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Original article

Both the 5′ and 3′ LTRs of FIV contain minor RNA encapsidation determinants compared to the two core packaging determinants within the 5′ untranslated region and *gag*

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Abstract

This study was undertaken to address the role of feline immunodeficiency virus (FIV) long terminal repeats (LTR) as potential packaging determinants. A number of studies in the recent past have clearly demonstrated that the core packaging determinants of FIV reside within at least two distinct regions at the 5′ end of the viral genome, from R in the 5′ LTR to ~150 bp within the 5′ untranslated region (5′ UTR) and within the first 100 bp of *gag*; however, there have been conflicting observations as to the role of the LTR regions in packaging and whether they contain the principal packaging determinants of FIV. Using a semi-quantitative RT-PCR approach on heterologous non-viral vector RNAs in an in vivo packaging assay, this study demonstrates that the principal packaging determinants of FIV reside within the first 150 bp of 5′ UTR and 100 bp of *gag* (the two core regions) and not the viral 5′ LTR. Furthermore, it shows that in addition to the 5′ LTR, the 3′ LTR also contains packaging determinants, but of a less significant nature compared to the core packaging determinants. This study defines the relative contribution of the various regions implicated in FIV genomic RNA packaging, and reveals that like other primate lentiviruses, the packaging determinants of FIV are multipartite and spread out, an observation that has implications for safer and more streamlined design of FIV-based gene transfer vectors.

Keywords: Feline immunodeficiency virus; Retrovirus; RNA packaging

1. Introduction

The feline immunodeficiency virus (FIV) is a non-primate lentivirus that is being explored as a potential vector system for gene delivery into humans [1–3]. Being of feline origin, FIV was considered a safer alternative to vector systems based on the primate lentiviruses such as human and simian immunodeficiency viruses (HIV and SIV). However, the safety of FIV-based vector systems was called into question when the promiscuity of FIV was demonstrated by its ability to be cross-packaged by even distantly related lentiviruses such as HIV and SIV [4]. This study highlighted the importance of

Abbreviations: FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; MLV, murine leukemia virus; ASLV, avian sarcoma-leukosis virus; LTR, long terminal repeats; UTR, untranslated region; mSD, major splice donor; PBS, primer binding site; hCMV, human cytomegalovirus; BGH, bovine growth hormone; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SV-Hygro, simian virus 40 promoter/enhance hygromycin gene cassette; RPE, relative packaging efficiency; SIN vectors, self-inactivating vectors; RRE, Rev responsive element.

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creating further modifications into FIV-based gene transfer vectors to make them safer for gene delivery into humans. One such modification could be elimination of the packaging determinants from the transfer vectors after delivery of the vector RNA into the target cells, a process known as ''self-inactivation'' that utilizes the unique properties of the retroviral reverse transcriptase to convert the viral RNA into DNA [5–11]. Thus, mapping the packaging determinants becomes vital if the next generation of FIV vectors are to be constructed where high titers of FIV particles bearing the therapeutic gene are to be generated with little ability to be packaged or cross-packaged into any retroviral proteins that may accidentally be present in the patient.

An enormous body of work over the past two decades has clearly shown that the 5′ end of the retroviral genome is crucial for specific incorporation of the retroviral genetic material into the virus particle (reviewed in [12–18]). This process requires the recognition of specific sequence/structural elements within the genomic RNA (gRNA) by the nucleocapsid portion of the retroviral Gag polyproteins. With increasing understanding, the emerging picture of retroviral RNA encapsidation suggests that successful RNA packaging is a multi-dimensional process that not only requires the specific RNA/protein interactions, but is also dependent upon the special properties of each retrovirus. Thus, in the case of the simple α retroviruses such as avian leukosis-sarcoma virus (ALSV), where the packaging signal lies upstream of the major splice donor (mSD) and hence is part of both genomic and subgenomic mRNAs, specific incorporation of gRNA is aided by the physical separation of pools of spliced and unspliced mRNAs within the cell with the spliced mRNA sequestered within the rough endoplasmic reticulum, and the unspliced mRNA expressed in the cytoplasm [19,20]. The γ retroviruses such as murine leukemia virus (MLV), on the other hand, contain their packaging signal downstream of the mSD so that only gRNA contains the packaging signal [21], and this process is facilitated by the sorting of the gRNAs into two pools—one destined for translation, while the other reserved for packaging [22].

Among the lentiviral group of retroviruses, the packaging determinants of HIV-1 are the most extensively studied, presenting a complex picture upon which most investigators agree. The HIV-1 packaging signal contains sequences both upstream and downstream of the mSD and extends into *gag* (reviewed in [13,17]). It is multipartite and known to have higher order RNA structures extending from the transcriptional start site and the TAR loop to the 5′ end of *gag* [23–29]. Three complex stem loops are present within the 5′ LTR, while sequences downstream of the primer binding site (PBS) fold into a cluster of four stem loops (SL1–4) of which SL3 is thought to contain the major packaging determinants of HIV [17]. SL1–4 are not sufficient in themselves in vivo for packaging and other sequences upstream to SL1 are also required such as sequences within the U5-PBS between the poly A stem loop and SL1, as well as GU-rich sequences in the lower stem of poly A and the U5-PBS stem loops [29–31]. Additionally, mutation in the stem of the TAR element affect packaging significantly [29,32]. Since the major packaging determinants of HIV-1 reside within SL3, their presence on full-length genomic RNA ensures specific incorporation into the virions over and above the spliced mRNAs. Interestingly, in contrast to MLV, the gRNA in HIV-1 can be used both for translation and packaging [22].

The packaging determinants of FIV have started to emerge with earlier studies implicating sequences within the 5′ end of the viral genome up to the first 350 bp of *gag* that were sufficient to allow efficient transduction of marker genes carried by FIV-based vectors [2,4,33]. These studies were followed by more systematic analyses of the FIV 5′ LTR/non-LTR containing untranslated regions (UTR) and *gag* sequences for packaging determinants. They revealed that the FIV packaging signal consisted of at least two discontinuous core regions, one located within the 5′ LTR/UTR upstream of the mSD from R/U5 to the first ∼150 bp of UTR, and the second within the first 100 bp of *gag*, whereas the region downstream of the mSD and beginning of *gag* was dispensable for packaging [34–37]. However, the relative contribution of each region to FIV RNA packaging remains unclear with some studies suggesting that sequences within the R/U5 contain the principal packaging determinant [34,37], while others suggesting otherwise [35,36]. The present study was undertaken to clarify the role of the FIV packaging determinants that may be present within the FIV LTR and determine their relative contribution to RNA packaging. A systematic analysis of the various regions of the FIV genome implicated in packaging reveals the true nature of the FIV packaging signal, demonstrating that the key FIV packaging determinants are indeed localized to the two core regions defined earlier [34–37] and not the R/U5. Furthermore, substantially weaker packaging determinants are present not only within the 5′ but also the 3′ LTR. These results suggest potential ways of creating safer, yet efficient, FIV-based vectors for gene delivery using self-inactivation and/or other strategies.

