Short Communication

Delineation of sequences important for efficient packaging of feline immunodeficiency virus RNA

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We have used systematic deletion analysis of the 5′ untranslated region (UTR) of the feline immunodeficiency virus (FIV) genome, both in the presence and absence of various amounts of *gag*, to define the *cis*-acting sequences responsible for efficient RNA packaging. Our analyses revealed that the primary FIV packaging signal consists of two essential core elements located within the first 90–120 bp of the 5′UTR and the first 90 bp of the *gag* gene. Interestingly, the region between the major splice donor (SD) and *gag*, including ∼130–160 bp upstream of the SD, is dispensable for encapsidation. Finally, other determinants of packaging were found to be present in the viral LTR and/or within the 3′ end of the viral genome. Taken together, our results suggest that the primary packaging determinants of FIV are multipartite and discontinuous, composed of two elements within the 5′UTR and *gag* gene.
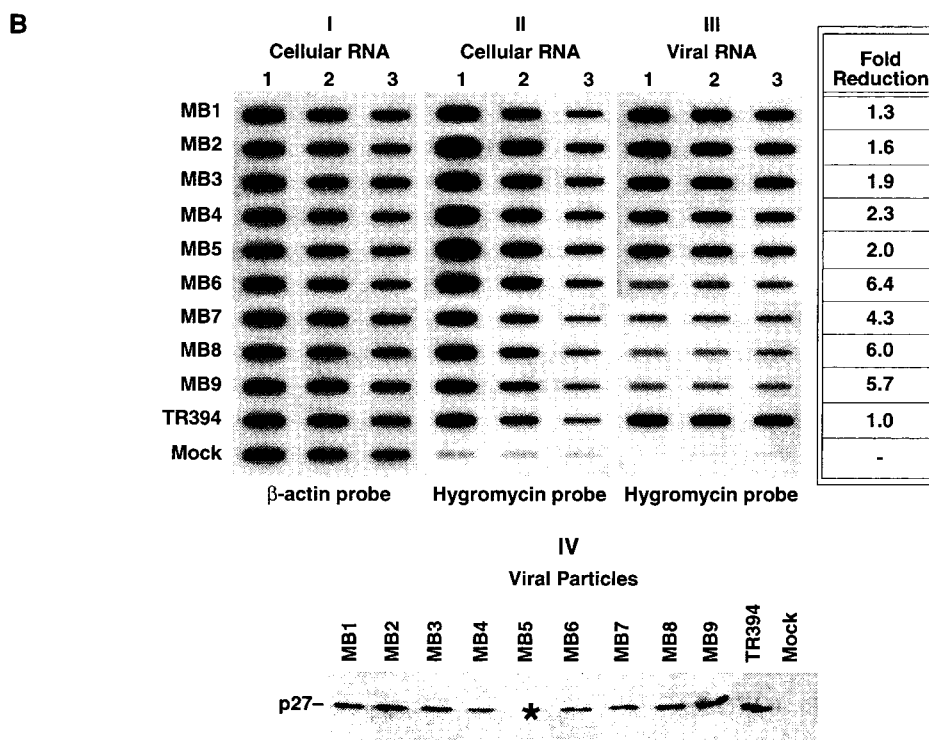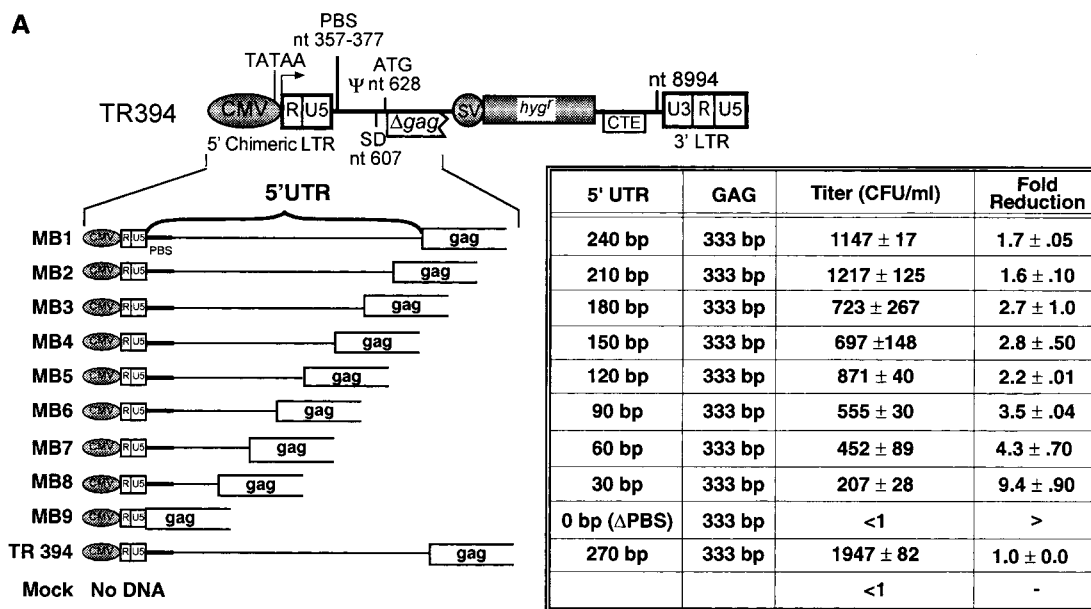
The packaging signal (Ψ) of feline immunodeficiency virus (FIV) remains undefined. Previous studies have revealed that the 5′ untranslated region (UTR) and sequences within the first 350 bp of *gag* are sufficient to allow efficient transduction of marker genes carried by FIV-based vectors (Poeschla *et al.*, 1998; Johnston *et al.*, 1999; Browning *et al.*, 2001). However, analysis of the minimum sequence requirements for efficient FIV RNA packaging has not been done. In this study, we have mapped the FIV Ψ using systematic deletion analysis of the 5′UTR, both in the presence and absence of *gag*.

