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Sequences within the *gag* gene of feline immunodeficiency virus (FIV) are important for efficient RNA encapsidation

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Abstract

Feline immunodeficiency virus (FIV)-based retroviral vector systems are being developed for human gene therapy. Consequently, it has become important to know the precise sequence requirements for the packaging of FIV genome so that such sequences can be eliminated from transfer vectors post-transduction for improved safety. Recently, we have shown that sequences both within the 5′-untranslated leader region (UTR) and the 5′-end of *gag* are required for efficient packaging and transduction of FIV-based vectors. However, the extent of *gag* sequence important in the encapsidation process is not clear as well as their relative contribution to packaging. In this study, using a biologically relevant packaging system, we demonstrate that at the most 100 bp of *gag* sequences are sufficient for efficient RNA packaging in conjunction with the 5′-UTR and no other sequences within the next 600 bp of *gag* exist that affect packaging. In addition, we show that sequences within *gag* do not simply act as spatial elements to stabilize other structural determinants of packaging located within the 5′-UTR, but are important in themselves for the encapsidation process.

Keywords: Feline immunodeficiency virus; RNA packaging; Packaging signal; *gag*; Retroviral vectors; Human gene therapy

1. Introduction

Retroviral RNA packaging is a highly specialized process that involves the specific incorporation of two copies of genomic RNA into the nascent virus particles (reviewed in Swanstrom and Wills, 1997). This process requires an interaction between a *cis*-acting sequence element, "the packaging or encapsidation signal" (ψ or *E*) located at the 5′-end of the viral genome with the virally encoded *gag* precursor proteins (reviewed in Lever, 2000). Although, the precise sequence requirement for packaging varies among different retroviruses, in general, the packaging signal includes sequences within the 5′-untranslated leader region (UTR) and sometimes sequences within the *gag* gene itself. For many retroviruses, such as spleen necrosis virus (SNV; Watanabe and Temin, 1982), avian leukosis-sarcoma virus (ALSV; Banks et al., 1998), feline leukemia virus (FeLV; Burns et al., 1996), simian immunodeficiency virus (SIV; Rizvi and Panganiban, 1993) and human immunodeficiency virus type 2 (HIV-2; McCann and Lever, 1997; Poeschla et al., 1998a; Kaye and Lever, 1999), the core packaging signal seems to reside within the 5′-UTR. In other retroviruses, such as murine leukemia virus (MLV; Bender et al., 1987; Adam and Miller, 1988), HIV-1 (Hayashi et al., 1992; Luban and Goff, 1994; McBride and Panganiban, 1996, 1997; reviewed in Lever, 2000) and bovine leukemia virus (BLV; Mansky et al., 1995; Mansky and Wisniewski, 1998), sequences in both the 5′-UTR and *gag* have been shown to be involved in efficient packaging. Sequences elsewhere in the viral genome have also been shown to affect RNA packaging, such as TAR loop and R/U5 sequences in HIV-1 (McBride et al., 1997; Helga-Maria et al., 1999; Harrich et al., 2000), as well as sequences in

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the 3′-end of the viral genome in Rous sarcoma virus (RSV; Sorge et al., 1983) and MLV (Yu et al., 2000).

The core packaging signal of feline immunodeficiency virus (FIV) is also thought to be located at the 5′-end of the viral genome since presence of the 5′-UTR and the first 350 bp of the *gag* gene are sufficient for efficient packaging of FIV vector RNAs (Poeschla et al., 1998b; Johnston et al., 1999; Browning et al., 2001). However, the precise sequence requirement for FIV RNA packaging remains unknown. Recently, we have shown that simultaneous presence of sequences within both the 5′-UTR and *gag* is crucial for efficient packaging in FIV (Browning et al., 2003). In this study, we determined the importance and extent of sequences within *gag* for FIV RNA packaging. Our results demonstrate that the first 100 bp of FIV *gag* sequences are sufficient for efficient RNA encapsidation and these sequences do not serve as mere spacer elements to stabilize other encapsidation determinants in the 5′-UTR.

2. Materials and methods

2.1. Numbering system

Nucleotide designations for $FIV_{Petaluma}$ (34TF10) strain are based on GenBank accession number M25381 (Talbott et al., 1989).

2.2. Plasmid construction

2.2.1. FIV expression constructs and control transfer vector

FIV packaging construct, MB22 (Fig. 1), expresses the *gag/pol* genes and has been described before (Browning et al., 2001). The vesicular stomatitis virus envelope glycoprotein (VSV-G) expression construct, MD.G (Fig. 1), was kindly provided by Dr. Didier Trono (Salk Institute, La Jolla, CA; Naldini et al., 1996). The control vector, TR394 (Fig. 1), contains the entire 5′-UTR and 333 bp of FIV *gag* in addition to *cis*-acting sequences needed for RNA propagation and has been described before (Browning et al., 2001).

2.2.2. FIV transfer vectors with incremental insertions of gag

MTB (Fig. 2A) contains the entire FIV 5′-UTR, but no *gag* sequences and was constructed by using a derivative of TR394, MTA, in which the hygromycin gene expressed from the SV40 promoter (SV-Hyg^r cassette) was inserted at the *Cla*I site (Browning et al., 2003). MB15–21 (Fig. 3) contain increasing amounts of *gag* sequences from the start of the *gag* gene to 700 bp in *gag* in 100 bp increments. MB15–21 were created by a modification of MTB in which the region between *Xho*I (nt 501) and the artificially created *Not*I site (immediately upstream of the SV-Hyg^r cassette) was replaced with PCR products containing part of the 5′-UTR and 100 bp incremental additions of *gag* starting from 100 to 700 bp. These PCR products were generated using 34TF10 as the template, OTR436 as the sense oligonucleotide and individual antisense oligonucleotides listed in Table 1. Each PCR product was digested with *Xho*I and *Not*I and ligated into *Xho*I site at FIV (nt 501) and *Not*I site located immediately 5′ to the SV-Hyg^r cassette of MTB.