2. Methods

2.1. Plasmid construction

All plasmids were constructed using the FIV Petaluma 34TF10 strain (GenBank accession number M25381) [38]. Vectors AG026-AG028 (Figs. 1 and 2) were cloned by modifying pcDNA3 where the region between *Nde*I site (nt 485) and the artificially created *Cla*I site (nt 951) was removed and replaced by the region between the *Nde*I site and the *Cla*I site of AG007 through AG009 (consisting of R/U5 + 90, 120, or 150 bp of FIV 5′ UTR + 100 bp *gag*, respectively). This was followed by insertion of the SV-hygro cassette at the *Xba*I site (nt 984) of pcDNA3, generating the final clones. AG040, AG041 and AG042 (Figs. 1 and 2) were created by replacing the region between *Kpn*I site (nt 900) and the artificially created *Cla*I site (nt 951) of pcDNA3 with sequences between the *Kpn*I (just after the end of U5) and *Cla*I sites of AG007 through AG009 (which contain only the 90, 120, or 150 bp of 5′ UTR and 100 bp of *gag*, respectively), followed

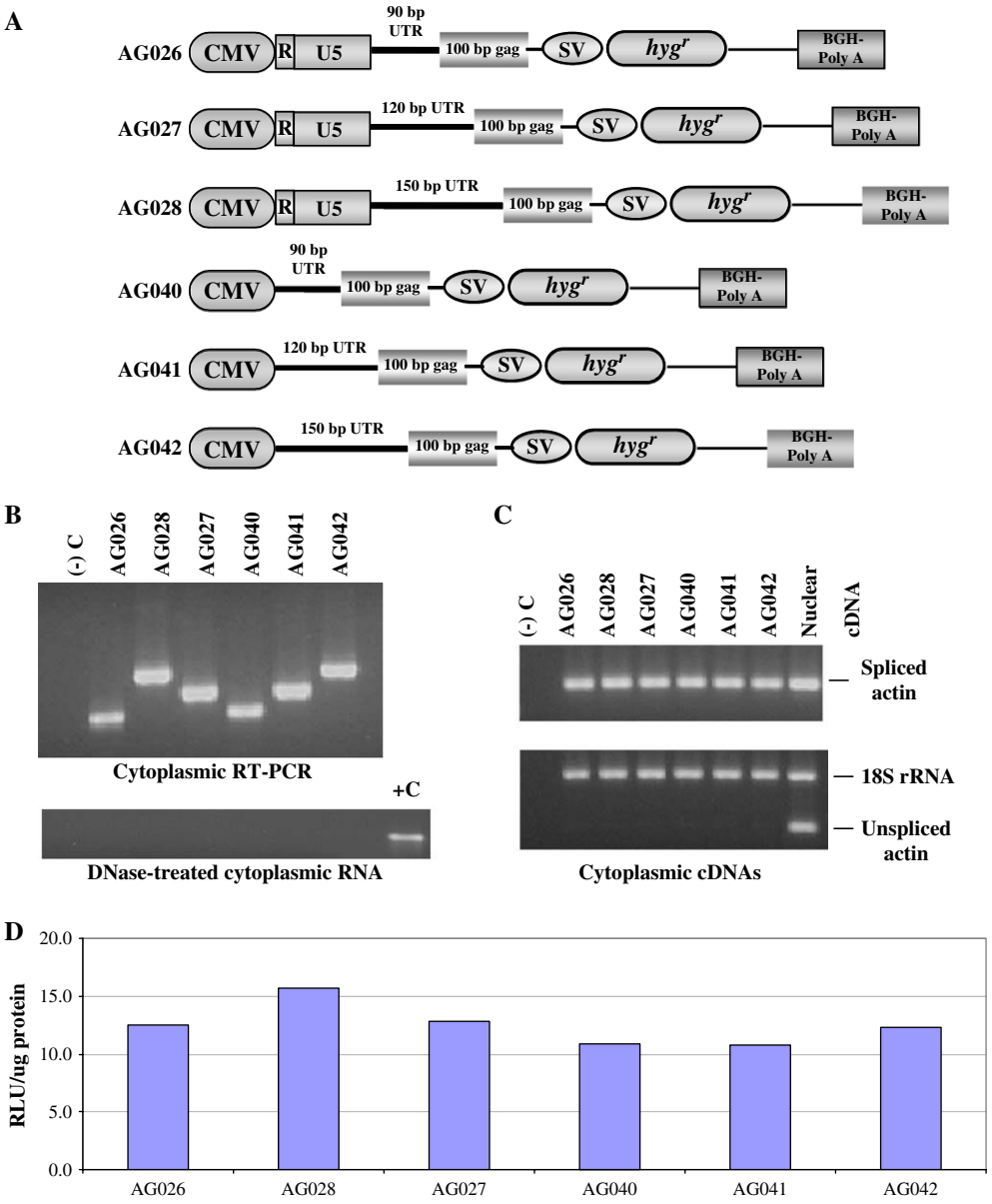

Fig. 1. Test of the FIV R/U5 region towards RNA packaging. (A) Schematic representation of the transfer vectors with and without R/U5 in addition to the two core regions in the heterologous context. (B) RT-PCR of cytoplasmic cDNAs amplified using OTR467 (PBS) and OTR503 (*gag*). The bottom gel shows the DNase-treated cytoplasmic RNA which was amplified using plasmid specific primers OTR 366 and 367; +C, plasmid DNA as a positive control. (C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA and 18S ribosomal RNA as a control for the presence of cDNA. (D) Transfection efficiency observed for different constructs assessed by the luciferase activity from the co-transfected pGL3 Control DNA using the Dual Luciferase Assay kit. RLU, relative light units/microgram protein.

by the insertion of the SV-Hygro cassette at the *Xba*I site (nt 984) of pcDNA3.

AG029 (Fig. 3) is a transfer vector containing the R/U5 region alone in pcDNA3. The R/U5 region was amplified using pCMV/RU5 as a template [39], OTR399 as the sense primer and OTR459 as the antisense primer (see Table 1 for primer details). The resulting PCR product was digested with *Hind*III and *Not*I, ligated to the similarly digested large fragment of pcDNA3, followed by insertion of the SV-Hygro cassette at the *Xba*I site. AG021 (Fig. 3) was constructed by deleting the region between the *Sfo*I (nt 359 of FIV just after the end of U5) and *Not*I sites of MTB [35,36], thus deleting the entire 5′ UTR and religating the backbone on its own, which now contains only the R/U5 region in the presence of the SV-Hygro cassette and the 3′ FIV LTR.

