Sequences within Both the 5’ UTR and Gag Are Required for Optimal In Vivo Packaging and Propagation of Mouse Mammary Tumor Virus (MMTV) Genomic RNA

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Abstract

Background: This study mapped regions of genomic RNA (gRNA) important for packaging and propagation of mouse mammary tumor virus (MMTV). MMTV is a type B betaretrovirus which preassembles intracellularly, a phenomenon distinct from retroviruses that assemble the progeny virion at cell surface just before budding such as the type C human and feline immunodeficiency viruses (HIV and FIV). Studies of FIV and Mason-Pfizer monkey virus (MPMV), a type D betaretrovirus with similar intracellular virion assembly processes as MMTV, have shown that the 5’ untranslated region (5’ UTR) and 5’ end of gag constitute important packaging determinants for gRNA.

Methodology: Three series of MMTV transfer vectors containing incremental amounts of gag or 5’ UTR sequences, or incremental amounts of 5’ UTR in the presence of 400 nucleotides (nt) of gag were constructed to delineate the extent of 5’ sequences that may be involved in MMTV gRNA packaging. Real time PCR measured the packaging efficiency of these vector RNAs into MMTV particles generated by co-transfection of MMTV Gag/Pol, vesicular stomatitis virus envelope glycoprotein (VSV-G Env), and individual transfer vectors into human 293T cells. Transfer vector RNA propagation was monitored by measuring transduction of target HeLaT4 cells following infection with viral particles containing a hygromycin resistance gene expression cassette on the packaged RNA.

Principal Findings: MMTV requires the entire 5’ UTR and a minimum of ~120 nucleotide (nt) at the 5’ end of gag for not only efficient gRNA packaging but also propagation of MMTV-based transfer vector RNAs. Vector RNAs without the entire 5’ UTR were defective for both efficient packaging and propagation into target cells.

Conclusions/Significance: These results reveal that the 5’ end of MMTV genome is critical for both gRNA packaging and propagation, unlike the recently delineated FIV and MPMV packaging determinants that have been shown to be of bipartite nature.

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Introduction

The flanking long terminal repeats (LTRs) of retroviruses serve as important control regions that are responsible for regulating many aspects of retroviral replication, from gene expression (promoters, enhancers, negative regulatory elements, hormone-inducible elements, and polyadenylation sites) to reverse transcription (strand switching using “Repeat (R)” regions), and integration (attachment-att sites) [1]. In addition, for at least some retroviruses, it has been shown that the R-U5/U3-R regions at the 5’ and 3’ ends of the genomic RNA (gRNA) and their adjoining regions play important roles in RNA packaging (packaging signals, π), dimerization (dimerization initiation site-DIS), and gRNA stability and transport (such as the constitutive transport element-CTE) [1–8]. Successful and specific gRNA packaging/encapsidation determines the fidelity of viral genome incorporation into the progeny virions. Studies over the past two decades have revealed the importance of the 5’ untranslated region (UTR) and beginning of the gag gene as crucial regions for augmenting retroviral gRNA encapsidation into the assembling particle (reviewed in [4–8]). For some retroviruses such as the human and simian immunodeficiency viruses (HIV and SIV), the packaging signal comprises of contiguous sequences within this region [9–20], while for others, the packaging signal seems to have a bi-partite nature, as has been
observed for feline immunodeficiency virus (FIV) and Mason-Pfizer monkey virus (MPMV) [20–24].

Recently, there has been an increasing interest in studying mouse mammary virus (MMTV) replication with the hope of developing MMTV-based vectors for human gene therapy [25]. This is because of the presence of hormone inducible promoters, making it an excellent candidate for tissue-specific and hormone-inducible gene therapy (reviewed in [25,26]). Being a non-primate retrovirus, MMTV-based vectors are likely to obviate potential safety concerns (that might result from the use of primate retroviral vectors) such as cross- and co-packaging of the transfer vector RNA by related primate retroviruses as has been observed between many retroviruses ([27–30] and references therein). However, the recent observation of the cross-packaging abilities of MMTV with a non-human primate retrovirus, MPMV [26], highlights the need to further enhance our understanding of the primary and secondary gRNA packaging determinants among retroviruses to establish their key contributing nature during gRNA packaging and/or cross-packaging processes.

Very little is known about the packaging determinants of MMTV that allow the virus to specifically incorporate its gRNA into the virus particle from a milieu of cellular and spliced RNAs. However, early MMTV studies showed that MMTV genome containing deletion in the envelope gene was competent for gRNA packaging [31], while MMTV vectors in which the 5’ MMTV LTR was swapped by that from Rous sarcoma virus (RSV) LTR [32] were defective for packaging, suggesting the importance of the 5’ region of the MMTV genomic RNA during the packaging process. Recently, we have shown that MMTV gRNA sequences from the first nucleotide (nt) in R up to 400 nts into gag are sufficient to allow efficient MTMV gRNA packaging and propagation, revealing the general significance of this region (R-U5-UTR-GAG) to the MMTV gRNA packaging process [33].

In order to further map the minimum viral sequences important for MMTV gRNA packaging and transfer vector RNA propagation, a systematic deletion approach was employed to delineate the extent of 5’ UTR and gag sequences important in these processes. Using several series of MMTV sub-genomic vectors in a biologically relevant in vivo packaging and transduction assay, our results reveal that unlike retroviruses such as FIV [20,21,23] and MPMV [22,24], but similar to HIV and SIV [9–19], the entire 5’ MMTV UTR is critical for both gRNA packaging and propagation. However, the 5’ UTR by itself is not sufficient and requires additional sequences, at least 120 nt of gag, for efficient gRNA packaging and propagation of the packaged transfer vector RNA into the infected cells.

Results

Experimental Approach and in vivo Packaging and Propagation Assay

To study MMTV RNA packaging, the newly developed in vivo RNA packaging and propagation assay for MMTV was employed that utilizes an MMTV gag/pol expression construct (JA10) for the production of viral structural and enzymatic proteins [33], a heterologous vesicular stomatitis virus glycoprotein (VSV-G) expression vector, MD.G, for the infection of target cells with pseudotyped MMTV particles [34], and MMTV sub-genomic transfer vectors serving as substrates for RNA packaging [33]. A commercially available luciferase expression plasmid, pGL3, was added to all the transfections to monitor and normalize for transfection efficiency as described previously [23]. Seventy-two hours post transfection, culture supernatants containing infectious pseudotyped virions carrying the transfer vector RNAs were harvested and a portion was used to infect human HeLaT4 cells to determine their propagation efficiency or pelleted via ultracentrifugation on a sucrose cushion for isolating packaged transfer vector RNA. If the assembling VSV-G-pseudotyped MMTV particles were able to package the transfer vector RNAs with the different deletions, successful transduction events could be scored by the appearance of hygromycin resistant colonies 10–12 days post infection upon hygromycin selection. To calculate the relative packaging efficiency for each transfer vector RNA, the amount of gRNA present in the pelleted virions was quantified by reverse transcriptase (RT) and real time PCR using β -actin as an endogenous control. Finally, the values were normalized with the luciferase expression to control for transfection efficiency.