2.2.3. Transfer vectors with heterologous sequences juxtaposed to the 5′-UTR

MB23–29 (Fig. 4) were also created using the base vector, MTB. The heterologous non-coding sequences were obtained from pCDNA3 (Invitrogen, Carlsbad, CA) and inserted into the region between the start of the *gag* ORF and the beginning of the SV-Hyg^r cassette in MTB. The oligonucleotides used to amplify the heterologous sequences contained flanking *Not*I sites, allowing PCR products to be ligated into the *Not*I site of MTB. Each reaction used the antisense oligonucleotide OTR480 and individual sense oligonucleotides listed in Table 1. PCR products were digested with the flanking *Not*I sites and ligated into the *Not*I site of MTB in the correct orientation.

All PCR amplifications were conducted on the Perkin–Elmer 9600 thermocycler using the GeneAmp PCR reagent kit (PE Applied Biosystems, Foster City, CA). The plasmids were constructed by standard molecular cloning techniques through several stages of cloning, generating a number of intermediate clones, the details on the design and construction of which can be obtained from the authors upon request.

2.3. Transfections and infections of cells

Cos cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone, Logan, UT), while HeLa CD4$^+$ cells were grown in DMEM supplemented with 7% calf serum (Hyclone). For details on the transfection of Cos cells and infection of HeLa CD4$^+$ cells, see Browning et al. (2001).

2.4. Western blot and slot blot analyses of viral particles

Virions were purified from the clarified supernatants of transfected cultures (9 ml) by ultracentrifugation as described previously (Browning et al., 2001). Viral proteins were analyzed by Western blot analysis using the Enhanced Chemiluminescence kit (Amersham, Arlington Heights, IL) as described (Rizvi et al., 1997) using a polyclonal antisera from cats infected with the Petaluma strain of FIV. The antisera were kindly

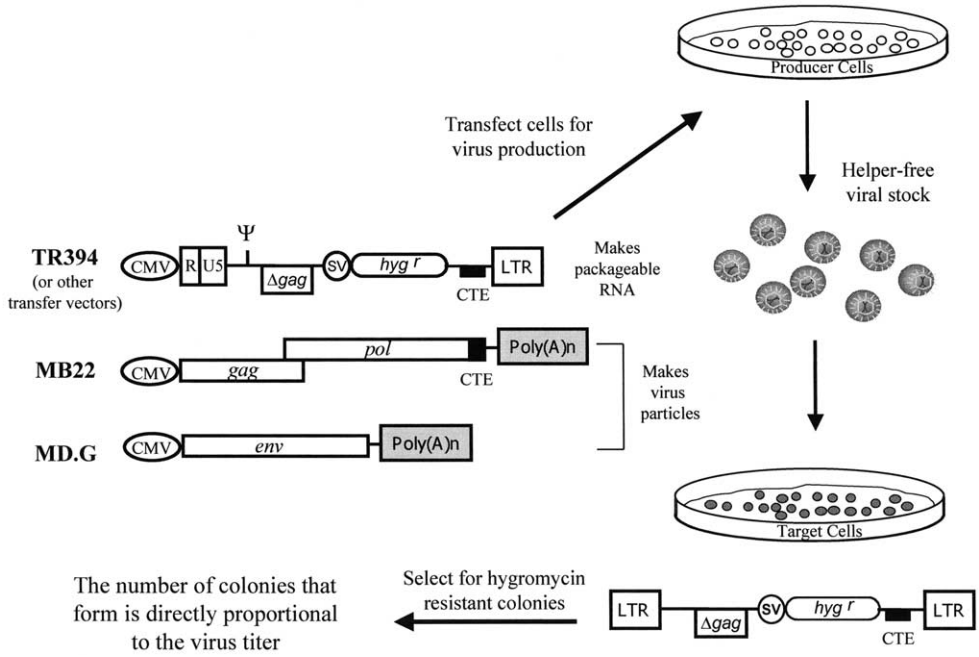


Fig. 1. Design and rationale of FIV 3-plasmid packaging and transduction assay. The retroviral genome is packageable by virtue of the presence of the packaging signal, ψ, and structural proteins that encapsidate the genomic RNA. RNA expressed from the transfer vector, TR394, or other similar vectors, is the packageable component due to the presence of ψ and contains Hyg^r gene as a selectable marker. TR394 contains a chimeric LTR promoter to allow its expression in human and primate cells. During the process of reverse transcription, the U3 region from the 3′-LTR is copied into the chimeric 5′-LTR, reconstituting the complete LTR at the 5′-end. The *gag*/*pol* expression construct, MB22, expresses the structural proteins, and the *env* expression construct, MD.G, expresses VSV-G necessary for viral particles to infect cells expressing the corresponding receptor. The three plasmids are transfected together in the "producer" Cos cells that generate "helper-free" virus stocks containing the transfer vector RNA which are subsequently used to infect target cells. Since these virions have only the transfer vector RNA as the genome, they are deficient for further rounds of replication, resulting only in a single round of replication. The successfully infected cells are selected by the use of hygromycin with the Hyg^r colonies obtained being proportional to the virus titer. Individual colonies can then be further analyzed for the amount of RNA packaged.

provided by Dr. Ellen Collisson (Texas A&M University, College Station, TX).