AG031 (Fig. 4) was constructed by deleting the region between the *Eco*RV (nt 951) and *Not*I sites (nt 966) of pcDNA3 and replacing it by the region between *Sfo*I (just after the end of U5) and *Not*I sites of MB15 (described in [35]) that contains 270 bp of 5′ UTR and 100 bp of *gag* followed by insertion of the SV-Hygro cassette at the *Xba*I site (nt 984) of pcDNA3. AG039 (Fig. 4) was constructed by subcloning the region between *Sfo*I (nt 359, just after the end of U5) and *Eco*RI of MB15, and recloning the region between *Not*I and partial

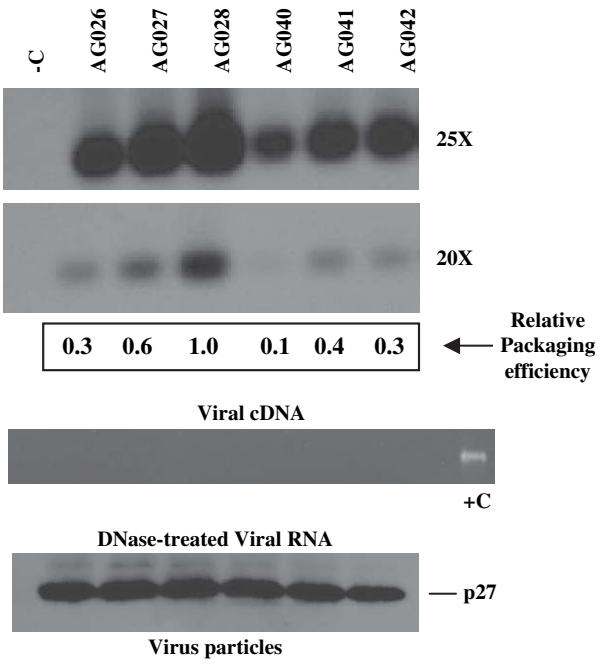

Fig. 2. The R/U5 region increases the packaging efficiency of heterologous FIV transfer vector RNAs. Semi-quantitative RT-PCR of viral cDNAs amplified using OTR467 (PBS) and OTR662 (90 bp of 5′ UTR) for 20 and 25 rounds of PCR and probed using the 90 bp probe. The panel below shows the DNase-treated viral RNAs which were amplified using plasmid specific primer OTR 366 and 367 (hygro primers). The bottom panel shows the Western blot analysis of equivalent amounts of the remaining one-fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats.

*Sac*I into the same sites of MB23 (a vector with 270 bp UTR but no SV-Hygro cassette), followed by insertion of the SV-Hygro cassette at the *Cla*I site.

In AG051, AG052, and AG053, the appropriate regions of the FIV LTR were PCR amplified using pCMV/RU5 as the template, OTR657 as the sense primer and three different antisense primers, OTR642, OTR658, and OTR659 (Table 1). The resulting PCR products were digested with *BamH*I and *Cla*I and cloned into the same sites of pcDNA3 to generate the final clones (Fig. 5).

2.2. Transfections of cells

Virus-producing 293T cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Hyclone, Logan, UT) and transfected in triplicate using the calcium phosphate method described in Mustafa et al. [39].

2.3. Luciferase assays and normalization of packaging efficiency

The transfection efficiency was determined by detection of luciferase activity from the cotransfected luciferase expression vector, pGL3 Control, as described previously [39]. The relative packaging efficiency (RPE) was calculated as follows: RPE = [(specific OD in test vector − Bkgrd OD)/RTE in test culture]/[(specific OD in vector being compared to − Bkgrd OD)/RTE in culture being compared to], where specific OD is the OD observed in the specific RT-PCR product from the test vector, Bkgrd OD is the OD observed in the no template control lane, RTE is the relative transfection efficiency = (LUC units in test culture/per μg protein)/(LUC units in highest expressing culture/per μg protein).

2.4. Virus isolation

Virions were isolated from the transfected 293T culture supernatants by first clarifying the supernatants from the cellular debris via low-speed centrifugation at 4000 rpm for 30 min, followed by pelleting the viral particles by ultracentrifugation using SW28 rotor at 24,000 rpm for 2 h at 4 °C. Viral pellets were resuspended in 125 μl of TNE buffer (50 mM Tris−Cl, pH 7.4, 100 mM NaCl, and 1 mM EDTA, pH 8.0), and one-third (∼40 μl) was saved for analysis of virus particles by Western blots, while the remaining two-thirds (∼80 μl) was used to isolate virion RNA.

2.5. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Cytoplasmic and viral RNAs were isolated from the transfected cells as described previously [39]. Transfected cells were fractionated into nuclear and cytoplasmic fractions as described by Qiagen (Germany). Cytoplasmic (∼2.5 μg) or viral (one-fifth of the total) RNA was DNase treated and reverse transcribed [39] and 1 μl of the cDNA was tested by PCR to confirm the absence of DNA. The cDNAs were amplified using primers listed in Table 1. The PCRs for unspliced actin [40] were performed as a multiplex in the presence of primers for 18S ribosomal RNA primers as a control for cDNA addition (18S Quantum competimer control, Ambion, TX). The resulting products were analyzed on 2−4% agarose gels in the presence of ethidium bromide, photographed, and scanned using the Biometra gel documentation system (Whatman Biometra, Gottingen, Germany). Some of the gels were further processed for Southern blot analysis.

2.6. Southern blot analysis

Southern blot analysis was conducted using a classical alkaline denaturation protocol followed by capillary transfer onto Hybond N+ membranes (Amersham Biosciences, UK). UV irradiation of the membranes was conducted twice at 70,000 μJ (UV crosslinker BLX/254, Invitrogen Life Technologies) and hybridization was performed using the AlkPhos Direct Labeling Kit (Amersham, Arlington Heights, IL) as per the manufacturer's directions. DNA for probes was generated by PCR and labeled with alkaline phosphatase as specified in the kit using 250−300 ng of purified PCR fragment. Finally, blots were developed using the CDP-Star chemiluminescent detection reagent, exposed to Fuji Medical X-ray film (HR-G30, Germany) for various lengths of times, digitized using