MMTV gRNA Packaging and Propagation Studies Using Subgenomic Transfer Vectors Containing Wild Type LTRs

Briefly, MMTV-based retroviral vectors were created that contain the wild type 5’ MMTV LTR, a hygromycin resistance gene driven from the simian virus 40 (SV40) promoter as a marker for successful propagation, a truncated MMTV env gene, and the 5’ MMTV LTR (Figure 1A). Different permutations of the 5’ UTR and gag sequences were introduced into the MMTV sub-genomic vector (Figure 1B) and tested using the in vivo packaging and propagation assay. The first series of vectors tested the entire 160 nt of the 5’ UTR in the presence of incremental amounts of gag up to the first 400 nt of gag (Figure 1B). The second series of vectors tested incremental amounts of 5’ UTR in the absence of any gag sequences, while the third series tested the same incremental 5’ UTR sequences in the presence of 400 nt of the gag (Figure 1B).

Initial test of the wild type LTR-based MMTV vectors revealed very low transfer vector RNA propagation efficiency as measured by the transduction of the target cells with hygromycin resistance gene (Figure 1C). This translated to viral titers of ~1×10^1–2.5×10^5 colony forming units/ml (CFU/ml) even with the vector DA07 containing the entire 5’ UTR and 400 nt of gag (Figure 1B). In fact, none of the transfer vector RNAs containing incremental amounts of the 5’ UTR showed any ability to be propagated (>1 CFU/ml) except NS06 with ~40 ± 20 CFU/ml (Figure 1C).

Next, the packaging ability of the DA vector series (containing the entire 5’ UTR along with incremental amounts of gag) that could be propagated, although inefficiently, was tested. Reverse transcriptase (RT) PCR of the DA series of vector RNAs packaged by the virus particles revealed that the vector RNAs could be packaged, but quite inefficiently with some of the samples requiring Southern blotting for visualization of the packaged RNA (Figure 1D). Southern blotting of the resultant PCR products revealed that at least 120 nt of gag were required to observe efficient RNA packaging (Figure 1D), which corroborated with the RNA propagation data for these vectors (Figure 1C). The inefficient RNA packaging and propagation abilities could be attributed to the low expression of the transfer vector RNAs perhaps due to the use of wild type 5’ MMTV LTR promoter that was used for their transcription in human cells. The MMTV LTR is a well-known hormone inducible promoter that has low basal promoter activity and requires induction with glucocorticoid hormones such as dexamethasone [35,36]. However, the gRNA packaging and propagation results obtained were following induction of the transfected cultures with 10^{-4} M dexamethasone (Figure 1C and 1D), suggesting an inherent MMTV promoter caveat while using human cells.

Besides possible promoter restrictions, the poor gRNA packaging and propagation results obtained could have been due to the inefficient export of the transfer vector RNAs out of the nucleus in
Figure 1. Experimental design to determine the role of the 5' untranslated region (UTR) and gag sequences in MMTV RNA packaging. (A) Schematic representations of the complete MMTV genome and the MMTV long terminal repeats (LTRs)-based transfer vectors used as RNA packaging substrates in the study. In these transfer vectors, the wild type (WT) MMTV LTRs were maintained and therefore following transfection the RNA transcription was initiated by the promoter sequences within the U3 region of the 5' LTR. Region encompassing most of the structural and enzymatic genes (gag, pro, pol, part of env and rem) were replaced by the SV-40 hygromycin resistance gene cassette as a marker for successful transduction of the target cells by packaged transfer vector RNA. (B) Three series of deletion mutations at the 5' end of MMTV transfer vector.
vector sequences were introduced to monitor their effect on RNA packaging and propagation. The first series of deletion mutants (DA07–12) contained the entire 5' UTR in the presence of incremental amounts of gag sequences, the second series of mutants (FA07–12) contained deletions in the 5' untranslated region (UTR) sequences in the absence of any gag sequences, while the third series of mutants (NS01–06) contained the same incremental amounts of 5' UTR sequences in the presence of 400 nucleotides (nt) of gag. (C) Table showing viral titers observed post transduction of HeLaT4 target cells by transfer vector RNAs tested. None of the transfer vector RNAs containing deletions in the 5' UTR in the absence (FA07–FA12) or the presence (NS01–NS05) of gag sequences could be propagated (<1 CFU/ml) except NS06. *JA10, MMTV packaging Gag/Pol expression construct; MD.G, vesicular stomatitis virus (VSV-G) Env expression plasmid; pGL3, luciferase expression plasmid. **Propagation of the transfer vector RNA expressed as hygromycin resistance colony forming units (CFU)/ml of viral supernatant that was used to infect target cells. ***The entire UTR refers to 160 nt excluding 17 nt of primer binding site (PBS). The data represents mean of at least three independent transfection and infection experiments testing all mutants and was derived after normalization to the transfection efficiencies observed by luciferase expression from a co-transfected luciferase expression vector. SD, standard deviation. (D) Reverse transcriptase (RT) PCR analysis of the MMTV LTR-based DA series of transfer vectors containing incremental amounts of gag sequences in the presence of the entire 5' UTR followed by Southern blotting. The probe was prepared by PCR amplification of a 142 nt long R/UR/5' UTR region (nt 1179–1321) common to all the transfer vectors, as described in Materials and Methods. Amplification was carried out for either 25 (lower panel) or 30 cycles (upper panel) using transfer vector-specific primers OTR671 and OTR 672. doi:10.1371/journal.pone.0047088.g001

the absence of an intact Rem/Rem Responsive Element (RmRE) regulatory export pathway. This pathway has recently been described in MMTV [37,38], and was perturbed during cloning of these subgenomic vectors. Specifically, an intact RmRE is maintained; however, the Rem protein that binds to RmRE (facilitating the efficient export of unspliced MMTV gRNA) is not expressed in these transfer vectors due to a truncation in the Rem open reading frame during the cloning process (Figure 1A). Therefore, it is possible that the poor gRNA packaging and propagation results obtained could also have been in part due to the inefficient export of the transfer vector RNAs out of the nucleus in the absence of an intact Rem/RmRE regulatory export pathway.