The slot blots were performed on virion and cellular RNAs isolated from transfected Cos cultures 72 h post-transfection using QiaAmp viral RNA Kit (Qiagen, Valencia, CA) and Qiagen RNeasy Total RNA Kit, respectively. Both virion and cellular RNAs were treated with RNase-free DNase I (Life Technologies, Gaithersburg, MD) prior to blotting on slot blots for analysis as described previously (Rizvi et al., 1996b, 1997). The membranes, hybridized with radiolabelled probes, were exposed to X-ray film (Kodak X-OMAT) for variable lengths of time (Browning et al., 2001). Band intensities in the slot blots were determined by electronically scanning the autoradiographs and quantitating the optical density in individual bands using the Molecular Analyst software (version 1.1) from BioRad Laboratories (Hercules, CA). Packaging efficiency of each vector RNA was determined by first subtracting background densities observed in mock transfections from test values, and dividing each with the background-subtracted values obtained from the control vector, TR394. Next, values obtained from the three dilutions of each RNA sample were averaged and the packaging efficiency normalized to the amount of RNA loaded and transfer RNA expressed as described in Schmidt et al. (in press). Finally, the fold reduction in packaging efficiency was obtained by dividing the packaging efficiency obtained for each construct with that of TR394.

Similarly, vector titer in Fig. 2 was normalized to the amount of virions produced as described in Schmidt et al. (in press). Briefly, optical density observed in the Western blot of virus particles in culture expressing MTB was divided with that observed for TR394 after subtraction of background. Next, the raw titer observed for MTB was divided by the factor obtained to calculate the normalized viral titers. Finally, fold reduction in viral titer was obtained by dividing the normalized viral titers with that observed for TR394.

3. Results

3.1. Sequences in gag are important for packaging in FIV

To establish the role of *gag* sequences in FIV genomic RNA encapsidation, we utilized the classical three-plasmid *trans* complementation assay developed earlier

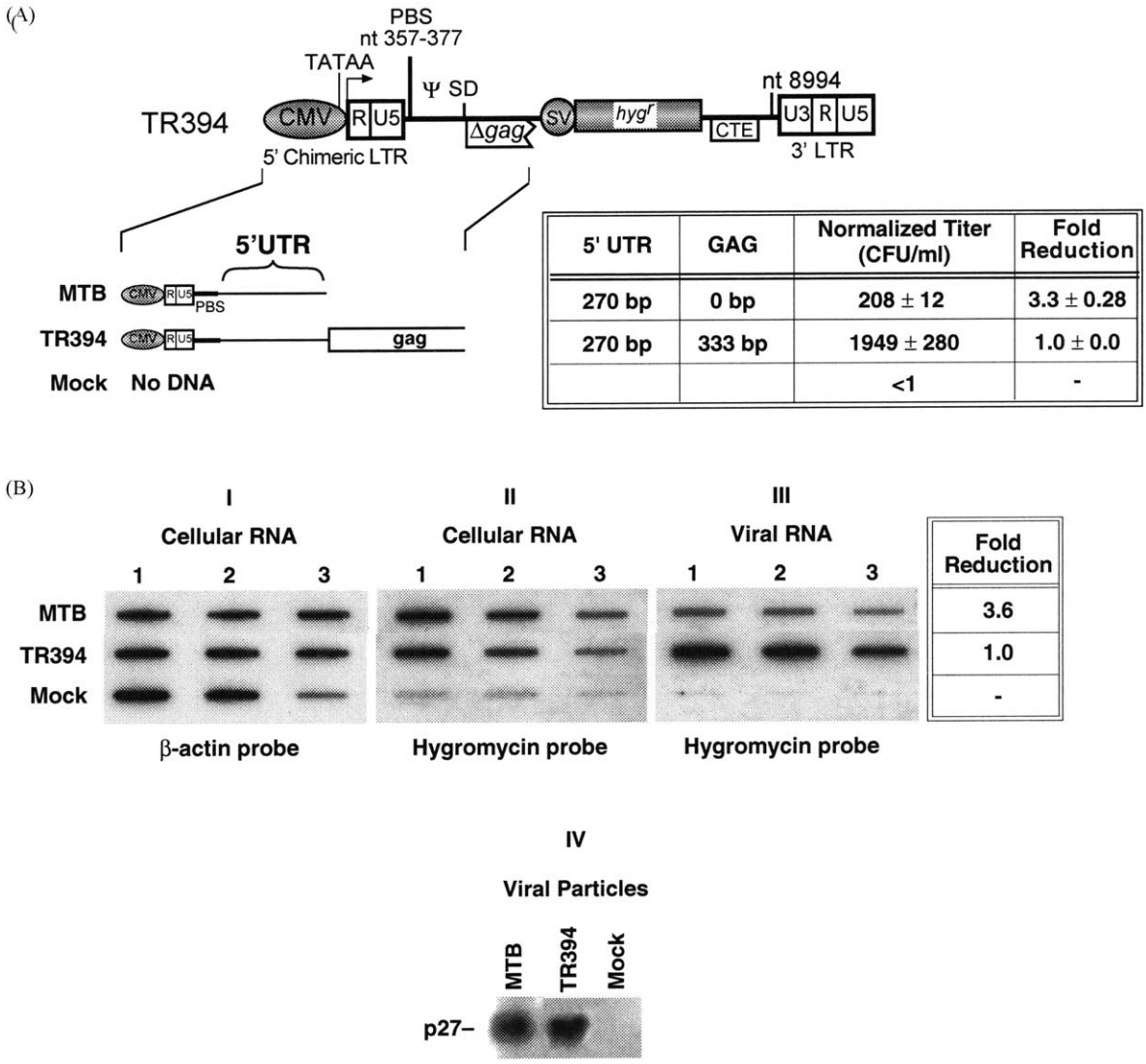
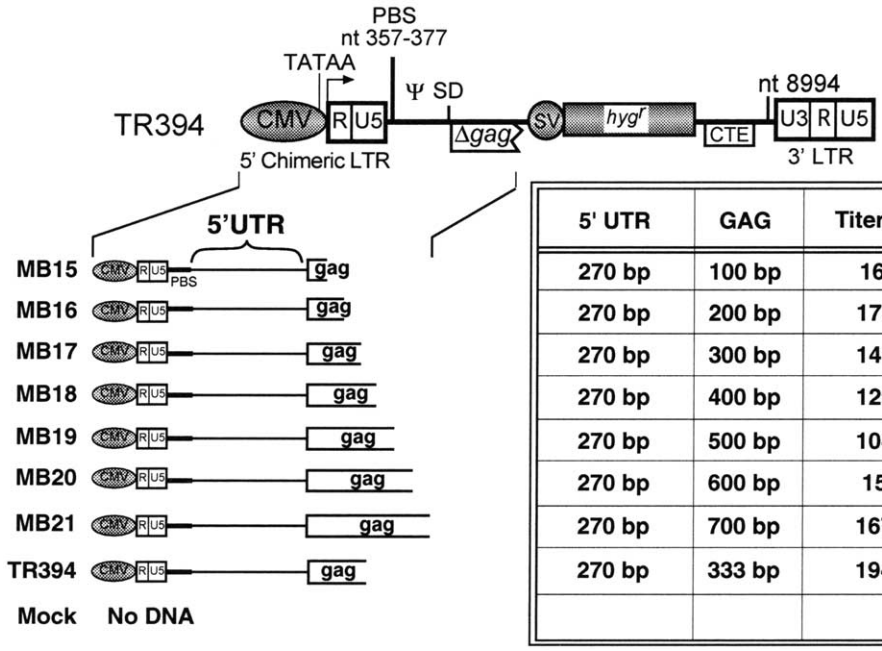