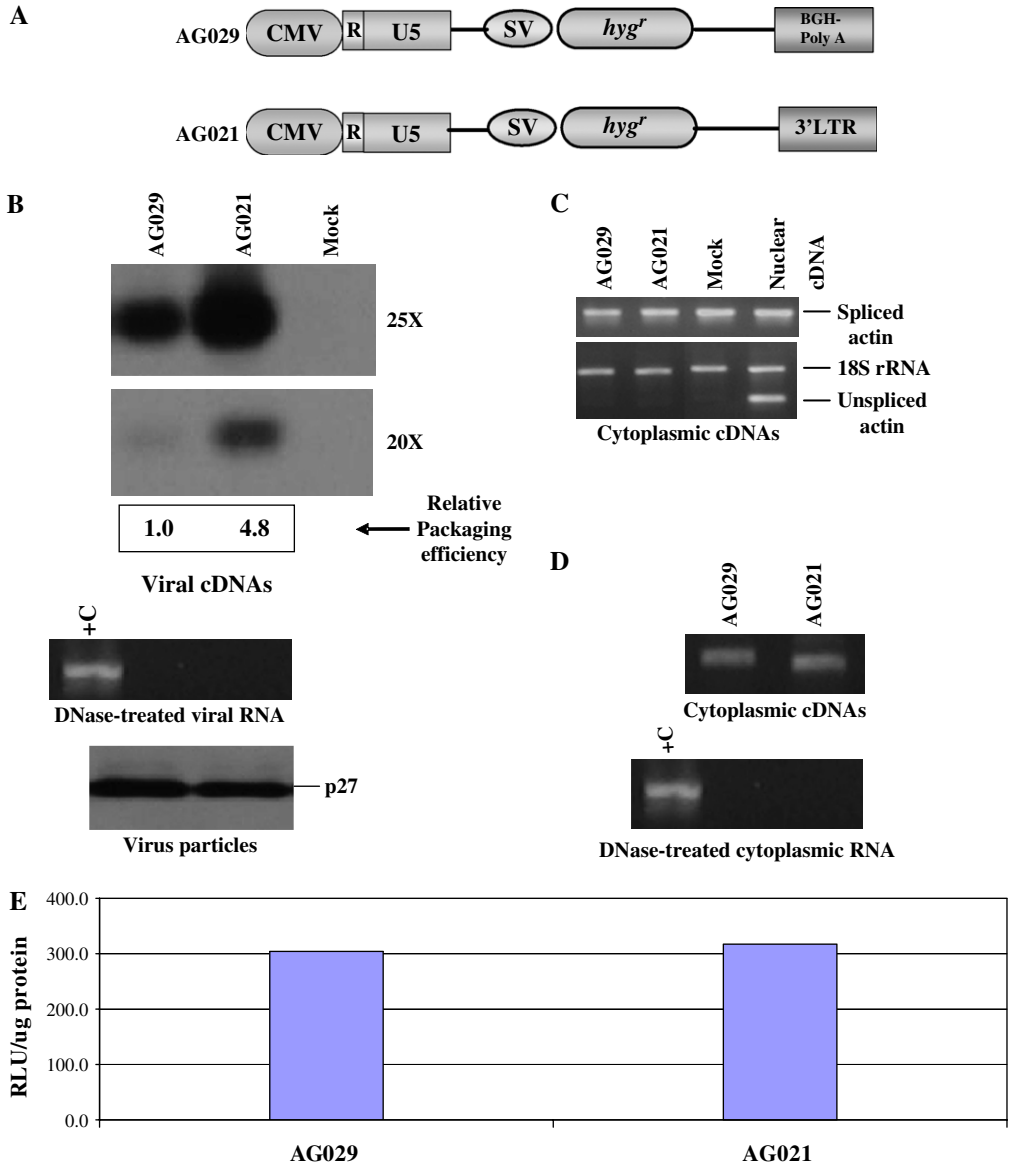

Fig. 3. The 3′ LTR increases the packaging efficiency of FIV RNA in the absence of UTR/*gag*. (A) Schematic representation of the transfer vectors with and without the 3′ LTR. (B) Semi-quantitative RT-PCR of viral cDNA amplified using OTR516 (R) and OTR459 (end of U5) primers for 20 and 25 rounds of PCR and probed using the R/U5 probe. The panel below shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR 366 and 367 (hygro primers). +C, plasmid as a positive control. The bottom panel shows the Western blot analysis of equivalent amounts of the remaining one-fourth purified virions harvested from transfected cultures using the FIV antiserum from infected cats. (C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin, while the bottom panel is a multiplex RT-PCR for the exclusively nuclear unspliced β-actin mRNA and 18S ribosomal RNA as a control for the presence of cDNA. (D) RT-PCR of cytoplasmic cDNA amplified using OTR516 (R) and OTR459 (end of U5) primers. The bottom panel shows the DNase-treated cytoplasmic RNA, which was amplified using plasmid specific primer OTR 366 and 367. (E) Transfection efficiency as assessed by the luciferase activity from the co-transfected pGL3 Control DNA using the Dual Luciferase Assay kit. RLU, relative light units/microgram protein.

the Biometra gel documentation system, and analyzed for optical densities using the BioDoc Analyze software version 2.0.

2.7. Western blot analysis

Western blot analyses on harvested virions were conducted using the QB2 fraction (1:2500 dilution) of the convalescent polyclonal antisera isolated from cats infected with the FIV Petaluma strain (a gift of Dr Ellen Collisson, Texas A&M University, College Station, TX). The blots were developed using the Super Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL), and exposed to Fuji Medical X-ray film for various lengths of time.

3. Results

3.1. Experimental design

To address the role of FIV LTRs in packaging, we utilized a modified version of the 3-plasmid in vivo packaging assay

Table 1
Primers used in RT-PCR and PCRs

Primer	Sequences[a]	Description
OTR399 (+)	5′ cccaagcttgagctcTGTGAAACTTCGAG GAGTCTC 3′	FIV 5′ LTR (U3/R junction) from nt 203–223
OTR459 (−)	5′ aaaaaagcggccgcACTGCGAAGTTCTCGG CCCGG 3′	End of U5 (nt 357–337) before the FIV primer binding site (PBS)
OTR657 (+)	5′ gcggatccTGGGATGAGTATTGGAACCCT 3′	FIV nt 9120–9140
OTR642 (−)	5′ ccatcgatTGCGAAGTTCTCGGC CCGGATTC 3′	FIV nt 9474–9452
OTR658 (−)	5′ ccatcgatGGTTCAATCTCA AATATTTATTG 3′	FIV nt 9409–9387
OTR659 (−)	5′ ccatcgatCTGTGGGA GCCTCAAGGG 3′	FIV nt 9383–9366
OTR366 (+)	5′ CGGCACTTTGCATCGGCC 3′	Nt 423–440 of hygromycin gene
OTR367 (−)	5′ CGGCGATCCTGCAAGCTC 3′	Nt 1010–993 of hygromycin gene
OTR580 (+)	5′ TGAGCTGCGTGTGGCTCC 3′	Spliced actin mRNA-specific primer (S) [40]
OTR581 (−)	5′ GGCATGGGGGAGGGCATACC 3′	Spliced/unspliced actin mRNA-specific primer (A) [40]
OTR582(+)	5′ CCAGTGGCTTCCCCAGTG 3′	Unspliced actin mRNA-specific primer (S-1) [40]
OTR600 (+)	5′ GAACCCTGTCGAGTATC GTG 3′	FIV 5′ LTR nt 286–306
OTR601 (−)	5′ CTGGTTGCTGACTAATTGAG 3′	pcDNA3 nt 1840–1821 (Invitrogen Life Technology's map)
OTR660 (+)	5′ GAGGACTTTTGAGTTCTCCCTTGAGGC 3′	FIV nt 230–256
OTR467 (+)	5′ aaaaaagcggccgcGTTGGCGCCCGAACAGGGACT 3′	Nt 356–376 of FIV 34TF10. Used for 90 bp 5′ UTR probe
OTR662 (−)	5′ AGCAGGAGTTCTGCTTAACAGCTTTC 3′	FIV nt 440–415. Used for 90 bp 5′ UTR probe
OTR516 (+)	5′ ttcgaatttgggcccGAGTCTCTTTGTTGAGGACTTTTG 3′	Nt 9336–9359 of FIV 34TF10 3′ LTR. Used for R/U5 probe
OTR460 (−)	5′ aaaaaagcggccgcAAGTCCCTGTTCGGGCGCCAA 3′	Nt 377–357 of FIV 34TF10. Used for R/U5 probe PCR
OTR627 (+)	5′ TGGGATGAGTATTGGAACCC 3′	Nt 1–20 of FIV Petaluma 5′ LTR. Used for FIV U3 probe
OTR517 (−)	5′ gggcccaaattcgaaCTCGAAGTTTCACAAAGCACTGGT 3′	Nt 9335–9312 of FIV 34TF10 3′ LTR. Used for FIV U3 probe
OTR574 (+)	5′ aaaaaagcggccgcATGGGGAATGGACAGGGGCGA 3′	Nt 628–648 of FIV gag. Used for FIV gag probe
OTR503 (−)	5′aaaaaagcggccgcTCCCTTCTCCAAATTTTTTACTCTTCC3′	Nt 727–701of FIV gag. Used for FIV gag probe

[a] Sequences included for cloning purposes are shown in lowercase followed by FIV sequences shown in uppercase.

that has been used previously to map the packaging determinants of FIV [4,35,36]. Unlike the 3-plasmid system, this assay lacked the vector that would provide the envelope proteins since there was no need for the infection of target cells as most of the transfer vectors did not contain the sequences necessary for reverse transcription, integration or propagation into the target cells. The *gag/pol* expression construct, MB22 [4] provided the structural proteins for virion formation, while the transfer vectors provided the RNA substrates to be tested for packaging. The transfer vectors containing specific regions of FIV in the non-retroviral, heterologous context were created by modifying the expression vector, pcDNA3; thus, the packaging determinants were expressed from the human cytomegalovirus (hCMV) promoter/enhancer for transcript initiation and the bovine growth hormone (BGH) polyadenylation (poly A) signal for transcript termination. Since small regions of the FIV genome were being tested, a ~2 kb Hygromycin resistance gene cassette (SV-Hygro) was inserted in the transfer vectors to increase the size of the packageable RNAs and make them relatively the same size for easier relative comparison.

