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# Sequences Intervening between the Core Packaging Determinants Are Dispensable for Maintaining the Packaging Potential and Propagation of Feline Immunodeficiency Virus Transfer Vector RNAs

Farah Mustafa,[1] Akela Ghazawi,[2] Preethi Jayanth,[2] Pretty Susan Phillip,[2] Jahabar Ali,[2] and Tahir A. Rizvi[2]*

*Departments of Biochemistry[1] and Medical Microbiology,[2] Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates*

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**The packaging determinants of feline immunodeficiency virus (FIV) consist of two discontinuous core regions, extending from R to ~150 bp of the 5′ untranslated region and the first ~100 bp of *gag*. However, the role of sequences intervening between the core regions in packaging has not been clear. A mutational analysis was conducted to determine whether the intervening sequences played a role in FIV RNA packaging, using an in vivo packaging assay complemented with semiquantitative reverse transcriptase PCR. Our analyses reveal that the intervening sequences are dispensable not only for vector RNA packaging but also for propagation, confirming the discontinuous nature of the FIV packaging signal.**

A series of recent studies have suggested that the packaging determinants of feline immunodeficiency virus (FIV) are complex and multipartite, consisting of at least two discontinuous core regions, one located upstream of the major splice donor sequence from R/U5 at the 5′ end to the first ~150 bp of the 5′ untranslated region (UTR), while the other is within the first 100 bp of *gag* (2, 3, 6, 7). To determine whether the region intervening between the core determinants was required for maintaining the stability of the FIV packaging signal, we modified our previously described vector, MB15 (2), and constructed a series of transfer vectors, AG002 to AG004, that maintained the R/U5 and 100 bp of *gag* but kept only the first 90, 120, or 150 bp of the 5′ UTR, generating incremental deletions between the core packaging determinants (Fig. 1). In a second series, AG013 to AG015, the deletions were replaced with heterologous sequences of the same lengths to further determine whether the deleted/substituted region had a role at the structural level or acted only as a spacer (Fig. 1).

The two series of mutant vector RNAs were tested for their ability to be packaged by FIV proteins, using our well-established in vivo packaging assay along with TR394, which contains the entire 270-bp UTR with a 333-bp *gag* sequence (1–3). The amount of transfer vector RNA packaged into viral particles was analyzed by reverse transcriptase PCR (RT-PCR) (10) for variable cycle numbers, and the resulting products were Southern blotted and hybridized using an R/U5 probe. All mutant vector RNAs, either with deletions or deletion/substitution, were packaged into the virus particles but with different efficiencies, while the control vectors, MB15 and TR394, were packaged at similar levels (Fig. 2A). This was despite the fact that all cultures produced similar levels of viral particles and the transfection efficiencies were within twofold of each other (Fig. 2B and C). Since the PCRs were conducted across the deleted or deleted/substituted region, the size of the PCR fragment varied with each construct, confirming that correct packaging constructs were being expressed in each transfection.

To determine the packaging efficiencies of the various constructs accurately, RT-PCRs were repeated using primers within the R/U5 region that would result in same-sized fragments. Optical densities observed in the various bands were normalized to the amount of luciferase expression observed from the cotransfected luciferase expression vector, and the packaging efficiencies obtained were compared to that for MB15, which was assigned a value of 1. The transfer vector RNAs with only 90 bp of UTR, AG002 and AG013, were least efficiently packaged (0.2 and 0.1, respectively) compared to MB15 (Fig. 2D). The encapsidation efficiency increased substantially with the increase of 30 bp at the 5′ UTR with AG003 and AG014, which contain 120 bp of UTR (0.6 and 0.4, respectively), and increased to nearly the same as that of MB15 by 150 bp of UTR with AG004 and AG015 (0.8 and 0.9, respectively) (Fig. 2D).

The reduced effect on packaging observed for AG013 over and above the lack of sufficient 5′ UTR sequences is probably due to the 180-bp heterologous sequences that were inserted to maintain the spacing between the two core regions. Other than for AG013, the packaging efficiencies between the vectors with deletions were similar to those between the vectors with deletions/substitutions, suggesting that sequences in between the core packaging determinants were not needed as spacers and the core elements could fold into functional packaging determinants independently of the intervening region.