Fig. 2. FIV *gag* sequences are important for the efficient packaging of FIV genome. (A) Schematic representation of FIV transfer vector MTB containing the entire 270 bp of the 5′-UTR in the absence of any *gag* sequences. The relative titers obtained as colony-forming units per milliliter (CFU/ml) of virus stock upon transduction of the transfer vectors into the target cells are indicated to the right. The value of each titer represents a mean of three independent experiments performed in duplicate along with the standard deviation. No Hyg^r colonies were observed for any of the transfer vectors or packaging and *env* expression constructs when transfected alone. (B) RNA slot blot and Western blot analyses of virus particles and cellular RNAs isolated from Cos cells transfected with MTB along with the packaging (MB22) and *env* (MD.G) expression contructs from one representative experiment. Panels I and II are slot blots of cellular RNAs, while panel III is slot blot of virion RNA isolated from transiently transfected Cos cells. RNAs were DNase-treated, diluted and subjected to slot blot analysis. Panel I represents a 1:2 dilution of 0.5 μg of cellular RNA (0.5, 0.25 and 0.125 μg) probed with a fragment of a housekeeping gene, β-actin, while panel II represents a 1:3 dilution of 1.5 μg of cellular RNA (1.5, 0.5 and 0.167 μg) probed with a fragment of the hygromycin gene. Panel III represents a 1:2 dilution of purified virion RNA isolated from the equivalent of 3/4 portion of 9 ml media containing virus particles, also probed with a fragment of the hygromycin gene. The column to the right of panel III refers to the fold reduction in packaging efficiency relative to TR394 after normalization for RNA loading and transfection efficiency (see Sections 2 and 3). Panel IV shows Western blot analysis of equivalent amounts of the remaining 1/4 portion of the purified virions harvested from each of the transfected cultures in the corresponding *trans* complementation assay using FIV antiserum from infected cats. Mock, cells transfected without DNA.

to study retroviral replication (Browning et al., 2001; Naldini et al., 1996; White et al., 1999) using the Petaluma strain of FIV (Talbott et al., 1989). Briefly, the assay used two expression plasmids, MB22 (Browning et al., 2001) and MD.G (Naldini et al., 1996), to provide the FIV *gag*/*pol* and VSV-G, respectively (Fig. 1). A simple FIV vector, TR394, containing minimum *cis*-acting sequences required for packaging, reverse transcription, and integration, as well as the gene of choice to be transduced, served as a source of the packageable vector RNA (Browning et al., 2001). Co-transfection of the three plasmids into producer cells resulted in the generation of FIV particles containing the vector RNA capable of transducing the marker gene



5' UTR	GAG	Titer (CFU/ml)	Fold Reduction
270 bp	100 bp	1675 ± 65	1.16 ± .12
270 bp	200 bp	1723 ± 160	1.13 ± .06
270 bp	300 bp	1419 ± 122	1.37 ± .08
270 bp	400 bp	1296 ± 216	1.50 ± .04
270 bp	500 bp	1040 ± 498	1.87 ± .84
270 bp	600 bp	1584 ± 96	1.23 ± .10
270 bp	700 bp	1675 ± 171	1.16 ± .05
270 bp	333 bp	1949 ± 280	1.0 ± 0.0
		<1	

Fig. 3. Additional sequences in *gag* after 100 bp do not further enhance the packaging efficiency of FIV RNA. Schematic representation of FIV transfer vectors MB15–21 containing incremental increases in *gag* starting from 100 to 700 bp in the presence of the entire 270 bp of 5′-UTR. The relative titers obtained as colony-forming units per milliliter (CFU/ml) of virus stock upon transduction of the transfer vectors into the target cells are indicated to the right. The value of each titer represents a mean of three independent experiments performed in duplicate along with the standard deviation. Mock, cells transfected without DNA.

into target cells with no further generation of replication competent virus (Fig. 1).