Each transfer vector was co-transfected with MB22 into 293T cells [39]; virus was harvested 72 h post transfection and processed for RNA and protein extraction, while a portion of the transfected cultures was fractionated into nuclear and cytoplasmic fractions for isolation of cytoplasmic RNA. To monitor for variations in transfection efficiencies quantitatively, the luciferase expression vector, pGL3 Control, was included in the DNA transfection cocktail and a portion of the transfected cultures was used to determine luciferase activity.

3.2. R/U5 region increases the packaging efficiency of heterologous FIV transfer vector RNAs

To test the role of the FIV R/U5 region towards packaging, a series of transfer vectors were created that incrementally maintained the previously identified core region within the 5′ UTR [34–37,41] with either 90, 120, or 150 bp of the 5′ UTR and the first 100 bp of *gag* either in the presence (AG026, AG027, and AG028) or absence (AG040, AG041, and AG042) of R/U5 (Fig. 1A). To distinguish between 5′ untranslated sequences within the LTR and those outside the LTR, the term "5′ UTR" has been used to designate only "non-LTR" untranslated sequences at the 5′ end of the viral genome in this study. First, the expression of the transfer vector RNAs in the transfected cultures was analyzed to determine if the vector RNAs were efficiently expressed and transported to the cytoplasm. Towards this end, equal amounts of the cytoplasmic RNA from each transfected culture was DNase-treated, reverse transcribed, and PCR amplified using OTR 467/OTR 503, primers within the PBS and the *gag* region (Fig. 1B and Table 1). As shown, all transfer vector RNAs were expressed efficiently in the cytoplasm of the transfected cells, demonstrating that RNAs from these transfer vector were stably expressed and successfully transported out of the nucleus and into the cytoplasm. Since we amplified across the 5′ UTR, the size of the PCR fragment differed between the different constructs and was indicative of the particular transfer vector used for transfection. As a control, a portion of the DNase-treated RNA was tested by PCR using plasmid-specific hygromycin primers to monitor for DNA contamination (Fig. 1B). All the samples were negative (except for the plasmid DNA sample that acted

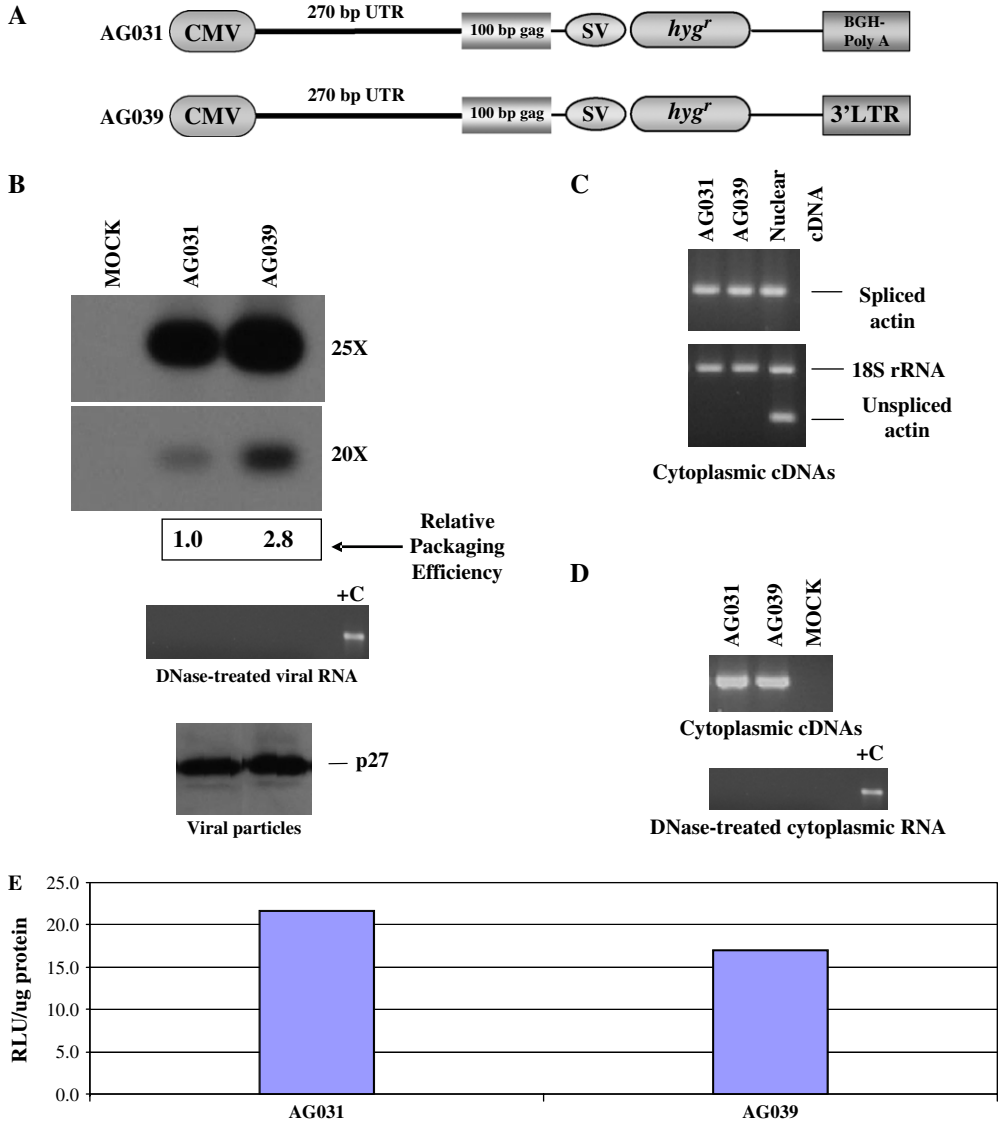

Fig. 4. The 3′ LTR increases the packaging efficiency of FIV RNA in the presence of the core packaging determinants. (A) Schematic representation of the transfer vectors with and without the 3′ LTR in the presence of the entire 5′ UTR and 100 bp of *gag*. (B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR467 (pbs) and OTR503 (*gag*) for 20 and 25 rounds of PCR amplification and probed using the *gag* probe. The panel below shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR 366 and 367 (hygro primers). +C, plasmid as a positive control. The bottom panel shows Western blot analysis of equivalent amounts of the remaining one-fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. (C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. (D) RT-PCR of cytoplasmic cDNAs amplified using OTR467 (pbs) and OTR503 (*gag*). The bottom panel shows the DNase-treated cytoplasmic RNA which was amplified using plasmid specific primer OTR 366 and 367 (hygro primers). +C, plasmid as a positive control. (E) Transfection efficiency as assessed by the luciferase activity from the co-transfected pGL3 Control DNA using the Dual Luciferase Assay kit. RLU, relative light units/microgram protein.

as a positive control, see last lane), assuring that there was no plasmid DNA carryover from the transfections contaminating our RNA samples (Fig. 1B).