Improvement of Transfer Vector Design

To circumvent any potential problems with poor MMTV promoter efficiency in human cells and RNA export defects, the 5' MMTV LTR was modified by replacing the U3 region (containing the promoter) with the constitutively active human cytomegalovirus (hCMV) promoter that has been classically used as a high efficiency promoter in mammalian cells (Figure 2A). A similarly constructed chimeric CMV/RU5 promoter has been successfully used to express MMTV [33,39] and FIV-based systems for HIV [43], SIV [44] FIV [41], and MMTV [33].

Role of MMTV 5' UTR and gag Sequences in MMTV gRNA Packaging and Propagation

The modified transfer vectors (Figure 2B) were co-transfected individually along with the packaging construct, JA10 and the env expression vector MD.G for virus production into 293T cells. Following transfection, virus particles were isolated from each transfected culture to quantify the effect of the introduced mutations on both gRNA packaging and propagation. However, before quantifying the viral RNA contents in the viral particles, it was imperative to determine whether each vector RNA was expressed stably and transported to the cytoplasm successfully.

Stable cytoplasmic expression of all transfer vector RNAs. In order to ascertain stable expression and efficient nuclear export of transfer vector RNAs, nuclear and cytoplasmic RNA fractions were prepared from the transfected cells and DNase-treated to ensure removal of any contaminating plasmid DNA (Figure 2C, panel III). This was followed by their conversion into cDNA by reverse transcriptase to allow amplification of the original transfer vector RNAs. Next the integrity of the RNA fractionation technique was tested to ensure that there was no leakage of the nuclear fraction into the cytoplasmic fraction by taking advantage of differential distribution of the unspliced and spliced β-actin mRNA message in the nuclear and cytoplasmic fractions, respectively [45] as described previously [23]. Attempts to amplify unspliced β-actin mRNA in a multiplex RT-PCR did not show its detectable amplification in the cytoplasmic fractionation (Figure 2G, panel I), in contrast to the presence of spliced β-actin and 18S rRNA mRNA in the cytoplasm, as expected (Figure 2G, panels I and II), further authenticating the amplificability of our cDNA preparations.

Development of a relative quantification assay using real time PCR. The relative expression of the various transfer vector RNAs in the cytoplasm was tested using a custom-made MMTV Taqman real time PCR assay in combination with a commercially-available-endogenous β-actin Taqman assay (Applied Biosystems). The custom MMTV Taqman assay was designed within the U5 region of the transfer vector RNAs, enabling detection of all mutant transfer vector RNAs via a single primer/probe combination. To allow a relative comparison of the transfer vector RNA expression using β-actin as an endogenous control, we determined whether the amplification efficiencies of the two assays were equivalent. This was accomplished by first determining the value of the threshold cycle, Ct (Figure 3A and 3B), followed by ΔCt values (ΔCt values of the MMTV assay – Ct value of the β-actin endogenous control) (Figure 3C and 3D). Finally, we analyzed how the ΔCt values varied with the amount of input template cDNA as a sensitive indicator of relative amplification efficiencies of the two assays. The value of the slope of log input amount verses ΔCt should be approximately zero (<0.1) for the two assays to have similar amplification efficiencies (User Bulletin #2, ABI PRISM 7700 Sequence Detection System). As can be observed from Figure 3E, the slope under our experimental conditions was calculated to be 0.0126, validating the assay for the relative quantification analysis.

Test of the cDNAs prepared from the cytoplasmic RNA fractions revealed that the transfer vector RNAs were stably expressed between Ct values of 23.5–27.5 and efficiently exported to the cytoplasm (Figure 3A). Alongside vector RNA expression in the cytoplasm, the expression of the endogenous β-actin mRNA was also monitored as a control for the amount of the input sample tested in the assay (Figure 4B). As observed, all samples expressed β-actin mRNA at very similar levels, within one Ct value of each other (Figure 4B). Normalization of the transfer vector RNA expression with the level of endogenous β-actin and exogenous luciferase expression revealed that all transfer vector RNAs were expressed in the cytoplasm within approximately 2–3 folds of each other (Figure 4C). These ancillary controls were incorporated into our experimental setup to confirm the integrity of the RNA
Figure 2. Improvement in the design of MMTV LTR-based transfer vectors and their test in the in vivo packaging assay. (A) Schematic representation of the second set of MMTV transfer vectors in which the U3 region containing the promoter within the 5' LTR was replaced with the promoter sequences of human cytomegalovirus (hCMV) generating a chimeric CMV-R/U5 LTR. In addition, the constitutive transport element (CTE) from the Mason-Pfizer monkey virus (MPMV) was inserted between the Δenv and the 3' LTR to facilitate efficient nuclear export of viral genomic RNA in the absence of an intact MMTV Rem/RmRE export pathway. (B) Schematic representation of the three series of transfer vectors tested in the study containing chimeric 5' MMTV LTR and therefore following transfection the RNA transcription was initiated by the hCMV promoter sequences. The first series of transfer vectors (DA19–24) tested the entire 5' UTR in the presence of incremental amounts of gag sequences, the second series (FA21–26) tested only incremental amounts of 5' UTR sequences in the absence of any gag sequences, while the third series (NS07–12) tested the same incremental amounts of 5' UTR sequences in the presence of 400 nt of gag. (C) RT-PCR of cytoplasmic RNA fractions to ensure the integrity of the fractionation technique and conventional PCR on RNA samples to monitor the absence of any contaminating DNA in the RNA preparations. Panel I: Multiplex PCR amplification of cytoplasmic cDNAs with 18S and unspliced β-actin primers; Panel II: PCR amplification of cytoplasmic cDNAs with spliced β-actin primers; Panel III: PCR amplification of DNase-treated RNA with GAPDH primers. Lane numbers 1–18 correspond to the numbers of the three series of chimeric LTR transfer vectors (DA, FA, and NS) in sequential order, whereas lanes 19 and 20 correspond to cytoplasmic fraction from Mock (containing the packaging construct, JA10 + the VSV-G-Env expression plasmid, MD.G + luciferase-expression vector, pGL3) and no DNA transfected cultures. +C, positive control (cDNA from cellular mRNA); M, molecular weight markers.