Finally, propagation of transfer vector RNAs by transduction of the hygromycin marker gene to target cells was determined by infecting HeLa cells with virions produced by the 293T cells. Colonies observed upon hygromycin selection of the infected cells were adjusted to the luciferase expression

* Corresponding author. Mailing address: Department of Medical Microbiology, Faculty of Medicine and Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, United Arab Emirates. Phone: (971) 3-713-7514. Fax: (971) 3-767-1966. E-mail: tarizvi@uaeu.ac.ae.

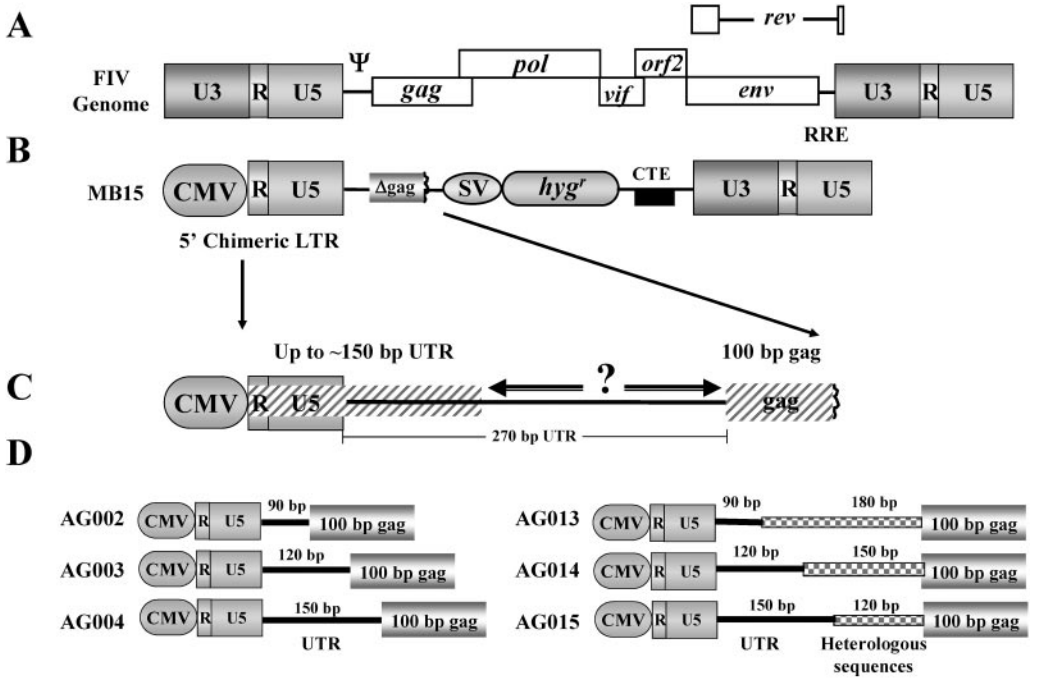


FIG. 1. Test of the region intervening between the core packaging determinants for effects on packaging. Schematic representation of (A) complete FIV genome, (B) MB15, the control transfer vector, (C) two core elements required for efficient RNA packaging (shown as hatched boxes; double-line arrows highlight the intervening region between the two core elements), and (D) transfer vectors with deletions between the two core elements (AG002 to AG004) and vectors with simultaneous deletions/insertions between the two core elements (AG013 to AG015). The heterologous sequences were PCR amplified from the expression vector, pcDNA3. The transfer vectors were constructed through several stages of cloning, details of which can be obtained from the authors upon request.

observed to normalize for various transfection efficiencies and amounts of virions produced in the supernatant. Overall, the transfer vector RNAs were propagated with ~1- to 6-fold less efficiency than MB15 (Table 1). (Similar observations were made when Cos cells were used as virus-producing cells; data not shown.) Transfer vector RNAs with the UTR deletions, AG002 and AG004, were propagated with ~2- to 4-fold-reduced efficiency, while those with the deletion/insertions were propagated with a 1- to 6-fold reduction compared to MB15 (Table 1). Consistent with the packaging data, vectors with only 90 bp of UTR (AG002 and AG013) exhibited the greatest reduction in titers (~4- and 6-fold), reflecting their poor packaging efficiency, while vectors with 120 (AG003 and AG014) or 150 (AG004 and AG015) bp of UTR resulted in near-wild-type titers (within 1- to 2.5-fold) either with or without insertions, reflecting the improved packaging efficiencies observed with these vectors (Table 1).