To ensure that the nucleocytoplasmic fractionation technique was effective, cytoplasmic RNAs were tested for the presence of the unspliced β-actin mRNA by RT-PCR. Unspliced β-actin mRNA is found exclusively within the nucleus, while the spliced form is found both in the nucleus and the cytoplasm [40]. As can be seen, spliced β-actin mRNA was abundantly found in the cytoplasm (Fig. 1C, upper half), while the unspliced form was absent from the cytoplasmic portion with 30-cycle PCR, and present only in the nuclear fraction (Fig. 1C, lower half, last lane). The unspliced β-actin PCR was conducted in the presence of 18S ribosomal RNA primers, which acted as an internal control for the presence of amplifiable cDNAs in each sample (Fig. 1C, lower half).

Once it was confirmed that all transfer vector RNAs were stably expressed and efficiently transported to the cytoplasm, we went on to analyze the amount of RNA packaged into the actual virus particles. Similar to cytoplasmic RNAs, the viral RNAs were DNase treated, reverse transcribed and used in PCR amplifications. To make sure that the amplifications

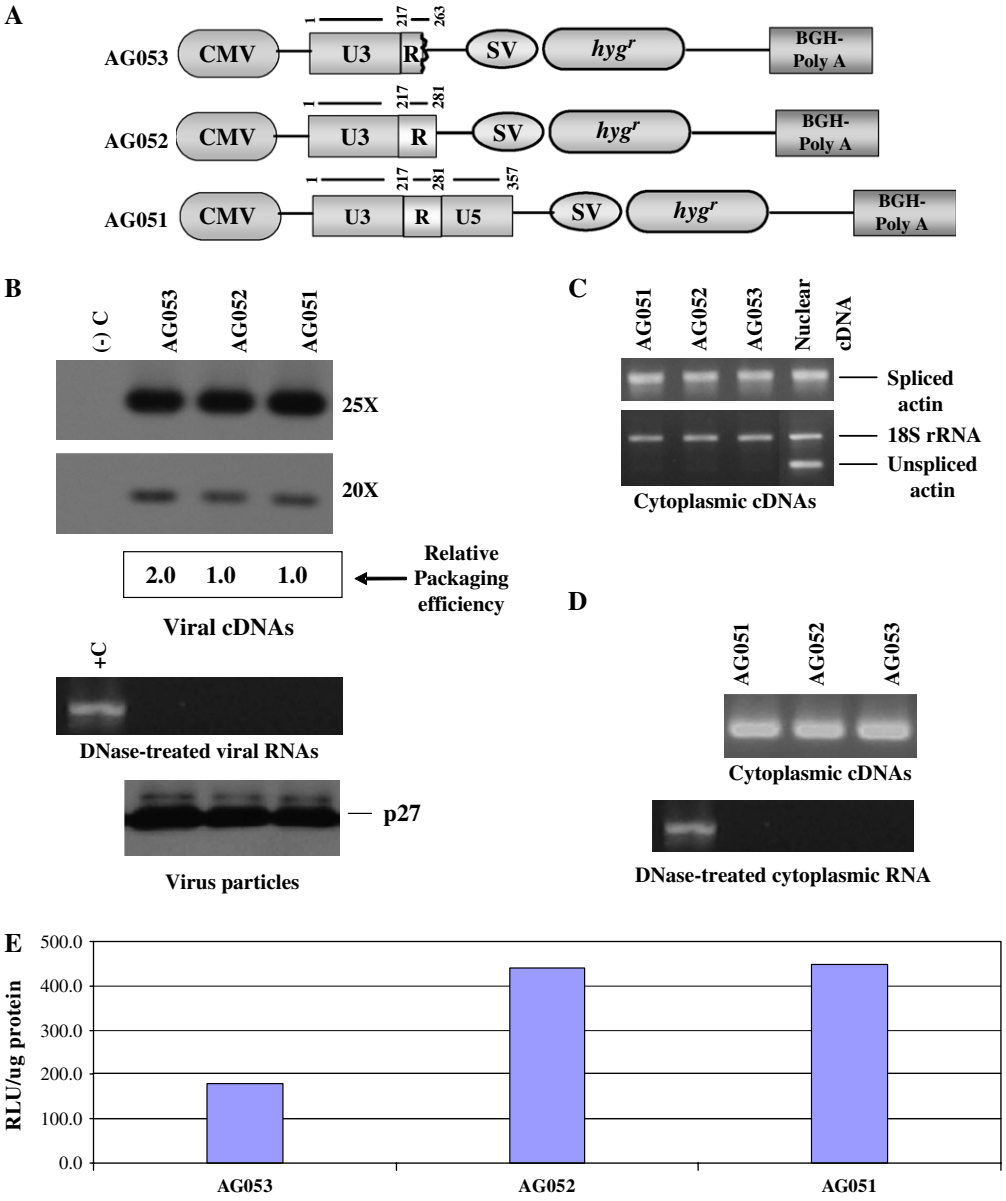

Fig. 5. The U3/partial R sequences contribute to FIV RNA packaging. (A) Schematic representation of the transfer vectors with U3/R/U5 or U3/R or U3/partial R. (B) Semi-quantitative RT-PCR of viral cDNAs amplified using OTR627 (beginning of U3) and OTR517 (end of U3) primers for 20 and 25 rounds of PCR and probed using the U3 probe. The panel below shows the DNase-treated viral RNA which was amplified using plasmid specific primer OTR 366 and 367 (hygro primers). +C, plasmid as a positive control. The bottom panel shows the Western blot analysis of equivalent amounts of the remaining one-fourth purified virus harvested from transfected cultures using the FIV antiserum from infected cats. (C) Control for nucleocytoplasmic fractionation technique. The upper panel is the RT-PCR on cytoplasmic RNA for spliced actin, while the bottom panel is a multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear and 18S ribosomal RNA as a control for the presence of cDNA. (D) RT-PCR of cytoplasmic cDNAs amplified using OTR627 (beginning of U3) and OTR517 (end of U3) primers. The bottom panel shows DNase-treated cytoplasmic RNA amplified using plasmid specific primers. (E) Transfection efficiency as assessed by the luciferase activity from the co-transfected pGL3 Control DNA using the Dual Luciferase Assay kit. RLU, relative light units/microgram protein.

were in the linear range of detection, PCRs were conducted for 20, 25, and 30 cycles and the resulting products were size fractionated on agarose gels containing ethidium bromide. Ethidium bromide staining was sufficient to visualize the bands within the 25 and 30 cycle PCRs, but Southern blotting was required to visualize many of the bands in the 20 cycle range. For better quantification of the signal in each band, the PCR fragments were transferred to nylon membranes, and hybridized with an FIV probe (90 bp UTR in this case) using a non-radioactive alkaline phosphatase labeling method. The chemiluminescent signals emitted were captured on X-ray films that were digitized and the optical density in the various bands quantitated using the Biometra gel documentation system. Finally, the optical densities obtained were normalized to the amount of luciferase expression observed in each of the transfected culture and expressed relative to AG028, the transfer vector that contains from R/U5 to 150 bp 5′ UTR and 100 bp of *gag*, that was given a value of 1.