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Figure 3. Validation of the real time PCR assay developed for relative quantification of transfer vector RNA expression using the Ct slope method. Determination of the threshold cycles (Ct) of the cDNA prepared from 293T cytoplasmic RNA expressing transfer vector DA24 tested in triplicates as 10- and 2-fold dilutions by the (A) custom-made MMTV Taqman assay, and (B) the β-actin Taqman assay. ΔRn is the target gene-specific fluorescence signal (FAM for MMTV-specific and VIC for β-actin-specific sequences) normalized to the signal for the internal passive control, ROX (Normalized Reporter or Rn) from which the baseline target fluorescence has been subtracted (ΔRn = Normalized Reporter (Rn) - baseline). Standard curves of the (C) MMTV and (D) β-actin Taqman assays were generated to determine their ΔCt values (Ct values of the MMTV assay – Ct value of the β-actin endogenous control) that were needed to allow comparison of amplification efficiencies of the two assays. (E) Relative amplification efficiencies of the two assays as determined by the analysis of ΔCt value variations with the amount of input template cDNAs. For the two assays to have similar amplification efficiencies, the value of the slope of log input amount versus ΔCt should be approximately zero (0.1), which under our experimental conditions were calculated to be 0.0126, thus validating the assay for the relative quantification analysis.

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Figure 4. Relative expression of transfer vector RNAs within the transfected cells. Real time PCR analysis of (A) cytoplasmic transfer vector RNAs and (B) cytoplasmic β-actin RNA expression expressed as log2Rn verses cycle number. ΔRn is the target gene-specific fluorescence signal (FAM for MMTV-specific and VIC for β-actin-specific sequences) normalized to the signal for the internal passive control, ROX (Normalized Reporter or Rn) from which the baseline target fluorescence has been subtracted (ΔRn = Normalized Reporter (Rn) - baseline). (C) Relative cytoplasmic transfer vector RNA expression in 293T cells after normalization with β-actin and luciferase expression. MK, Mock, transfected cultures with packaging construct, JA10 + the VSV-G-Env expression plasmid, MD.G + luciferase-expression vector, pGL3 except the transfer vector. RQ, Relative Quantification in log10 units. The primers for detecting the transfer vectors were designed within the U5 region of the MMTV LTR, a region common to all transfer vector RNAs. Each sample was tested in duplicates with MMTV- and β-actin-specific probes and primers as described in Materials and Methods.
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packaging and propagation assay and to further validate that the results obtained were bonafide.

The Entire MMTV 5’ UTR and ~ 120 nt of gag are Needed for Optimal MMTV gRNA Packaging

Next, the packaging efficiency of the transfer vector RNAs into the pseudotyped MMTV particles was assessed by the same custom-made real time PCR assay as described above. Towards this end, the first question asked was whether a cellular housekeeping β-actin mRNA could be used as an endogenous control in our assays since it is not a recognized virion-associated mRNA. It has now been well-established that cellular RNAs can be packaged into retroviral particles as has been shown for several RNAs such as 7SL RNA, U6 snRNA, tRNA, or mRNAs for cyclophilin A and for several types of ribosomal proteins, and even ribosomal RNA itself [18,46–51].

Therefore, we tested whether β-actin mRNA could be packaged into the MMTV particles and at similar levels irrespective of the transfer vector packaging efficiency (Figure 5A). As observed, β-actin mRNA could indeed be detected quite consistently inside the virus particles within 2 Ct values of each other, starting from 33.5 Ct-35.5 Ct, irrespective of whether transfer vector RNAs were packaged efficiently or poorly within the virus particles (Figure 5B). This observation is consistent with the finding of Ruli and colleagues who observed that a majority of the cellular RNAs packaged in the MLV and HIV-1 virus particles were packaged non-selectively and merely represented the proportions in which they were found in the cytoplasm of the virus-producing cells [51]. Thus, this enabled the use of β-actin mRNA as an endogenous control while calculating relative packaging efficiency of the transfer vector RNAs in our custom-made real time PCR assay.

As opposed to β-actin mRNA, all transfer vector RNAs were encapsidated with widely different packaging efficiencies, encompassing raw Ct values between 23–36, depending upon the amount and type of sequence present at the 5’ end of the sub-genomic RNAs (see boxed region in Figure 5B). The DA series of vectors (DA19–DA24) were packaged between Ct values of 25 to 28, the FA series of vectors (FA21–FA26) were packaged between Ct values of 30–34, while the NS series of vectors (NS07–NS12) were packaged between Ct values of 32–34 with the exception of NS12 that was packaged at a Ct value of 24 (Figure 5B). The mock-transfected samples obtained Ct values of 32–34 with the exception of NS12 that was packaged at a greater than 10-fold (1 log) drop in RNA packaging efficiency (by about 0.7 logs compared to DA24; p<0.01) and was similar to DA22 and DA23, vectors with 120 and 150 nt of gag sequences, respectively (Figures 2B and 5C). The NS series vector was cloned in a different manner and contains an Sp6 site that was introduced between the 5’ UTR and beginning of gag sequences for the cloning process that is absent in the DA series. It is possible that presence of the Sp6 site may have perturbed the higher order structure, if any, of this important region in some subtle manner, resulting in lower packaging efficiencies. The same may hold true for DA07 and NS06, although we did not directly test the packaging efficiency of NS06 vector RNA (Figure 1). Taken together, these results suggest that the entire 5’ UTR and approximately 120 nt of gag seem to be minimally required for efficient packaging of MMTV transfer vector RNAs.

The 5’ MMTV UTR is Critical but not Sufficient for Efficient MMTV RNA Packaging

Analysis of the FA series of vectors (FA21–FA26 containing incremental amounts of 5’ UTR sequences in the absence of any gag sequences, Figure 2B) revealed that the entire 5’ UTR is not sufficient for efficient RNA packaging since the RNA of FA26 containing the entire 5’ UTR but no gag sequences could be barely packaged within the viral particles (Figure 5C). However, the 5’ UTR was observed to be important for RNA encapsidation since even a 32 nt deletion at the 3’ end of the 5’ UTR in the presence of 400 nt of gag (NS11) resulted in greater than 100-fold (~2 log) drop in RNA packaging efficiency compared to the vector RNA with the entire 5’ UTR, NS12 (Figure 5C). Similarly, the vector with the entire 5’ UTR in the absence of gag (FA26) was packaged at a greater than 10-fold (1 log) lower efficiency than NS12 and greater than 60-fold (~1.8 log) lower efficiency than DA24, vectors containing the entire 5’ UTR in the presence of 400 nt of gag (p<0.01), confirming that gag sequences are indeed required during MMTV gRNA packaging (Figure 5C). As observed with MMTV vectors with the wild type LTRs, ~120 nt of gag were required for improving the RNA packaging efficiency in the presence of the entire 5’ UTR by 10-fold, as opposed to ~30–90 nt of gag which did not enhance packaging efficiency significantly (compare FA26 with DA19–DA22 in Figure 5C and DA11 with DA08–DA10 in Figure 1D).

