences necessary for RNA packaging and propagation were co-transfected with TR301, an MPMV Gag/Pol expression plasmid (Browning et al., 2001) and MD.G, an envelope expression plasmid (Naldini et al., 1996) into 293T cells using calcium phosphate method as described earlier (Mustafa et al., 2005a). In a converse experiment, MPMV transfer vector, KAL011 (Fig. 1a; Browning et al., 2001) was co-transfected with HIV-1 (CMVΔR8.2) and SIV (SIV pack) Gag/Pol expression plasmids (Naldini et al., 1996; White et al., 1999) and MD.G to determine whether HIV-1 and SIV proteins can also cross-package MPMV RNA. As a control, HIV-1, SIV and MPMV transfer vectors were co-transfected with the homologous Gag/Pol expression plasmids and the envelope expression plasmid, MD.G.

RNAs from the transfected cells were isolated 72 h post transfection and fractionated into cytoplasmic and nuclear fractions. Cytoplasmic RNAs were confirmed for the absence of any contaminating plasmid DNA, followed by cDNAs preparation and amplification using vector specific primers as described earlier (Mustafa et al., 2005b; Ghazawi et al., 2006). These amplifications confirmed that vector RNAs were stably expressed and transported to the cytoplasm and that the integrity of the nuclear membrane was not compromised during the fractionation process (data not shown). Next, viral RNAs were prepared from virions that were pelleted using a sucrose cushion, DNase treated as described earlier (Mustafa et al., 2005b; Ghazawi et al., 2006), and amplified using virus specific primers, which ensured that there was no contaminating plasmid DNA in the viral RNA preparations that may have been carried over from the transfected cultures (data not shown). This was followed by cDNAs preparation and amplification for 30 cycles using transfer vector specific primers. The results of these experiments revealed that both HIV-1 and SIV RNAs could be cross-packaged by MPMV proteins (Fig. 1b and c). In a reciprocal manner, HIV-1 and SIV proteins were found to cross-package MPMV RNA within detectable range (Fig. 1b and c), suggesting that the ψ of these retroviruses were readily recognized by the NC domain of Gag proteins, which further indicate that molecularly divergent and phylogenetically distant retroviral proteins are promiscuous in their ability to pseudotype each other's RNA. Although not quantitative, the cross-packaging between HIV and MPMV seemed to be more efficient than the cross-packaging between SIV and MPMV



(d)

Transfer Vectors	Titers CFU/ml		
	SIV Particles	HIV-1 Particles	MPMV Particles
MB41 (SIV)	> 30,000	ND	< 1
MB58 (HIV-1)	ND	> 30,000	< 1
KAL011 (MPMV)	< 1	< 1	> 30,000

Fig. 1. HIV-1 and SIV RNAs can be cross-packaged by MPMV proteins. (a) Schematic representation of SIV, HIV-1 and MPMV transfer vectors used in the cross-packaging study and have been described previously (Browning et al., 2001). RT-PCR of viral cDNAs amplified using transfer vector specific primers for 30 cycles showing the reciprocal cross-packaging of (b) HIV-1 and MPMV and (c) SIV and MPMV. For HIV-1, SIV, and MPMV primer sets OTR462/463, OTR465/466, OTR112/320 were used, which amplified 784, 839, and 187 bp fragments, respectively. (d) Propagation of SIV, HIV-1 and MPMV transfer vectors RNA by homologous and heterologous proteins. The table shows the number of hygromycin resistant (Hyg^r) colony forming units per milliliter (CFU/ml) of non-concentrated supernatant from the transfected cultures from three experiments performed in duplicates. <1 indicates <1 Hyg^r colonies in the target cells.

(Fig. 1b and c). It has recently been reported that retroviral Gag proteins can non-specifically encapsidate cell non-coding RNAs as efficiently as viral RNAs especially under conditions in which the native packaging sequences are absent (Berkowitz et al., 1996; Onafuwa-Nuga et al., 2005; Rulli et al., 2007). Therefore, as an ancillary control to exclude such non-specific encapsidation of viral or cellular RNA, we attempted to package an overly expressed non-specific RNA by these retroviral proteins. Our results indicated that MPMV, HIV-1 and SIV proteins encapsidated the heterologous and homologous RNAs in a specific manner as these proteins did not encapsidate (Fig. 1b and c) a non-specific RNA generated from a control vector (TR174) described earlier (Rizvi and Panganiban, 1993) that lacks viral sequences at the 5′ end or if encapsidated, the level of encapsidation was below the level of detection.