Test of the transfer vectors RNAs packaged into the viral particles by semi-quantitative RT-PCR revealed that all transfer

vector RNAs were packaged, but to various extents (Fig. 2). Overall, the constructs with the R/U5 region were packaged two- to threefold more efficiently than those lacking the R/U5 region. Thus, after normalization with the transfection efficiencies (Fig. 1D), vectors RNAs with R/U5, AG026–AG028, were packaged with a relative packaging efficiency (RPE) of 0.3, 0.6, and 1.0 compared to AG028, while constructs lacking the R/U5 region (AG040–AG042), were packaged with a relative packaging efficiency of 0.1, 0.4, and 0.3, respectively (Fig. 2). This is despite the production of similar levels of viral particles in the transfected cultures (Fig. 2). As expected, the R/U5-containing construct with only 90 bp of UTR was packaged least efficiently (RPE of 0.3), followed by the construct with 120 bp UTR (RPE of 0.6), while 150 bp UTR enhanced packaging the most (RPE of 1.0) since 150 bp UTR is required for optimum packaging [34,35,41]. Similar observations were made for AG040–AG042, though in this case, the 120 bp and 150 bp constructs were packaged with similar relative packaging efficiencies in the absence of R/U5 (Fig. 2). These results were reproducible with different primer pairs (data not shown) and reveal that the core packaging determinants identified earlier (150 bp 5′ UTR/100 bp *gag* or an increment thereof) can function as a packaging determinant: (1) independently in a heterologous context, (2) despite the large (120–150 bp) intervening deletion in the 5′ UTR and, (3) in the absence of any R/U5 sequences. Furthermore, this level of packaging can be nearly doubled or more by the presence of R/U5 sequences at the 5′ end, demonstrating that the R/U5 sequences also contain packaging determinants.

3.3. Role of the 3′ LTR on the packaging efficiency of FIV RNA

To study the role of the 3′ LTR as containing potential packaging determinants, two transfer vectors were constructed to determine whether 3′ LTR could enhance the packaging efficiency of transfer vector RNAs in the absence of most FIV sequences other than R/U5 (Fig. 3A). AG029 and AG021 contain the FIV R/U5 region at the 5′ end and either the BGH poly A signal or the 3′ FIV LTR downstream of the SV-Hygro cassette in the background of pcDNA3. Semi-quantitative RT-PCR analysis of the transfer vector RNAs packaged into the virions showed that AG021 with the 3′ LTR was packaged with a nearly fivefold enhanced efficiency than AG029 after normalization with the transfection efficiency (Fig. 3B and E), an observation repeatable with different primer pairs and in different experiments (data not shown). This difference was not due to differences in the levels of virions produced in the transfected cultures (Fig. 3B), or due to the presence of contaminating plasmid DNA since no amplification could be observed when the DNased RNAs were used as templates in PCR reactions with hygro primers (Fig. 3B). Similarly, the transfected cells were fractionated appropriately and no unspliced β-actin mRNA could be observed in the cytoplasm (Fig. 3C), while the vector RNAs were expressed efficiently in the cytoplasm (Fig. 3D). These results reveal that not only does the 3′ LTR have packaging determinants, but that the R/U5 on its own can act as a packaging determinant as well without the need for sequences within the 5′ UTR/*gag* or the 3′ LTR.

Taking these analyses further, two more transfer vectors were constructed and tested to determine the role of 3′ LTR in packaging; however, this time in the absence of R/U5, but presence of the core packaging determinants to determine if similar effects of the 3′ LTR could be observed upon packaging when the core packaging determinants were present in their natural context (Fig. 4A). AG031 and AG039 represent transfer vectors that express the 270 bp UTR/100 bp *gag* in the presence or absence of 3′ LTR, respectively, without the R/U5 sequences. Test of these constructs in the in vivo packaging assay revealed that both constructs were efficiently expressed in the cytoplasm of the transfected cells (Fig. 4D), and the cells were fractionated appropriately (Fig. 4C). Semi-quantitative RT-PCR analysis of the RNA packaged in the virions revealed that the AG039, the vector with the 3′ LTR, was packaged nearly threefold better than the vector RNA without 3′ LTR, after normalization for differences in the transfection efficiency (Fig. 4B). Once again, these results were confirmed using an independent primer pair (data not shown). This was despite the fact that both vectors were transfected with similar efficiencies, and similar amounts of virions were produced in each culture (Fig. 4B and E). Thus, together, these data clearly show that the 3′ LTR harbors packaging determinants that can substantially increase the packaging efficiency of heterologous vectors similar to the R/U5 sequences in the 5′ LTR.

3.4. The U3/R portion of the LTR also contains packaging determinants

To determine whether the increase in the packaging efficiency was due to the presence of the U3/R element, the region of the 3′ LTR that is present in the actual viral transcript, we tested three additional transfer vectors that expressed either the entire U3/R/U5 (AG051), U3/complete R (AG052), or U3/partial R (up to the poly A signal at nt 253 in the LTR) (AG053) from the hCMV (Fig. 5A). AG051, AG052, and AG053 were packaged at relatively the same packaging efficiency (Fig. 5B–E). Since the FIV LTR when present at the 3′ end is transcribed until the poly A signal in the R region (nt 253), these constructs also revealed that there was essentially no difference in the packaging of RNAs when only U3/partial R sequences were present in the transfer vector RNAs (AG053) versus U3/complete R (nt 281) (AG052), or the entire U3/R/U5 (up to nt 357) (AG051) (Fig. 5A). Together, these results suggest that at the 3′ end, the FIV RNA packaging determinants are present within the U3/partial R region.

3.5. The packaging determinants within the UTR and gag constitute the core packaging determinants of FIV

Finally, using our already constructed vectors, we asked as to which of the regions of FIV contain the primary packaging

determinant, R/U5 at the 5′ end, 150 bp UTR/100 bp *gag*, or the 3′ LTR. Since we were limited by the FIV-specific primer pairs we could use for a simultaneous analysis of the constructs, vectors were chosen that contained R/U5 in common (Fig. 6A). Semi-quantitative RT-PCR analysis of these constructs revealed that R/U5 on its own (in AG029) was a weak packaging signal compared to the vector that contains the two core packaging determinants in addition to R/U5, AG028. AG029 was assigned a RPE of 1. The packaging efficiency increased by approximately fourfold in the presence of the 3′ LTR for transfer vector AG021, similar to the observations made earlier (see Fig. 3). With the addition of the suboptimal core packaging determinant (90 bp UTR/100 bp *gag*) to the R/U5-containing construct alone, the packaging efficiency increased to 5 which was only slightly more than that observed for the two-LTR-containing construct, AG021. A further incremental increase in the UTR to 120 bp nearly doubled the packaging efficiency to 9 compared to AG026, while maximum packaging efficiency of 12 was observed for the construct with the complete core packaging determinant, 150 bp 5′ UTR, AG028 (Fig. 6B). These data conclusively show that 150 bp 5′ UTR/100 bp *gag* region contains the primary packaging determinants of FIV and that these determinant(s) are at least ∼3 times as strong as the packaging determinants present within the 5′ and 3′ LTRs (compare AG021 with AG028 containing the two core determinants in addition to the LTRs).