To determine the effect of mutations on any putative secondary RNA structure that this region may assume, the region between R and the first 333 bp of FIV *gag* was folded using the RNAstructure program (8). Similar to the case with human immunodeficiency virus, simian immunodeficiency virus, and Mason-Pfizer monkey virus (4, 5, 9, 11), this region folded into several stem-loops, of which four were stable, named stem-loops 1 to 4 (SL1 to SL4). Folding of the mutant vector RNAs revealed that most of the conformation of the wild-type UTR was destroyed in the vectors with only 90 and 120 bp of the UTR, except for SL1, which was conserved in all transfer vector RNAs (Fig. 3C to F). However, since it was present both in RNAs that were packaged efficiently and in those that were packaged poorly, SL1 does not seem to play a significant role as a packaging determinant (Fig. 3C to H).

SL2, an unusually large stem-loop found within the first ~180 bp of the UTR (the region directly tested in our deletion analysis), was the most consistently formed stem-loop structure

TABLE 1. Sequences intervening between the core packaging determinants of FIV do not affect vector RNA propagation significantly

| Vector name | Length of 5′ UTR/*gag* maintained (bp) | Length of heterologous sequence (bp) | Normalized 293T titer (CFU/ml[a]) | Relative titer[b] | Fold reduction[c] |
|---|---|---|---|---|---|
| AG002 | 90/100 | | 600 ± 58 | 0.27 | 3.7 |
| AG003 | 120/100 | | 884 ± 112 | 0.40 | 2.5 |
| AG004 | 150/100 | | 1238 ± 103 | 0.56 | 1.8 |
| AG013 | 90/100 | 180 | 402 ± 69 | 0.18 | 5.5 |
| AG014 | 120/100 | 150 | 1,235 ± 93 | 0.55 | 1.8 |
| AG015 | 150/100 | 120 | 1,835 ± 73 | 0.82 | 1.2 |
| MB15 | 270/100 | | 2,229 ± 18 | 1.00 | 1.0 |
| TR394 | 270/333 | | 2,261 ± 219 | 1.01 | 1.0 |
| Mock | No DNA | | <1 | | |

[a] CFU per ml of supernatant in the transfected cultures (mean ± standard deviation). The raw titers were normalized to the luciferase expression observed in the transfected cultures as well as the amount of virus produced. These data are representative of two experiments performed in triplicate.

[b] Titer produced by each construct relative to that of MB15. No hygromycin-resistant colonies were observed for any of the transfer vector or packaging constructs when transfected alone.

[c] *n*-fold reduction in RNA propagation.