Using various modifications of the control transfer vector, TR394, in the in vivo packaging assay, we have recently shown that 5′-UTR on its own is not sufficient for efficient packaging in FIV (Browning et al., 2003). This was accomplished by generating a transfer vector, MTB, which contains the entire 5′-UTR in the complete absence of any *gag* sequences (Fig. 2). Virus from co-transfected Cos cells was harvested 72 h post-transfection and processed for virion RNA and proteins, as well as used to infect human HeLa $CD4^+$ cells, while the transfected Cos cells were processed for cellular RNA extraction. Finally, the infected cultures were selected with medium containing hygromycin B to identify successfully transduced cells by the appearance of hygromycin-resistant (Hyg^r) colonies 10 days post-infection (Fig. 1).

Test of MTB in the *trans* complementation assay revealed nearly a threefold loss in viral titers compared to TR394, the control vector that contains 333 bp of *gag* (Fig. 2A) (the propagation efficiency of TR394 was assigned a value of 1 to allow easy comparison among different constructs). This reduction in viral titers was observed despite the fact that similar amounts of viral particles were produced in each of the transfected cultures, as assessed by analysis of p27 *gag* proteins produced by purified virions in the culture supernatants (Fig. 2B, panel IV). A direct analysis of RNA packaged by the virions revealed that along with the threefold drop in viral titers, there was a concomitant loss in packaging in the absence of any *gag* sequences (Fig. 2B, panel III). The reduced packaging observed with MTB was not due to effects on RNA stability or expression of the transfer vector in the transfected cells, as assessed by slot blot analysis of dilutions of cellular RNAs probed either with a β-actin cDNA probe or hygromycin radiolabelled DNA fragments (Fig. 2B, panels I and II). Normalization of the packaging efficiency with the amount of cellular RNA loaded and the transfection efficiency of each construct revealed a 3.6-fold loss in packaging compared with the control vector, TR394, directly correlating with the reduction in the propagation efficiency of the transfer vector RNA. Once again, TR394 was assigned a packaging efficiency of 1 to allow for easy comparison among different constructs. These data revealed that *gag* sequences are indeed important for efficient packaging of FIV RNA.

3.2. The first 100 bp of gag is sufficient for efficient FIV RNA packaging and up to 600 bp further downstream do not affect this process

Next, we determined the extent of sequences in *gag* required for the encapsidation process. Towards this end, we generated a series of FIV transfer vectors, MB15–21, that incorporated *gag* sequences in MTB from 100 to 700 bp in 100 bp increments in the presence

Table 1
Primers used to introduce additional sequences into FIV transfer vectors

Vector	Primer	Sequence	Comments
MB15	OTR503	(AS; 5′-aaaaaagcggccgcTCCCTTCTCCAAATTTTTTACTCTTCC-3′; nt 727–901)	Antisense (AS) primers OTR499, OTR503–508 along with the sense (S) primer, OTR436, were used to introduce 100–700 bp of FIV *gag* in 100 bp increments, into MTB, creating MB15–21; lowercase denotes artificial sequences introduced into the primers for cloning purposes, while uppercase denotes FIV sequences
MB16	OTR504	(AS; 5′-aaaaaagcggccgcAAATCGCAAATAACCAACCTTAGT-3′; nt 827–804)	
MB17	OTR505	(AS; 5′-aaaaaagcggccgcCACCGTCATATTTAAAAGTCC-3′; nt 927–907)	
MB18	OTR506	(AS; 5′-aaaaaagcggccgcCCTGTGGAGGGCCTTCCTCTT-3′; nt 1027–1007)	
MB19	OTR499	(AS; 5′-aaaaaagcggccgcCCTCCTAGTCCTTCTCTTGCC-3′; nt 1127–1107)	
MB20	OTR507	(AS; 5′-aaaaaagcggccgcTTTATCTGCAGCGCACCCTGG-3′; nt 1227–1207)	
MB21	OTR508	(AS; 5′-aaaaaagcggccgcTTTCTGCTGCAGTAAAATAGGGTA-3′; nt 1327–1304)	
	OTR436	(S; 5′-TGACCTAAATAGGGAAGCAGT-3′; nt 440–460)	
MB23	OTR481	(S; 5′-aaaaaagcggccgcGGGATTTTGGTCATGAGATTA-3′; nt 4340–4360)	Sense primers OTR481–487, along with the antisense primer, OTR480, were used to introduce 100–400 bp of heterologous sequences in 50 bp increments from the cloning vector, pCDNA3, into MTB, creating MB23–29; the lowercase denotes artificial sequences introduced into the primers for cloning purposes, while the uppercase denotes pcDNA3 sequences
MB24	OTR482	(S; 5′-aaaaaagcggccgcTGATCTTTTCTACGGGGTCTG-3′; nt 4290–4310)	
MB25	OTR483	(S; 5′-aaaaaagcggccgcTTGCAAGCAGCAGATTACGCG-3′; nt 4240–4260)	
MB26	OTR484	(S; 5′-aaaaaagcggccgccTTCGGAAAAAGAGTTGGTA-3′; nt 4171–4189)	
MB27	OTR485	(S; 5′-aaaaaagcggccgcTTGGTATCTGCGCTCTGCTGA-3′; nt 4140–4160)	
MB28	OTR486	(S; 5′-aaaaaagcggccgcAGAGTTCTTGAAGTGGTGGCC-3′; nt 4090–4110)	
MB29	OTR487	(S; 5′-aaaaaagcggccgcGCAGCCACTGGTAACAGGATT-3′; nt 4040–4060)	
	OTR480	(AS; 5′-aaaaaagcggccgcgaattcCAAGTTTACTCATATATACTTTAGATT-GAT-3′; nt 4440–4411)	