4. Discussion

Studies presented here provide a clearer picture of the FIV packaging signal, demonstrating that it is multipartite and discontinuous primarily composed of two core regions with 150 bp of the 5′ UTR and 100 bp of *gag* comprising the principal packaging determinants of FIV. These results also show that the core packaging determinants do not require the intervening UTR regions for functionality as packaging signals, confirming our recent observations [41]. Additionally, these studies reveal that sequences within both the 5′ and 3′ LTRs also contribute to packaging, though to a lesser extent. In contrast, Kemler and colleagues found the R/U5 region of the 5′ LTR to have a key role in packaging, but only when present at the 5′ end of the viral RNA [34]. Our conclusions are further supported by the observation that despite the presence of R/U5 in AG026–AG028, packaging of the vector RNAs with 90 bp UTR/100 bp *gag* was substantially compromised compared to those containing 120 and 150 bp UTR (Figs. 2 and 6).

Several reasons may explain the discrepancies in the observations of the two studies, including our observation that the 3′ LTR also contains FIV packaging determinants. These reasons may relate to differences in construct design and methods employed for the packaging analyses. The Kemler study, for example, tested full-length genomic constructs, while our studies were conducted using non-viral heterologous or subgenomic

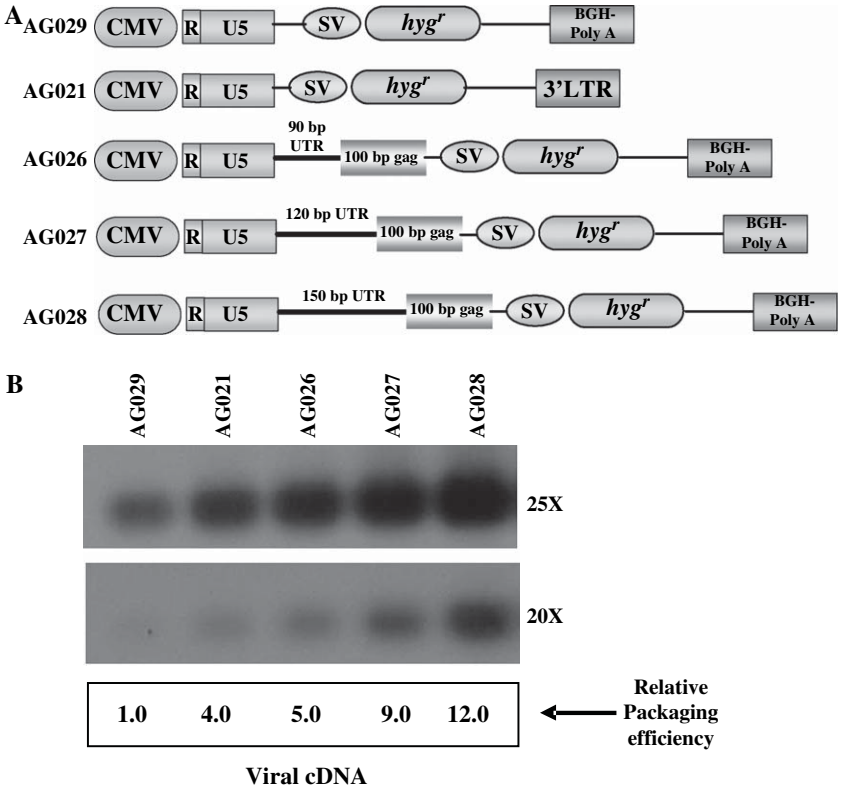

Fig. 6. The principle determinants of FIV RNA packaging lie within 150 bp of 5′ UTR and 100 bp of *gag*. (A) Schematic representation of the vectors tested. (B) Semi-quantitative RT-PCR of viral cDNA amplified using OTR660 (R) and OTR459 (end of U5) primers for 20 and 25 rounds of PCR and probed using the R/U5 probe.

constructs that lacked most of the sequences encoding for the structural genes. The presence of negative inhibitory elements within structural genes such as *gag*, *pol*, and *env* have been shown to affect expression of unspliced mRNAs (transport, stability, and translatability) and hence could theoretically affect the subsequent incorporation of the unspliced mRNA into virions [42]. Vector design itself can sometimes pose problems such as those observed by Poeschla and colleagues who reported that addition of *gag* sequences beyond nt 311 negatively impacted the ability of such transfer vector RNAs to be packaged into virions possibly related to the presence of the highly structured Rev responsive element (RRE) in their vectors [43]. The sensitivity of the assay used for the packaging analysis can also lead to different results. Kemler and colleagues used RNase protection assays to analyze packaging, a technique though more quantitative, but less sensitive than the semi-quantitative RT-PCR approach used here. Thus, it is possible that they could have missed the effects of 3′ LTR on packaging using their analyses.

This study reveals that FIV RNA packaging determinants are as complex as those observed for other lentiviruses, reflecting the multidimensional nature of how genomic RNAs are packaged by the assembling virion—a process that requires not only recognition of the RNA structural features by the Gag polyproteins to distinguish between sub-genomic and genomic mRNAs, but also events related to RNA processing. Since FIV-based self-inactivating vectors (SIN) would be good candidates for human gene therapy, the dispersal of FIV RNA packaging determinants may make this task somewhat tricky, especially the involvement of the R element in the 3′ LTR. Several strategies are available for making SIN vectors that exploit steps of reverse transcription to prevent generation of packageable vector RNAs (see [5–11] and summarized in [44]). These include the use of short stretches of repeat sequences or splice sites to remove the packaging signal during reverse transcription of the vector RNA as well as deletions within the U3 region. A combination of such strategies should lead to the development of effective FIV-based SIN vectors, including deletion of not only the core packaging determinants in the 5′ UTR and *gag*, but also deletion of as much of the U3 region of 3′ LTR as possible (permissible since the marker/therapeutic gene will use an internal promoter and will not need the viral promoter for expression). As demonstrated, we can delete major portions of the 5′ UTR without affecting vector RNA packaging significantly. Such deletions do not seem to be detrimental to the ability of the vector RNA to complete the reverse transcription and integration process as well [41]. Our prediction is that the remaining packaging determinants within the R region in the 3′ LTR in the presence of minimal U3 sequences should not be able to function as an effective packaging signal, or will have highly reduced packaging potential, resulting in the minimization of the packaging potential of the FIV transfer vector RNAs. If that is not the case, further modifications will need to be made (such as shortening of the R region) to disable the packaging determinants residing within the R region of the FIV LTR for safer FIV-based SIN vectors for human gene therapy.

In short, this study clarifies the role of the various regions of the FIV genome that have been implicated in the specific incorporation of the genomic RNA into the viral particles. It reveals that the primary packaging determinants of FIV are present as two discrete regions at the 5′ end of the virus genome, within the first 150 bp 5′ UTR and the first 100 bp of *gag*, and not the 5′ R/U5 region. Furthermore, this study shows that not only the 5′ LTR, but also the 3′ LTR contains packaging determinants, an observation with consequences for safer design of FIV-based vectors for gene therapy.

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