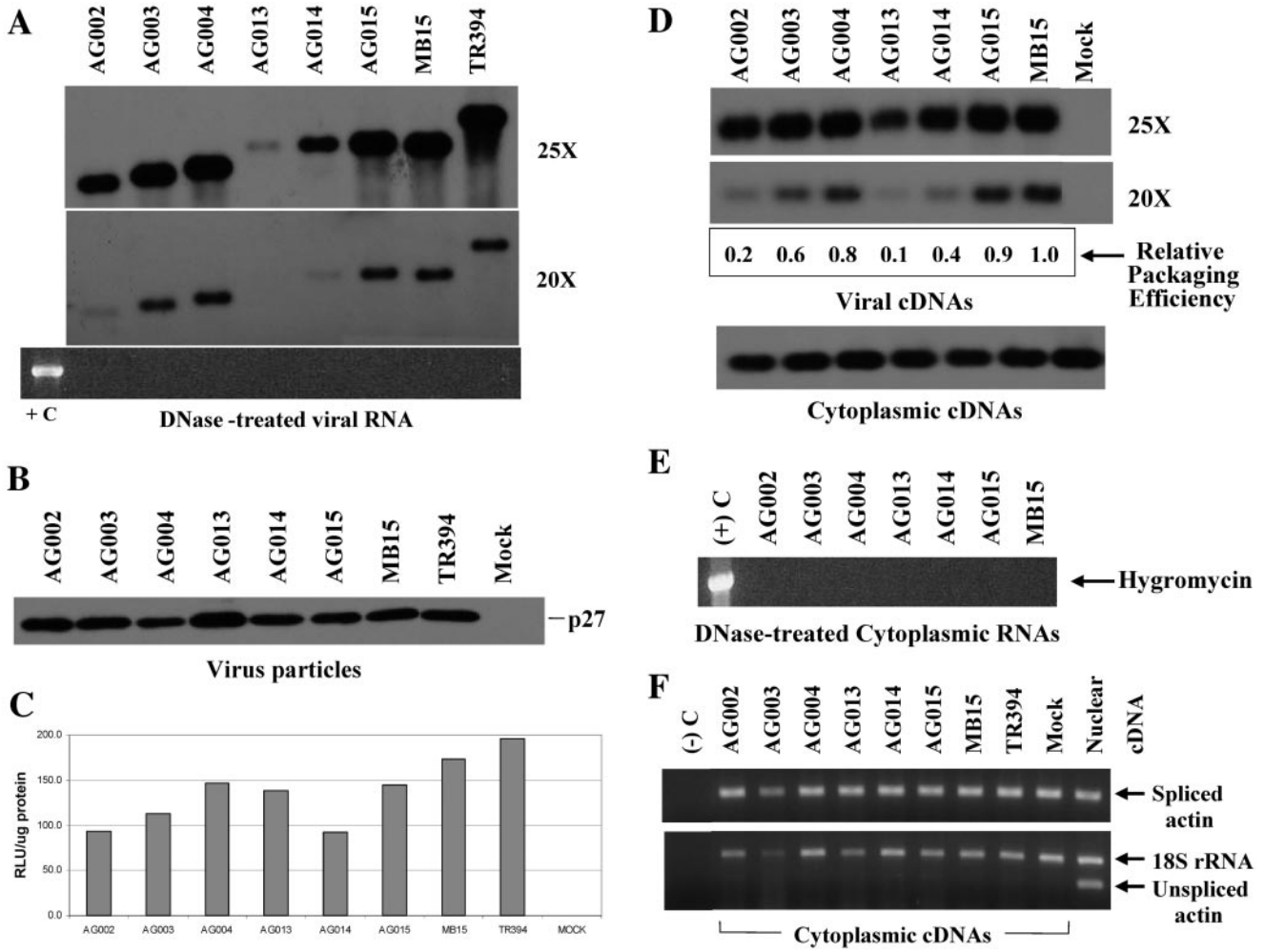


FIG. 2. The region intervening between the two core packaging determinants is not necessary for FIV RNA packaging. (A) RT-PCR of the viral cDNAs amplified using OTR600 (sense, 5′ GAACCCTGTCGAGTATCGTG 3′; FIV nucleotides 286 to 306 of FIV 5′ R region) and OTR601 (antisense, 5′ CTGGTTGCTGACTAATTGAG 3′; nucleotides 1840 to 1821 of pcDNA3 within the simian virus 40 promoter) and probed with an R/U5 probe using the nonradioactive AlkPhos direct labeling kit from Amersham (IL). To ensure that the amplifications were in the linear range of detection, PCRs were conducted for 20, 25, and 30 cycles. The bottom panel represents DNase-treated viral RNA which was amplified using hygromycin-specific primers to confirm the absence of plasmid DNA; +C, plasmid as a positive control. (B) Western blot analysis of equivalent amounts of the remaining one-third portion of purified virus harvested from transfected cultures using the FIV polyclonal antiserum, QB2, from infected cats. Mock, no DNA. (C) Transfection efficiencies observed for different constructs were assessed by the firefly luciferase activity from the cotransfected pGL3 control DNA using the Dual Luciferase assay kit. RLU, relative light units. (D) Semiquantitative RT-PCR of the viral cDNA amplified using OTR660 (sense, 5′ GAGGACTTTTGAGTTCTCCCTTGAGGC 3′; nucleotides 230 to 256 of FIV 5′ long terminal repeat) and OTR662 (antisense, 5′ AGCAGGAGTTCTGCTTAACAGCTTTC 3′, nucleotides 440 to 415 of FIV 5′ long terminal repeat), which gives the same size of fragments, and probed using the R/U5 probe. The chemiluminescent signals emitted were captured on X-ray films for various lengths of time and digitized using the Biometra gel documentation system (Germany). The lower panel shows semiquantitative RT-PCR conducted for the cytoplasmic RNAs using the same primer set as for the viral