The 5’ MMTV UTR is Critical but not Sufficient for MMTV Transfer Vector RNA Propagation and Requires at least 120 nt of gag as well

Test of the new series of transfer vectors in the propagation assay further revealed that the LTR promoter and CTE
Figure 5. Relative packaging efficiency of MMTV transfer vector RNAs into the pseudotyped MMTV particles. Real time PCR analysis of (A) packaged β-actin RNA into VSV-G-Env-pseudotyped MMTV particles and (B) packaged transfer vector RNAs into the VSV-G-Env-pseudotyped MMTV particles expressed as ΔRn verses cycle number. ΔRn is the target gene-specific fluorescence signal (FAM for MMTV-specific and VIC for β-actin-specific sequences) normalized to the signal for the internal passive control, ROX (Normalized Reporter or Rn) from which the baseline target fluorescence has been subtracted (ΔRn = Normalized Reporter (Rn) - baseline). (C) Relative RNA packaging efficiencies for each of the mutant transfer vector RNA after normalization with β-actin and luciferase expression. The box in panel B highlights the wide range of threshold cycle (Ct) values observed for each transfer vector in comparison to the similar amounts of β-actin packaged into the viral particles. The arrow in panel C highlights the threshold value of detection. MK, Mock, cells transfected with packaging construct, JA10 + the VSV-G-Env expression plasmid, MD.G + luciferase-expression vector, pGL3 except the transfer vector. RQ, Relative Quantification in log_{10} units. *, statistically significant differences between constructs are shown by the brackets (p<0.01). #, statistically significant difference between NS12 and DA24 (p<0.01). The primers/probe for detecting the transfer vectors were designed within the U5 region of the MMTV LTR, a region common to all transfer vector RNAs. Each sample was tested in triplicates with MMTV- and β-actin-specific probes and primers in panel B, while the β-actin samples shown in panel A were tested in duplicates, as described in Materials and Methods.

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modifications resulted in several folds increase in the pseudotyped virion titers as demonstrated by an increased appearance of hygromycin resistant colonies in the infected cultures (compare Table 1 with Figure 1C). However, despite the overall increase in vector RNA propagation (DA series of vectors), most of the vectors with incremental amounts of the 5′ UTR sequences with or without gag (the FA and NS series, Figure 1B) could not be successfully propagated except for NS12 (Table 1). This is a very unusual finding compared to what has been observed in similar vector systems of MPMV and FIV employing a similar deletion approach [20–23]. The DA-series of vectors that could be propagated successfully revealed that whereas 120 nt of gag were sufficient for vector RNA propagation, 400 nt increased that efficiency by two-folds (Table 1). These observations revealed that, 1) the 5′ end of MMTV RNA [R-U5-UTR-gag] contains elements critical for proper completion of steps in vector RNA propagation, such as RNA dimerization, encapsidation, reverse transcription, and integration since our readout assay for RNA propagation was dependent on the successful completion of these crucial steps in virus life cycle, and 2) the entire 5′ UTR itself is not sufficient and requires ~120–400 nt of gag for efficient vector RNA propagation. Overall, these results mimicked the RNA packaging and propagation results obtained with the wild type MMTV LTR vectors and further validated that the intact 5′ UTR is as critical for efficient RNA propagation as it is for successful RNA packaging (Figures 1, 5 and Table 1), but is not sufficient by itself for either of the two processes.

Discussion

It is a well-established fact now that the major determinants of retroviral gRNA packaging lie at the 5′ end of the retroviral genome downstream of R and extending into the gag gene (reviewed in [4–8]); however, specific residues within this region and their spatial organization may vary among different retroviruses. We have used a similar deletion analysis of the 5′ UTR and gag in MPMV [22,24] and FIV [20–23] to determine the precise boundaries of their packaging determinants. Our results have revealed that both MPMV, a simple retrovirus, and FIV, a complex lentivirus, have bipartite packaging determinants composed of regions at the flanks of the 5′ UTR and gag sequences tested (Figure 6). These packaging determinants have been shown to assume higher order structures important for RNA packaging and dimerization [24,42,52,53]. This is contrary to what we report here for MMTV where the entire MMTV 5′ UTR was found to be critical for both RNA packaging and propagation. However, our observations with MMTV are similar to what has been observed for HIV-1 and SIV [9–19], where most of the 5′ UTR has been shown to be important for packaging of the respective viruses (Figure 6).

The importance of the entire 5′ UTR in retroviral RNA packaging is not surprising and may reflect the presence of cis acting sequences/structural motifs important in other steps in viral life cycle as well such as genomic RNA dimerization, reverse transcription, transcriptional activation, nucleocytoplasmic transport of unspliced RNA, and efficiency of RNA translation [1–8,54,55]. The presence of so many cis/structural features with critical functions in a small region may make it difficult to identify a unique or minimal packaging determinant. For example, it has been shown that RNA dimerization and packaging are interlinked phenomenon with one dependent upon the other depending upon the different retrovirus [4–8,56–59]. Thus, perturbation of one may affect the other phenomena by default. For instance, the FIV DIS is present within its gag gene, a retrovirus in which a bipartite packaging signal has been observed [42,52,53], and for this reason parts of the 5′ UTR have been shown to be dispensable for FIV RNA packaging [23]. On the other hand, the HIV-1 DIS is present within the 5′ UTR [60], suggesting the necessity of the presence of the entire UTR for optimal packaging as has recently been shown by Summers and colleagues [19].