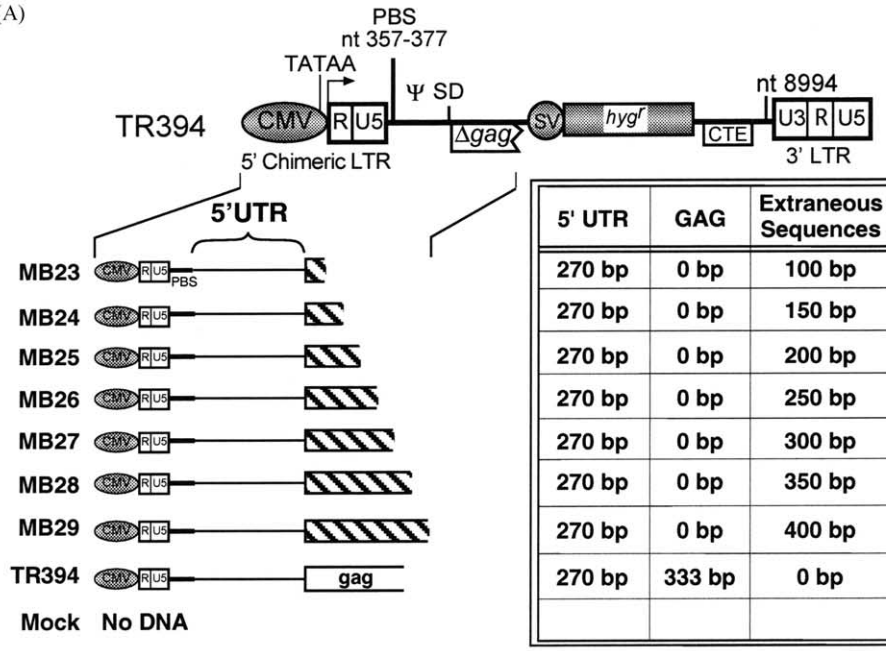
of the entire 5′-UTR (Fig. 3). Test of these constructs in the in vivo packaging and transduction assay revealed that the addition of 100 bp of *gag* restored the packaging efficiency of MB15 to that of the control vector, TR394, as assessed by the number of Hyg^r colonies observed (Fig. 3). Further incremental addition of up to 700 bp of *gag* did not increase or decrease the vector titers any further, within the standard deviation, revealing that there are no other sequence elements 700 bp downstream of the *gag* initiator codon that influence packaging in FIV. We did not directly analyze RNA packaged by the virions in this series of constructs since test of MTB (Fig. 2) and other FIV transfer vectors (Browning et al., 2003) have revealed that the transduction efficiency correlates directly with the packaging efficiency in our *trans* complementation assay in the presence of the complete 5′-UTR. Thus, these data demonstrate that the first 100 bp in *gag* is sufficient for efficient RNA packaging in FIV and no other sequences within the next 600 bp affect this process.

3.3. Sequences specific to gag and not irrelevant sequences juxtaposed to the 5′-UTR are required for efficient packaging of FIV RNA

Although the above results suggested that sequences in *gag* are important for FIV RNA packaging, the possibility remained that *gag* sequences themselves may not be involved in the encapsidation process, but perhaps may serve as spacer sequences important for the stability of a putative higher-order structure that the FIV 5′-UTR may assume. To address this possibility, we generated a series of transfer vectors, MB23–29, that contained the entire 5′-UTR juxtaposed to non-viral heterologous sequences from 100 to 400 bp in 50 bp increments (Fig. 4). Test of MB23–29 in the *trans* complementation assay revealed that the number of Hyg^r colonies did not increase with the addition of heterologous sequences of various lengths to the end of the 5′-UTR (Fig. 4A). Instead, there was a decline in titers from 9 ± 13- to 25 ± 21.3-fold with the addition of

Fig. 4. *gag*-specific sequences and not irrelevant sequences juxtaposed to 5′-UTR are required for efficient FIV RNA packaging. (A) Schematic representation of FIV transfer vectors MB23–29 containing heterologous sequences from 100 to 400 bp in 50 bp increments juxtaposed to the entire FIV 5′-UTR. The relative titers obtained as colony-forming units per milliliter (CFU/ml) of virus stock upon transduction of the transfer vectors into the target cells are indicated to the right. The value of each titer represents a mean of three independent experiments performed in duplicate along with the standard deviation, except in MB24 and 25 where the titer represents the average of two experiments performed in duplicate. (B) RNA slot blot and Western blot analyses of virus particles and cellular RNAs isolated from Cos cells transfected with the various transfer vectors as described in the legend to Fig. 2 from one representative experiment. Panels I and II are slot blots of cellular RNAs, while panel III is slot blot of virion RNA isolated from transiently transfected Cos cells. The column to the right of panel III refers to the fold reduction in packaging efficiency relative to TR394 after normalization for RNA loading and transfection efficiency (see Section 2). RNAs were DNase-treated, diluted and subjected to slot blot analysis as mentioned in the legend to Fig. 2. Panel IV shows Western blot analysis of purified virions harvested from each of the transfected culture supernatants as described in Browning et al. (2001). Mock, cells transfected without DNA.

(A)



5' UTR	GAG	Extraneous Sequences	Titer (CFU/ml)	Fold Reduction
270 bp	0 bp	100 bp	285 ± 53	9.0 ± .13
270 bp	0 bp	150 bp	283 ± 62	9.1 ± .44
270 bp	0 bp	200 bp	234	11.0
270 bp	0 bp	250 bp	185 ± 96	14.0 ± 6.8
270 bp	0 bp	300 bp	157	16.4
270 bp	0 bp	350 bp	124 ± 54	21.0 ± 6.9
270 bp	0 bp	400 bp	105 ± 68	24.6 ± 21.3
270 bp	333 bp	0 bp	2578 ± 457	1.0 ± 0.0
			<1	-

(B)