RNA to exclude the possibility that the variable packaging efficiency observed was due to poor expression, instability, or deficient nucleocytoplasmic transport of transfer vector RNAs. Cytoplasmic RNAs were tested by variable-cycle RT-PCR and found to be stably expressed and efficiently transported to the cytoplasm of the transfected cells. (E) DNase-treated cytoplasmic RNA amplified using hygromycin-specific primers to confirm the absence of plasmid DNA. (+) C, plasmid as a positive control. (F) Control for nucleocytoplasmic fractionation technique. To ensure that the nucleocytoplasmic fractionation technique was reliable, cytoplasmic RNAs were tested for the presence of unspliced β-actin mRNA found exclusively in the nucleus unless the nuclear membrane integrity has been compromised (13). Only the spliced β-actin mRNA was observed in the cytoplasmic fractions (upper panel), while the unspliced RNA was undetectable with 30 rounds of PCR even though it could be detected in the nuclear fraction (lower panel, last lane). To ensure that each cytoplasmic sample in the unspliced β-actin PCRs contained amplifiable cDNAs, the unspliced β-actin PCR was conducted in the presence of primers/competimer for 18S ribosomal RNAs as an internal control. The nucleotide sequences are based on the FIV Petaluma (34TF10) strain (GenBank accession number M25381) (12). (−) C, no template control.

found to be associated with enhanced packaging (Fig. 2 and 3). It was completely absent in vectors with 90 bp of UTR (AG002 and AG013) (Fig. 3C and D). However, in the presence of 120 bp of UTR in AG003 and AG014, the first few loops of SL2 started to emerge, irrespective of the insertion (Fig. 3E and F), while in the presence of 150 bp of UTR in AG004 and AG015, two-thirds of SL2 had reformed (Fig. 3G and H). Even restoration of the first two bulges of SL2 formed within 120 bp of