Prior to dimerization and packaging, gRNA must be efficiently exported to the cytoplasm and therefore it has been suggested that retroviral gRNA nuclear export, dimerization and packaging mechanisms are also interlinked [6,61–63]. Consistent with this, packaging signal of some retroviruses have been shown to play an important role in the nuclear export of viral gRNA [64,65]. For example, an internal loop in the packaging signal of HIV-1 closely resembles HIV Rev Responsive Element (RRE) and binds to Rev protein [61,66]. Mutations in this loop have been shown to reduce nuclear export of viral gRNA and packaging [62]. Similar to HIV-1 Rev/RRE, MMTV also contains a nuclear export pathway (Rem/Rem-Responsive Element (RmRE), facilitating the transport of gRNA, but not partially spliced env RNA [67–69]. Furthermore, MMTV RmRE can also assume a higher order structure with multiple loops where its cognate counterpart, Rem, can bind and multimerize to mediate RNA export [37,38,68,69]. Based on their mutual similarity, it has been hypothesized that MMTV may also contain two RmREs; one at the 5′ end (encompassing packaging sequences of MMTV gRNA) present only in unspliced RNA and the second one at the 3′ end present in all MMTV RNAs, facilitating nuclear export of the gRNA and translation of all mRNAs, respectively [69]. The recent study by Hohenadl et al., looking at the effects of 5′ UTR deletions on MMTV env mRNA expression, supports the existence of such an element at the 3′ half of the MMTV 5′ UTR [39]. Deletion of this region in our assay severely impacted RNA packaging (Figures 5C); however, we cannot assess its effect on mRNA transport since all our vector RNAs contained CTE which could have compensated for such transport defects, leading to the efficient export of these vector RNAs to the cytoplasm (Figure 4C). Experiments are currently underway to delineate the overlapping nature of MMTV packaging sequences with those of the recently hypothesized additional nucleocytoplasmic export regulatory element, RmRE.

The present study reveals that the MMTV region starting from R at the 5′ end up to ~120 bp in the gag gene is important for genomic RNA packaging. One of the earliest studies on MMTV RNA packaging tested the role of the 5′ LTR by substituting the 5′ MMTV LTR with that of the Rous sarcoma virus (RSV) LTR [32]. Genomic RNAs encoded by this recombinant virus were defective for RNA packaging despite the fact that they contained the entire 5′ UTR and the remaining viral genome. It is plausible that the impaired packaging observed could have been due to: 1) the role of R/U5 sequences, if any, in packaging in addition to 5′ UTR and gag sequences, 2) the insensitivity of the assays used to study RNA packaging. In their assay, RNA from a 100 ml of a wild type virus stock containing 2×10^9 virions/ml (2×10^9 virus particles total) was required to observe packaging efficiently on a dot blot assay. In contrast, in our real-time based PCR assay, we could efficiently observe packaged retroviral subgenomic RNAs from vectors that gave rise to even 25 CFU/ml (Figure 5C and Table 1). Since we did not investigate the R/U5 sequences in our mutational analysis, we cannot rule out the role of MMTV R and U5 sequences independently on MMTV RNA packaging (all our constructs contained this region unaltered). It has been well-established that R/U5 can harbor retroviral RNA packaging determinants ([70] and references therein). However, given the observation that our vectors that contained even a 32 nt deletion at the 3′ end of the 5′ UTR (FA25 and NS11, Figures 2B and 5C)
were essentially defective for RNA packaging despite containing R/U5 suggests that the role of R/U5 on RNA packaging is not significant at the primary sequence level, though its independent effect as part of a larger secondary structural element, if any, cannot be ruled out.

Interestingly, our similar and systematic deletion analysis of the 5′ UTR and gag regions of three different retroviruses, FIV [20,21,23,42,52,53], MPMV [22,24], and MMTV (the present study), has revealed that not only gag sequences are required for packaging, but that the amount of gag sequences needed are quite similar (100–120 nt) (Figure 6). Considering that 100–120 nt are the structural significance of this region may also explain why the Spe I site may have destabilized some sequence/structural motifs important for MMTV transfer vector RNA packaging and propagation (compare DA24 and NS12 in Figure 5C).

Over the years, it is becoming increasingly clear that retroviral RNA packaging and dimerization, regardless of the primary sequence, are dependent on RNA structural motifs of the packaging determinants and their interaction(s) at the structural level (RNA-RNA interactions during dimerization and RNA-protein interactions during packaging) [71]. The structural significance of this region may also explain why the Spe I restriction site insertion between the end of 5′ UTR and gag AUG had resulted in lower packaging efficiency of the NS12 vector compared to the DA24 (Figure 5C).

| Vector Name | Description of Transfer Vectors Used in Each Transfection | Other Plasmids Added in Each Transfection* | Normalized** CFU/ml ± SD |
|-------------|----------------------------------------------------------|--------------------------------------------|--------------------------|
| Mock        | No transfer vector                                       | JA10 + MD.G + pGL3                         | <1                       |
| DA19        | Entire UTR*** + 30 bp of gag                              | JA10 + MD.G + pGL3                         | 25±6                     |
| DA20        | Entire UTR +60 bp of gag                                  | JA10 + MD.G + pGL3                         | 28±6                     |
| DA21        | Entire UTR +90 bp of gag                                  | JA10 + MD.G + pGL3                         | 133±14                   |
| DA22        | Entire UTR +120 bp of gag                                 | JA10 + MD.G + pGL3                         | 635±48                   |
| DA23        | Entire UTR +150 bp of gag                                 | JA10 + MD.G + pGL3                         | 667±83                   |
| DA24        | Entire UTR +400 bp of gag                                 | JA10 + MD.G + pGL3                         | 1575±207                 |
| FA21        | PBS only                                                 | JA10 + MD.G + pGL3                         | <1                       |
| FA22        | 32 bp of UTR without gag                                  | JA10 + MD.G + pGL3                         | <1                       |
| FA23        | 64 bp of UTR without gag                                  | JA10 + MD.G + pGL3                         | <1                       |
| FA24        | 96 bp of UTR without gag                                  | JA10 + MD.G + pGL3                         | <1                       |
| FA25        | 128 bp of UTR without gag                                 | JA10 + MD.G + pGL3                         | <1                       |
| FA26        | Entire UTR without gag                                    | JA10 + MD.G + pGL3                         | <1                       |
| NS07        | PBS only +400 bp gag                                      | JA10 + MD.G + pGL3                         | <1                       |
| NS08        | 32 bp of UTR +400 bp of gag                               | JA10 + MD.G + pGL3                         | <1                       |
| NS09        | 64 bp of UTR +400 bp of gag                               | JA10 + MD.G + pGL3                         | <1                       |
| NS10        | 96 bp of UTR +400 bp of gag                               | JA10 + MD.G + pGL3                         | <1                       |
| NS11        | 128 bp of UTR +400 bp of gag                              | JA10 + MD.G + pGL3                         | <1                       |
| NS12***     | Entire UTR +400 bp of gag                                 | JA10 + MD.G + pGL3                         | 403±82                   |

*JA10, MMTV gag/pol packaging expression vector; MD.G, vesicular stomatitis virus (VSV-G) envelope expression vector; pGL3, luciferase expression vector.