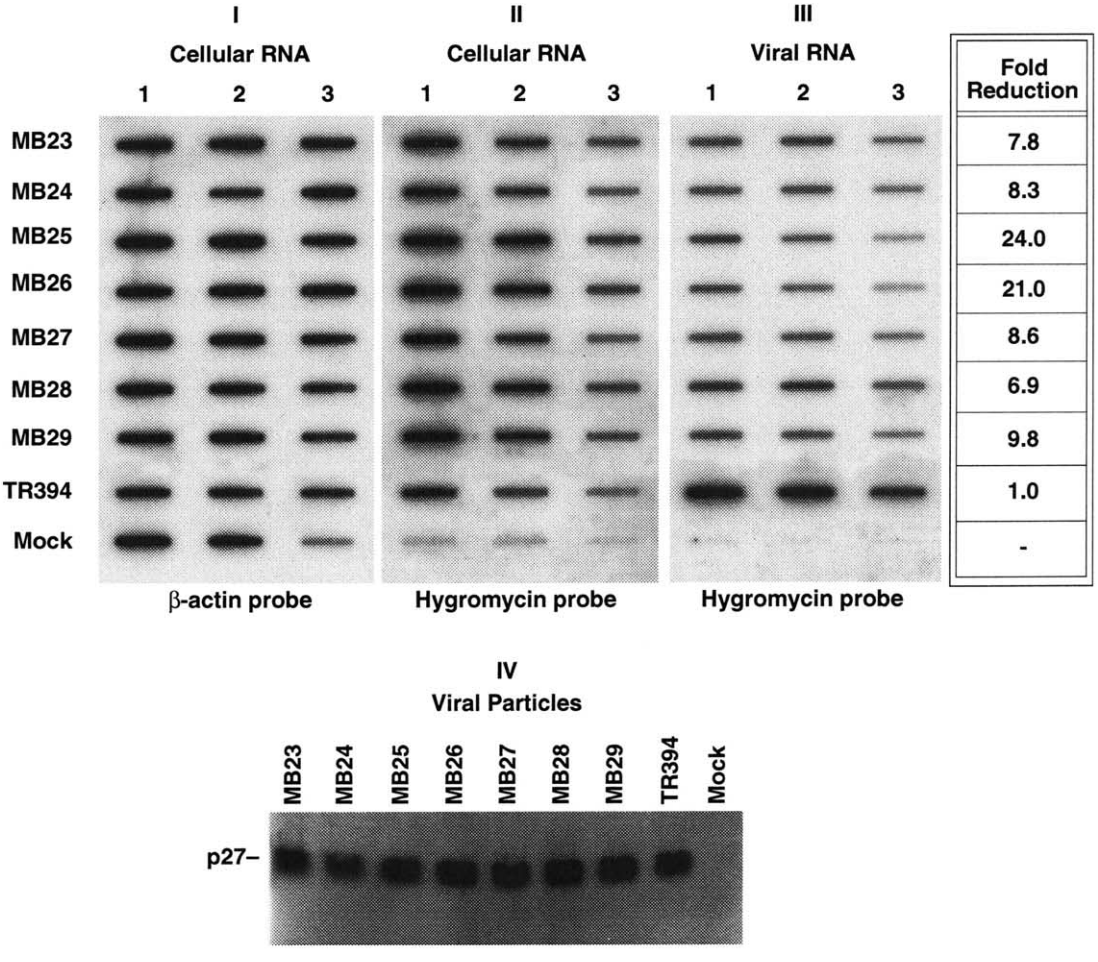


Fig. 4

extraneous sequences from 100 to 400 bp. This decline was approximately 3–4-fold more than what was observed with MTB, the vector that lacks any *gag* sequences in the presence of the entire 5′-UTR. The reduction in titers suggested that either packaging was being directly inhibited by the presence of heterologous sequences thereby affecting titers, or the heterologous sequences were interfering with other steps in vector RNA propagation.

To determine the effect of the heterologous sequences on packaging directly, the amount of virions produced in each transfection and the RNA packaged in these virions was assessed. Western blot analysis of virions purified by ultracentrifugation of clarified transfected culture supernatants revealed that similar amounts of viral p27 *gag* were produced in each of the transfected cultures (Fig. 4B, panel IV). Slot blot analysis of RNA packaged by the purified virus particles revealed that MB23–29 packaged lower levels of RNA into virions compared with TR394 (Fig. 4B, panel III). The reduction in RNA packaging corresponded to the loss in titers in the case of MB23 and 24, but not necessarily in the case of MB25–29. Other than MB25 and 26 that showed a sharp reduction in packaging efficiency (24- and 21-fold, respectively), MB23 and 24 and MB27–29 were packaged 7–10-fold less efficiently than TR394. In comparison, MTB, the vector that contained no *gag* sequences, was packaged with a 3.6-fold less efficiency than TR394 (Fig. 2). The 2–3-fold greater reduction in packaging efficiency observed with the vectors containing heterologous sequences suggests that part of the reduction in packaging observed was due to the lack of *gag* sequences required for efficient encapsidation, while the remaining reduction in packaging efficiency was due to the effects of the heterologous sequences on packaging itself, either directly or indirectly. These data further suggest that the variable reduction in viral titers observed despite a consistent level of packaging (except for MB25 and 26) is probably due to the inhibitory effects of the heterologous sequences on other steps in vector RNA propagation. Thus, contrary to expectations, our data reveal that the heterologous sequences had a negative effect on both packaging and other steps in retroviral RNA propagation. However, the lack of improvement in packaging efficiency with the inclusion of irrelevant sequences to the 5′-UTR suggests that FIV *gag* sequences play an important role in the process of encapsidation itself.

4. Discussion

This study demonstrates the importance of *gag* sequences for the encapsidation process of FIV genomic RNA. We show that the entire 5′-UTR on its own is not sufficient for efficient FIV RNA packaging (Fig. 2), and needs ∼100 bp of *gag* sequences to restore efficient packaging (Fig. 3). Additionally, we show that there are no other sequence elements within the next 600 bp in FIV *gag* gene that influence packaging and/or propagation of vector RNAs (Fig. 3). Finally, we show that sequences in *gag* important for packaging do not simply act as spacers to stabilize other secondary RNA structural elements of the packaging signal, but are important in themselves for the encapsidation process either at the primary sequence or the secondary RNA structural level (Fig. 4).