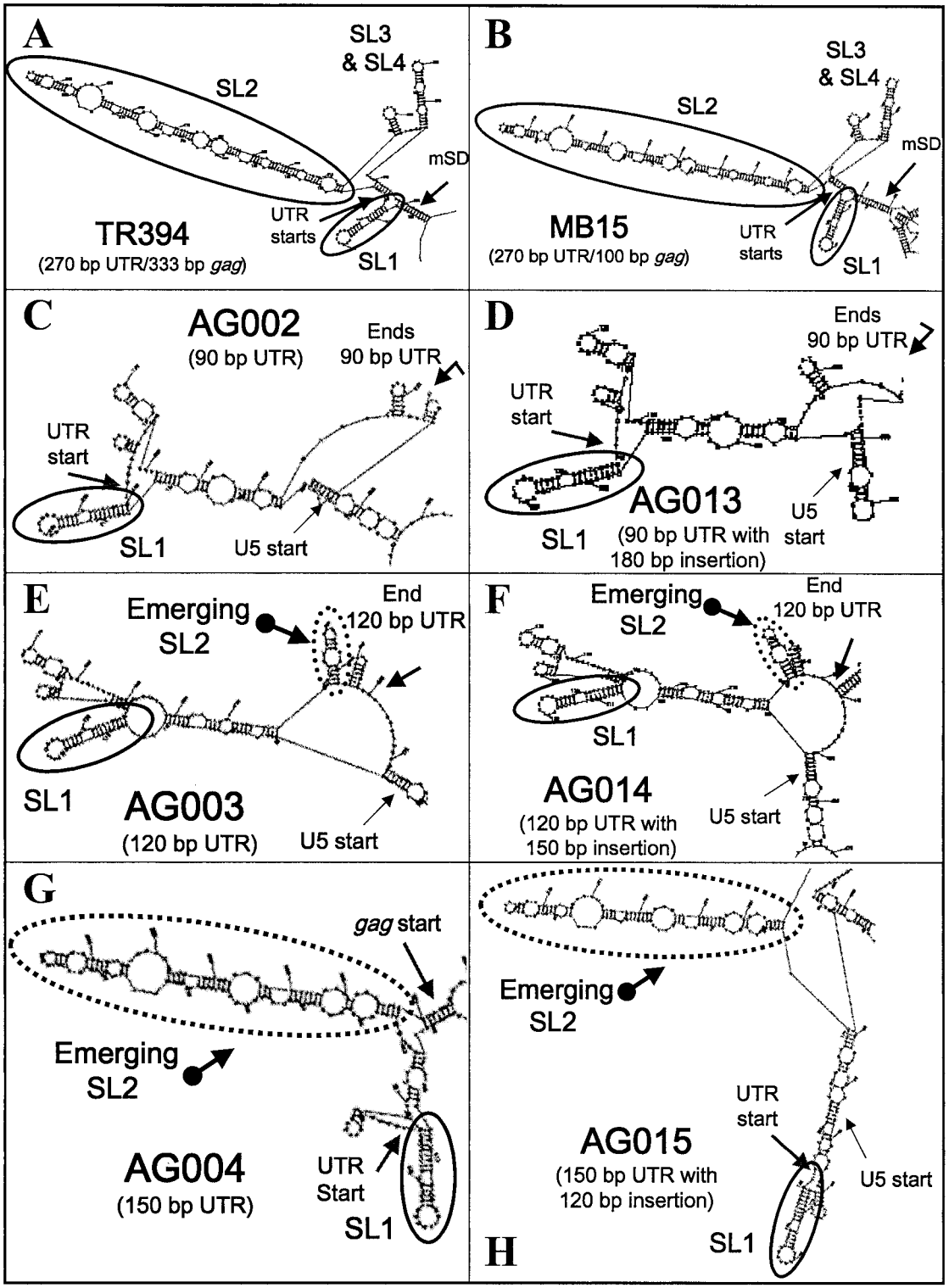


FIG. 3. Folding potential of the control and mutant transfer vector RNAs with either deletions or deletions/insertions in between the two core elements using the RNAstructure program (version 3.6) (8). SL1 and -2 have been highlighted by solid ovals. The emerging SL2 loop observed in AG003 and AG014 and in AG004 and AG015 has been encircled by dashed ovals and highlighted by the arrows with knobs at the end. A variable number of structures was predicted for each of the transfer vector RNAs (TR394, 4; MB15, 2; AG002, 5; AG003, 6; AG004, 2; AG013, 7; AG014, 6; and AG015, 4), of which the most stable structure is depicted. SL3 and -4 were not found to correlate with the functional packaging data, since they were found within a region deleted in our constructs.

UTR improved packaging significantly (0.62 and 0.40 for AG003 and AG014, respectively), while emergence of two-thirds of SL2 restored packaging nearly to that of MB15 (0.80 and 0.92 for AG004 and AG015, respectively) (Fig. 3G and H). The reformation of partial SL2 in vectors with 120- and 150-bp UTR sequences (irrespective of the insertions) suggests that FIV may not need the entire SL2 for packaging, but only a part of this structure that is probably sufficiently formed by the presence of sequences within ~150 bp of the 5′ UTR for efficient packaging.

Kemler et al. (6) mutated part of the region encompassing the fourth stem/bulge region of SL2 but observed no effect of the mutations on packaging (6). Our structural analysis indicates that their mutations did not really perturb the structure of SL2 enough to affect packaging due to its size and stability ($\Delta G = -75.5$) (data not shown), and perhaps more drastic mutations (large deletions) will be needed to affect RNA packaging significantly, such as those in AG002.

Together, this study reveals that the sequences intervening between the core packaging determinants are dispensable not only for efficient FIV RNA packaging but for vector RNA propagation as well. These data should enhance our understanding of how complex retroviruses package their genomic RNAs and help streamline the design of FIV-based transfer vectors for human gene therapy.

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