**Propagation of the transfer vector RNA expressed as hygromycin resistance colony forming units (CFU)/ml of viral supernatant that was used to infect target cells. The data represents the mean of at least three independent transfection and infection experiments testing all mutants and was derived after normalization to the transfection efficiencies observed by luciferase expression from a co-transfected luciferase expression vector. SD, standard deviation.

***Entire UTR refers to 160 bp excluding 17 bp of primer binding site (PBS).

****The differences observed in the RNA propagation abilities of DA24 and NS12, both containing same amounts of 5′ UTR and gag sequences could be attributed to an artificially introduced SpeI site in NS12 at the junction of 5′ UTR and gag during cloning. This SpeI site may have destabilized some sequence/structural motifs important for MMTV transfer vector RNA packaging and propagation (compare DA24 and NS12 in Figure 5C).

**Reference:**

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Table 1. Propagation efficiency of MMTV transfer vector RNAs containing chimeric 5′ LTRs.
motifs are essential during the MMTV RNA packaging process. Delineation of MMTV gRNA packaging determinants presented in this study should enhance our understanding of MMTV replication, which is imperative if MMTV vectors are to be used successfully for inducible and targeted human gene therapy.

**Materials and Methods**

**Plasmid Construction**

MMTV packaging construct, JA10, and the transfer vector, DA24, have been derived from HYBMTV, the molecular clone of MMTV [72] and their design and cloning strategies have been described earlier [33]. Briefly, JA10 expresses the MMTV gag/pol genes in the presence of the MPMV CTE using the hCMV promoter and the bovine growth hormone (BGH) poly A sequences. The transfer vectors were created as three series: the DA series with incremental deletions of gag sequences in the presence of the entire 5' UTR, the FA series with incremental deletions of the 5' UTR sequences in the absence of gag, and the NS series with incremental deletions of the 5' UTR sequences in the presence of 400 nt of gag (Figures 1B and 2B). Both the vector series with the wild-type MMTV LTR (Figure 1B) and the chimeric CMV/RU5 LTR (Figure 2B) were created through several stages of cloning using PCR amplification of the 5' end of the HYBMTV clone, from the first nucleotide in R to the various regions specified in the constructs using specific primers listed in Table 2. Initially, the three series of transfer vectors maintained the MMTV wild type LTR (Figure 1B); however, due to their low expression in human cells despite hormone induction, the U3 region of the LTR containing the MMTV promoter was replaced by the hCMV promoter via PCR-based cloning, as described earlier [33]. Specific details of the plasmid construction can be obtained from the authors upon request.

**Transfection and Infection of Cells**

The human epithelial kidney cell line, 293T, was used for the production of VSV-G-pseudotyped-MMTV virus particles containing RNA from the various constructs using a modified calcium phosphate method as described [73]. The transfections for each mutant transfer vector were carried out in 6-well plates with 1–2 plates per vector (depending upon the experiment) along with the control luciferase expression vector, pGL3 (Promega, USA), to monitor transfection efficiency as described earlier [23]. Transduction by the marker hygromycin resistance gene present on the transfer vector RNA was observed by infection of the human cervical cancer cell line (HeLaT4) by the pseudotyped virus particles and selection for hygromycin-resistant colonies, as described previously [41].

**Figure 6. Schematic representation of the packaging determinants observed empirically in different retroviral systems starting from nt +1 in R to the beginning of gag.** The figure compares the published data pertaining to the requirements of the 5' UTR and gag sequences for optimal RNA packaging in different retroviruses. The table provides further details of the RNA packaging determinants required as observed in various studies referenced in the last column.

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RNA Isolation, Nucleocytoplasmic Fractionation of Cells and RT PCR

Transfected cells were fractionated into nuclear and cytoplasmic fractions to allow separate analysis of RNA residing within the two cellular compartments as described earlier [23]. RNA from each fraction was isolated from transfected cells using the Trizol reagent, as per manufacturer’s directions (Invitrogen, USA). Integrity of the nucleocytoplasmic fractionation was monitored by the absence of the nuclear-only unspliced ß-actin mRNA in the cytoplasmic RNA fractions by reverse transcriptase PCR (RT-PCR). Briefly, following DNase treatment, equal RNA amounts (2.5 ug per sample for each fractions) were tested for DNA contamination by PCR amplification for 30 cycles using primers specific for a house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figure 2C, panel III). Lack of any amplifiable signal confirmed that the RNA preparations were not contaminated with DNA carried over from the transfected cultures. Next, the DNased RNA fractions were reverse transcribed and amplified using primers specific for unspliced and spliced ß-actin mRNA to confirm whether nuclear membrane integrity was maintained during fractionation or not. As an additional control, multiplex amplifications were performed in the presence of primers/competimer for 18S ribosomal RNA, ensuring that each sample in the unspliced ß-actin PCRs contained amplifiable cDNAs.

Southern Blot Analysis

Some of the packaged viral RNA samples were subjected to Southern blot analysis as described earlier [70] for which the probe was prepared by PCR amplification using primers within the 5’ R/U5/PBS region common to all the transfer vectors as follows: OTR671 (þ) GTC CTA ATA TTT CCG TCC AGC TCT GTG, nt 1179–1202 and OTR 672 (−) CTG TCC CGG CGC CAG CTT CCG CAG, nt 1298–1321. Hybridization was performed using the AlkPhos Direct Labeling Kit (Amersham, USA) as per manufacturer’s directions.

Real Time PCR Analysis of Transfer Vector Expression

To test the relative levels of transfer vector RNAs expressed within the cytoplasm and packaged into the viral particles, a real-time PCR method was employed. A series of primers specific for the MMTV LTR or the chimeric LTR containing CMV/R/U5 were designed to be used in multiplex PCR reactions. Table 2. provides a description of primers used for cloning of the DA, FA, and NS series of vectors, with either the MMTV LTR or the chimeric LTR containing CMV/R/U5.