Since we considered only the first 700 bp of *gag* in our deletion analysis, it is possible that other sequences important for packaging may be located either within the remaining sequences of the *gag* gene or outside it. Of the *gag* region analyzed, we did not observe any enhancement of vector RNA propagation whether 100 or 700 bp of *gag* was added to our transfer vectors (Fig. 3). The first 700 bp in *gag* represent the coding region for the entire matrix (MA) protein as well as the first 296 bp of the capsid (CA) protein of FIV. In comparison, the second important determinant of packaging in BLV was located within the CA domain of *gag* (Mansky and Wisniewski, 1998), a region immediately downstream of the MA coding sequences. Thus, if FIV has any packaging determinants in the remaining portion of the CA region, our analysis would have missed them.

The role of *gag* sequences outside the primary determinants for efficient packaging in other retroviruses is not clear. In BLV, addition of a larger portion of *gag* to BLV transfer vectors, in addition to the 5′-end of *gag*, did not increase vector RNA propagation significantly (Milan and Nicolas, 1991). Similarly, in Moloney murine leukemia virus (MoMuLV), inclusion of the first ∼418 bp of *gag* resulted in more efficient packaging of the genomic RNA into nascent virions (Bender et al., 1987), but a further inclusion of additional *gag* sequences did not lead to significant increase in vector titers (Armentano et al., 1987). The observation in HIV-1 is more controversial. Some investigators have found that sequences further downstream in the *gag* gene enhance packaging (Buchschacher and Panganiban, 1992; Luban and Goff, 1994), while others have observed no enhancement of vector propagation after nt 990, and in fact have reported reduction in the titers obtained of their transfer vectors upon the addition of sequences beyond 990 nt (Parolin et al., 1994). The reduction in titers observed could be due to the presence of *cis*-acting repressor sequences (CRS) reported in the *gag* gene that downregulate vector RNA expression post-transcriptionally (Maldarelli et al., 1991; Schwartz et al., 1992).

Sequences within *gag* were first found to be important for RNA packaging when it was inadvertently discovered that inclusion of such sequences in retroviral vectors enhanced viral titers significantly (Miller et al.,

1986; Armentano et al., 1987; Palmer et al., 1987). This effect was observed to be at the *cis*-level rather than *trans* where such sequences were found to enhance the amount of genomic RNA packaged by the virions (Bender et al., 1987). It has been shown further that, like the packaging determinants within the 5′-UTR, the sequences important for packaging in *gag* assume higher-order structure(s) that are important in the encapsidation process directly in *cis* and not due to some other *trans* effects (McBride and Panganiban, 1996; Mansky and Wisniewski, 1998). The same seems most likely to be true in the case of FIV since, in our parallel studies, we have observed enhanced effects of *gag* sequences on the actual amount of genomic RNA packaged in the virions and not due to effects on overall intracellular RNA levels or protein production (Browning et al., 2003). Our preliminary computer analysis of the 5′-end of FIV genome, including both the 5′-UTR and 5′-end of the *gag* gene, has shown that, like most retroviruses, this region in FIV assumes a complex secondary RNA structure (unpublished observations). We are currently in the process of determining the significance of this structure to RNA encapsidation by FIV.

Interestingly, instead of a neutral effect, we observed inhibition of vector RNA packaging and propagation with the addition of heterologous sequences to our transfer vectors (Fig. 4A and B). This inhibition could not have been due to the increase in size of the transfer vector RNAs since even larger additions of *gag* sequences did not lead to any inhibition in MB15–21 series of vectors (Fig. 3). It is conceivable that this inhibition could have resulted from the presence of sequences like CRSs in the heterologous DNA fragments used that could have prevented nucleocytoplasmic trafficking of the unspliced RNA molecules post-transcriptionally. However, this is unlikely in our case since we have the constitutive transport element (CTE) incorporated into all our transfer vectors, ensuring the smooth nucleocytoplasmic transport of viral RNAs (Bray et al., 1994; Tan et al., 1995; Rizvi et al., 1996a,b, 1997). McBride et al. (1997) have reported negative effects of the presence of foreign sequences on the encapsidation of their transfer vector RNAs. They speculate that this is probably a general effect on packaging rather than a more specific interaction and may be due to effects of the foreign sequences on the folding of the secondary RNA structure at the 5′-end of the viral genome. We feel that, more than likely, this scenario may be true in our case. As predicted for FIV, higher-order structures involving sequences both within 5′-UTR and *gag* have been observed in other retroviruses also such as MPMV (Harrison et al., 1995). Therefore, we feel that the juxtaposition of the heterologous sequences instead of *gag* to FIV 5′-UTR probably interfered with the ability of the 5′-end of the FIV genome to assume the appropriate secondary RNA structure important for efficient packaging and vector RNA propagation.

Knowledge of the packaging determinants of FIV should be valuable in the better design of FIV-based vectors for gene therapy. Elimination of sequences not required for efficient RNA packaging from transfer vectors post-transduction using "self-inactivation" strategies should improve the safety of these vectors by curtailing their further mobilization within the target cells following delivery of the gene of choice to the transduced cells (Dull et al., 1998; Miyoshi et al., 1998; Zufferey et al., 1998). Vector mobilization (Bukovsky et al., 1999) could happen if the transfer vector RNA comes in contact with endogenous retroviruses present within a patient, or if a patient is already infected with a human retroviral pathogen capable of packaging FIV vector RNA since cross-packaging of FIV RNA by primate lentiviruses has recently been demonstrated (Browning et al., 2001).

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