To determine whether the cross-packaged RNAs could be propagated by the heterologous proteins for further steps in retroviral life cycle, supernatants from the harvested cultures were used to infect HeLa CD4+ cells and monitored for the appearance of hygromycin resistant colonies as described earlier (Browning et al., 2001). As expected, HIV-1, SIV and MPMV transfer vectors, when packaged by the homologous proteins, were efficiently propagated (Fig. 1d), ensuring the integrity of our transfer vectors and transduction assay. However, when HIV-1 and SIV were cross-packaged by the MPMV proteins or vice versa, no hygromycin resistant colonies were obtained in the infected cultures (Fig. 1d), suggesting that their RNAs could not be propagated further. These results reveal that while the proteins of HIV-1, SIV, and MPMV are efficient at cross-packaging the heterologous RNAs, they are not capable of propagating these RNA substrates further. This indicates a potential block in post-packaging events of the retroviral life cycle such as reverse transcription and/or integration since our read-out assay was dependent on the successful expression of hygromycin gene from an integrated provirus. Thus, it is plausible that the reverse transcriptase and integrase of HIV-1, SIV and MPMV are not able to work on the respective targets (PBS/poly purine tract (PPT) and att sites, respectively) of the heterologous RNAs, and therefore these cross-packaged RNAs could not be propagated further.

Comparison of the PBS and PPT sequences of MPMV with similar sequences of HIV-1 and SIV showed that MPMV PBS is 44% and 39% homologous to HIV-1 and SIV, respectively, whereas MPMV PPT has 45% sequence homology to both HIV-1 and SIV (Browning et al., 2001). On the other hand, comparison of the att sites revealed that MPMV att has less than 30% sequence homology to HIV-1 (U3 att 30% and U5 att 20%) and 20% sequence homology to SIV (Browning et al., 2001). Therefore, the lack of propagation of these cross-packaged RNAs could be attributed to the inability of these RNAs to complete the process of reverse transcription and/or integration successfully due to the incompatibilities of the PBS/PPT and att sites. We have recently observed a similar phenomenon between MMTV and MPMV where the lack of propagation of the cross-packaged MPMV and MMTV RNAs was attributed to the heterogeneity in the cis acting sequences of these viruses (Al Dhaheri et al., 2009). Although, as mentioned above, the RNA propagation read-out assay in our system is based on the expression of