![Table 2. Description of primers used for cloning of the DA, FA, and NS series of vectors, with either the MMTV LTR or the chimeric LTR containing CMV/R/U5.](image-url)

- **Oligo Name**
- **Genomic Location**
- **Size**
- **S/AS***
- **Sequence (5’ to 3’)**
- **Description**

| Oligo Name | Genomic Location | Size | S/AS* | Sequence (5’ to 3’) | Description |
|------------|------------------|------|-------|---------------------|-------------|
| OTR 551    | From the start of28 HYBMTV 5’ U3 | S    |       | GCATCGATATGCAGCGCTGACAGA | 2 extra bp/Cla/nt 1–20 of 5’ U3 of HYBMTV |
| OTR 552    | Initial 400 bp of 33 HYBMTV Gag | AS   |       | CGACTAGTATGATCGCTCCTAGAAG | 2 extra bp/EcoRV/nt 1885–1867 HYBMTV Gag |
| OTR 553    | HYBMTV Env 40     | S    |       | GCATCTGATGCTACATAGTGGTCCGAAAAGATTCTCC | 2 extra bp/NheI/nt 7485–7509 HYBMTV Env |
| OTR 554    | End of HYBMTV 29  | S    |       | CAGGGTACCCTGCGAAGCTCGGCGACC | 3 extra bp/KpnI/nt 9877–9857 HYBMTV 3’ U5 end |
| OTR 555    | HYBMTV 160 bp 28  | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1485–1468 of HYBMTV 5’ UTR |
| OTR 556    | HYBMTV 30 bp 28   | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1515–1495 of HYBMTV Gag |
| OTR 557    | HYBMTV 60 bp 28   | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1544–1525 of HYBMTV Gag |
| OTR 558    | HYBMTV 90 bp 28   | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1574–1555 of HYBMTV Gag |
| OTR 559    | HYBMTV 120 bp 29  | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1604–1585 of HYBMTV Gag |
| OTR 560    | HYBMTV 150 bp 29  | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1634–1615 of HYBMTV Gag |
| OTR 561    | HYBMTV PBS only   | 28   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1324–1305 of HYBMTV PBS |
| OTR 562    | HYBMTV 32 bp 28   | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1354–1337 of HYBMTV 5’ UTR |
| OTR 563    | HYBMTV 64 bp 28   | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1388–1369 of HYBMTV 5’ UTR |
| OTR 564    | HYBMTV 96 bp 28   | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1420–1401 of HYBMTV 5’ UTR |
| OTR 565    | HYBMTV 128 bp 28  | AS   |       | CGACTAGTCTCCGCTACGTGAC | 2 extra bp/SpeI/nt 1452–1433 of HYBMTV 5’ UTR |
| OTR 617    | Start of HYBMTV35 | S    |       | CGCAAGGCTTGGCTAGTGAATACCTGCCGAGTCC | 3 extra bp/HindIII site/nt 1163–1182 of HYBMTV R |

*S/AS, sense or antisense.
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time PCR custom expression assay was developed using the minor groove binding (MGB)/FAM and MGB/VIC chemistry from Applied Biosystems, USA. Additionally, the ABI real time PCR assays use ROX as an internal fluorescence reference dye to which the target reporter dye signal (either FAM or VIC, in our case) is normalized during data analysis, to correct for fluorescence fluctuations caused by changes in concentration or volume. The PCR primers were designed to anneal within a region at the beginning of the MMTV U5 region (nt 1192–1259), a 68 nt region common to all the constructs, enabling their relative quantitative assessment using the same probe/primer combination concomitantly. The sequence of the probe and primers were as follows: Probe (MMTV-ILTR-SITEF) FAM: TCCGATCCGTTCTCC (16-mer; nt 1214–1229); Forward primer (MMTV-ILTR-SITEF): CGTCTCGTGTGTTTGTGTTTGTGTCTGT (22-mer; nt 1192–1213); Reverse primer (MMTV-ILTR-SITEF): CCTCCTGGAATGTGAAAGGATAATGGA (25-mer; nt 1259–1235). The assay was validated in situ using the Primer Express tool as well as the ABI design pipeline bioinformatic tools that ensure specificity, reproducibility, and amplification efficiency of the assay performance. Further validation of the PCR amplification efficiency was conducted by testing the assay using the online web tool “pcrEfficiency” [74] that confirmed it to be over 100%. As an endogenous control, the optimized β-actin MGB VIC-labeled assay (no 4326315E with limited primer concentration) from ABI was used (Applied Biosystems, USA). To determine which method to use for the Relative Quantification analysis, the standard curve or the comparative (ΔΔCt) method, a 10- and serial 2-fold dilutions of a cDNA prepared from 293T cells expressing DA24 were tested in non-multiplex RT-PCRs in triplicates assuming conservatively that 1 ug of input RNA results in ~100 ng of single stranded cDNA using random hexamers as primers and MMLV RT (Promega; Figure 3A and 3B). The resultant Ct values were plotted on a scatter plot against the input cDNA to determine the slope of the curve (Figure 3E).

For the final relative quantification analysis, each viral RNA sample was tested in triplicates in multiple assays using the Taqman Universal Master Mix (Applied Biosystems and the 7500 Real Time PCR System (Applied Biosystems, USA), while the cytoplasmic RNA was tested in duplicates. As mentioned earlier for the cytoplasmic RNA fractions, 1/3 of the total viral RNA preparations were first DNase-treated, confirmed for the absence of DNA by PCR using vector RNA specific primers, and then reverse transcribed into cDNA, as previously described [73]. Equal amounts of the resulting cDNAs were tested for vector expression in the cytoplasmic fractions as well as their packaging in the viral particles using the real time PCR assay and the following cycling conditions: an initial denaturation step of 10 minutes at 94°C followed by 40 cycles of denaturation and annealing/extension steps at 95°C for 15 secs and 60°C for 1 min. The relative packaging efficiency for each mutant transfer vector RNA was determined after normalization of the data with the endogenous control, β-actin, as well as the control for transfection efficiency, luciferase expression.

Statistical Analysis
To determine whether the observed differences in the normalized packaging efficiencies were statistically significant, a standard, paired, two-tailed Student’s t test was performed between various constructs using the Microsoft Excel software. A value of p<0.01 was considered statistically significant.

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Author Contributions
Conceived and designed the experiments: FM TAR. Performed the experiments: FM DA NA SA PJ PSP. Analyzed the data: FM TAR PJ PSP. Contributed reagents/materials/analysis tools: FM TAR. Wrote the paper: FM.

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