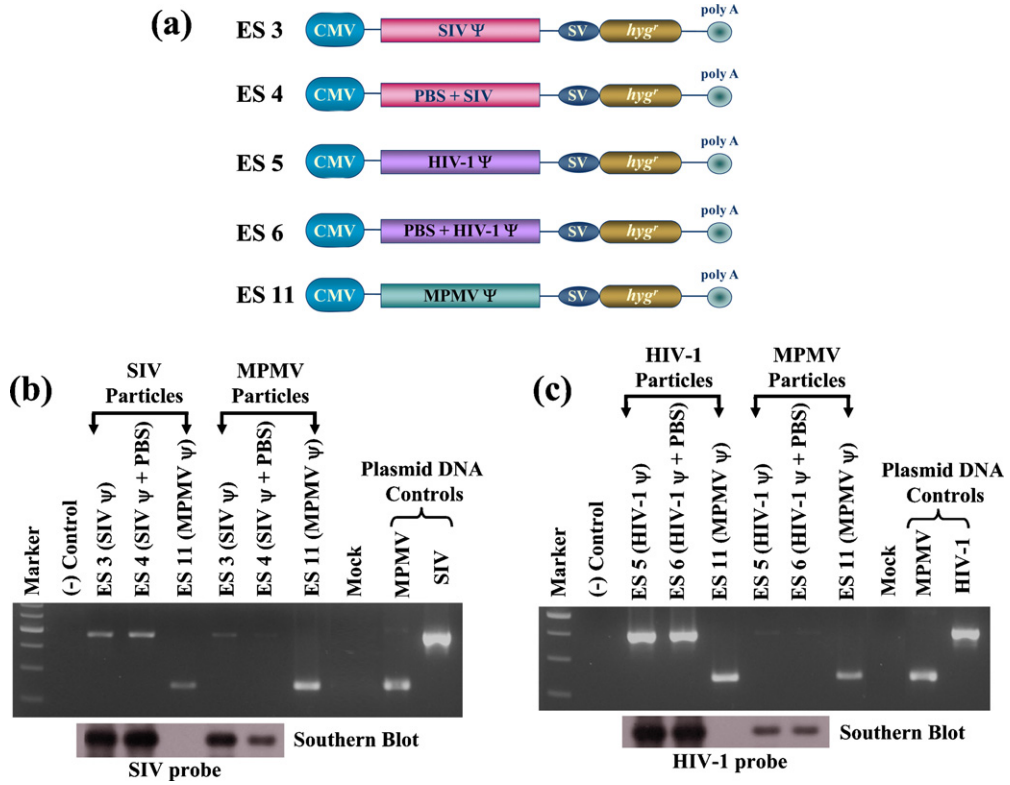


Fig. 2. Cross-packaging of non-viral RNAs containing SIV, HIV-1 or MPMV packaging signal. (a) Schematic representation of the expression plasmids containing SIV, HIV-1 or MPMV ψ. For SIV, the regions of SIV ψ spanning nucleotide (nt) 1101–1940 (without PBS) and nt 1079–1940 (with PBS) were cloned into a pcDNA3 based expression plasmid resulting in the generation of ES3 and ES4, respectively. A similar strategy was used to create expression plasmids containing HIV-1 ψ with and without PBS where the region spanning nt 659–1443 or nt 637–1443 were cloned into pcDNA3 based expression plasmid, resulting in the generation of ES5 and ES6, respectively. Another expression plasmid, ES11, was created by cloning the region spanning nt 350–778 of MPMV (without PBS) into pcDNA3 based expression plasmid. Therefore the regions spanning ψ are the same in the sub-genomic vectors as well as in these expression plasmids. Nucleotide designation for MPMV, SIV_{mac239}, and $HIV\text{-}1_{NL4\text{-}3}$ are based on GenBank accession numbers M12349 (Sonigo et al., 1986), M33262 (Kestler et al., 1990), and M19921 (Adachi et al., 1986), respectively. (b) Upper panel shows the reciprocal cross-packaging of the non-viral RNAs containing SIV and MPMV ψ by each other proteins. Southern blot of the amplified products for the corresponding number of cycle is also shown (lower panel) and (c) RT-PCR analysis showing the reciprocal cross-packaging of the non-viral RNAs containing HIV-1 and MPMV ψ by each other proteins (upper panel) along with Southern blot of the amplified product for the corresponding number of cycle (lower panel). Details of the primers used in RT-PCR, and Southern hybridization can be obtained from authors upon request.

hygromycin gene from an integrated provirus, the possibility of a fully reverse transcribed unintegrated provirus as reported earlier (Lewis and Emerman, 2008) cannot be ruled out in our study.

3. Non-viral RNAs containing SIV, HIV-1 and MPMV packaging sequences can be reciprocally cross-packaged by each other proteins

To verify whether the reciprocal cross-packaging of the HIV-1, SIV and MPMV RNAs was due to the specific recognition of ψ by the proteins of the assembling virus particles, the regions encompassing ψ (with and without PBS) of SIV (ES3 and ES4), HIV-1 (ES5 and ES6) and MPMV (ES11) were cloned into pcDNA3-based expression plasmids to generate non-viral RNA containing ψ of these viruses (Fig. 2a). These expression plasmids were co-transfected in a two-plasmid trans complementation assay with either homologous or heterologous Gag/Pol expression plasmids (CMVΔR8.2; SIV pack; TR301) and examined for RNA packaging.

RT-PCR analysis revealed that non-viral RNAs containing ψ were reciprocally cross-packaged by both the homologous and heterologous proteins (Fig. 2b and c) in the presence as well as in the absence of PBS, suggesting that the PBS did not significantly contribute to the cross-packaging of these non-viral RNAs containing ψ. The relative cross-packaging efficiency of the non-viral RNAs containing ψ (especially HIV-1) was lower compared to the transfer vector RNAs in the subgenomic context (MB41, MB58, KAL011) when packaged or cross-packaged by the homologous or heterologous proteins (compare Figs. 1b, c and 2b, c), since Southern blotting was needed to appreciate the cross-packaging after 30 cycles of PCR. This differential packaging pattern could be explained if these viruses contained sequences outside the ψ that could be involved in synergistically enhancing packaging of the transfer vectors, since such sequences were excluded from non-viral RNAs containing ψ. A somewhat comparable situation has been reported in the case of FIV using non-viral RNAs containing different regions of FIV genome (Ghazawi et al., 2006). Ghazawi and colleagues showed that U3 region of FIV 3′ LTR contains minor packaging determinants that work synergistically with the major packaging determinants present at the 5′ end to enhance RNA packaging. Similarly, it has also been observed that when bovine leukemia virus (BLV) RNA contained regions other than its packaging sequences, it was efficiently packaged into BLV particles (Jewell and Mansky, 2005). The cross-packaging of non-viral RNAs containing ψ in these experiments further substantiates our initial observation that the reciprocal cross-packaging of these retroviral RNAs was primarily due to the specific interaction between the NC proteins and the packaging sequences of these viruses. Although our results clearly demonstrate that the reciprocal cross-packaging of these molecularly divergent RNAs was due to the presence of the packaging

sequences on these RNAs, the possibility of non-specific RNA cross-packaging cannot be ruled out, especially in the light of recent observations showing encapsidation of overly expressed cellular RNAs (Berkowitz et al., 1996; Onafuwa-Nuga et al., 2005; Rulli et al., 2007). Therefore, it will be interesting to test whether similar cross-packaging of these distinct RNAs can take place in more controlled experimental protocols such as single copy transfection or infection like conditions that should negate the production of large amounts of RNA, which in turn maybe responsible for providing substrates facilitating non-specific RNA packaging.

Examination of the current data available in literature on the ability of different retroviruses to cross-package their genomes into heterologous nucleocapsid proteins revealed that retroviruses are actually quite promiscuous (Table 1 and further reviewed in Al Dhaheri et al., 2009). The lentiviruses (HIV, SIV, and FIV) seemed to be the most promiscuous since they have been observed not only to cross-package their genomes, but also to successfully reverse transcribe and integrate using heterologous proteins (Table 1). On the other hand, the SNV genome seemed the least promiscuous, revealing that it cannot be cross-packaged by either HIV or MLV NC proteins. Interestingly, unlike HIV or SIV RNAs, FIV RNA cannot be cross-packaged by MPMV proteins. These observations suggest that the inability of SNV RNA to be cross-packaged by either HIV-1 or MLV proteins can be explained by restrictions perhaps not at the RNA end, but at some other level, such as the NC and matrix proteins as has been suggested earlier for some retroviruses (Zhang and Barklis, 1995; Poon et al., 1998). Thus, the combined data predicts that the MLV NC may be highly restrictive in their ability to capture RNAs from other retroviral species, including SNV RNA that has a very similar PBS structure as that of MLV (data not shown). This may also hold true for the inability of FIV RNA to be cross-packaged by MPMV proteins as well as for other retroviruses.

Together, the results presented in this report reveal that SIV and HIV-1 can be cross-packaged reciprocally by MPMV proteins; however such pseudotyping is limited only to the RNA packaging event since the cross-packaged RNAs were not propagated, indicating a block in post-packaging events of the retroviral life cycle. This study reinforces the observation that retroviral ψ are highly promiscuous in nature and therefore the presence of such sequences must be minimized on retroviral vectors to be used for human gene therapy in order to minimize RNA cross packaging among retroviruses especially with human endogenous retroviruses. The data presented here further proposes the possibility that SIV and HIV-1 RNAs can also co-package with MPMV RNA, which could provide a substrate for genetic recombination that might lead to the generation of replication competent viral variants with unknown pathogenic potential. Experiments to determine co-packaging and exchange of genetic information between MPMV and lentiviruses such as SIV and HIV-1 are currently under way.

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