To assess the requirement of sequences within the 5′UTR for packaging, we generated a series of transfer vectors containing various amounts of the 5′UTR in the presence of 333 bp of *gag* (Fig. 1A). The 5′UTR was deleted in 30 bp increments from upstream of the *gag* initiator codon (nt 627) to the U5/UTR junction at the 5′ end of the primer binding site (PBS) (nt 357) using TR394, which has been shown to be packaged and propagated efficiently by FIV proteins (Browning *et al.*, 2001). The ability of RNA from transfer vectors MB1–9 to be packaged and propagated by the FIV packaging construct MB22, and the Env expression construct MD.G., were tested using our *trans*-complementation assay (Browning *et al.*, 2001).

Western blot analysis of purified virions produced by MB22 revealed that similar amounts of viral particles were produced in each of the transfected cultures (Fig. 1B, panel IV). Direct analysis of the amount of transfer vector

RNA packaged in the virions was done by slot-blot analysis of RNA isolated from purified virions (Fig. 1B, panel III). These analyses revealed that RNA from transfer vectors MB1–5 was packaged nearly as efficiently (within approximately 2-fold) as the control vector, TR394, when normalized for RNA loading and intracellular steady-state levels of transfer vector RNA (Fig. 1B, panels I and II).

However, the packaging efficiency dropped about 6-fold compared with TR394 when RNA was packaged from MB6, the vector that contained only 90 bp out of 270 bp of the 5′UTR (Fig. 1B, panel III), and remained at about the same level (∼4- to 6-fold reduction compared with TR394) irrespective of the remaining sequences in the 5′UTR (MB7–9). Thus, between 90 and 120 bp of the 5′UTR appear to be required for efficient packaging of FIV RNA. Interestingly, considerable packaging was observed with MB9, the vector that had lost even the PBS (Fig. 1B, panel III). The packaging observed with MB9 could not have been due to non-specific packaging of any hygromycin mRNA from the SV40 promoter because, along with these vectors, we have tested our control plasmid, TR174, in the *trans*-complementation assay (data not shown). TR174 expressed the hygromycin gene from the SV40 promoter and used polyadenylation sequences from the 3′LTR for transcript termination. It lacked the sequences necessary for vector RNA packaging and propagation and did not package RNA into virions or give rise to hygromycin-resistant colonies (Hyg[r]) with either human, simian or feline immunodeficiency viruses (HIV, SIV, FIV), or

A

TR394

PBS nt 357-377

TATAA

ATG Ψ nt 628

nt 8994

5' Chimeric LTR

SD nt 607

Δgag

CTE

3' LTR

5'UTR

MB1
MB2
MB3
MB4
MB5
MB6
MB7
MB8
MB9
TR 394
Mock No DNA

5' UTR	GAG	Titer (CFU/ml)	Fold Reduction
240 bp	333 bp	1147 ± 17	1.7 ± .05
210 bp	333 bp	1217 ± 125	1.6 ± .10
180 bp	333 bp	723 ± 267	2.7 ± 1.0
150 bp	333 bp	697 ±148	2.8 ± .50
120 bp	333 bp	871 ± 40	2.2 ± .01
90 bp	333 bp	555 ± 30	3.5 ± .04
60 bp	333 bp	452 ± 89	4.3 ± .70
30 bp	333 bp	207 ± 28	9.4 ± .90
0 bp (ΔPBS)	333 bp	<1	>
270 bp	333 bp	1947 ± 82	1.0 ± 0.0
		<1	-

B

	I Cellular RNA			II Cellular RNA			III Viral RNA			Fold Reduction
	1	2	3	1	2	3	1	2	3	
MB1										1.3
MB2										1.6
MB3										1.9
MB4										2.3
MB5										2.0
MB6										6.4
MB7										4.3
MB8										6.0
MB9										5.7
TR394										1.0
Mock										-

β-actin probe   Hygromycin probe   Hygromycin probe

IV
Viral Particles

MB1 MB2 MB3 MB4 MB5 MB6 MB7 MB8 MB9 TR394 Mock

p27–

Mason–Pfizer monkey virus (MPMV) proteins when tested in the *trans*-complementation assay (Rizvi & Panganiban, 1993; Browning *et al.*, 2001).

Successful transduction of most of the FIV 5′UTR deletion transfer vectors was indicated by the appearance of Hyg[r] colonies (Fig. 1A). The overall viral titres observed in the *trans*-complementation assay correlated well with the packaging efficiency. However, deletion of the first 30 bp

of the 5′UTR containing the PBS in MB9 resulted in a total loss of viral titre, despite only a 5·7-fold reduction in packaging. The complete absence of Hyg[r] colonies despite considerable packaging was expected in MB9, since the PBS is required for reverse transcription, a step necessary for the successful propagation of the transfer vector. Thus, the coordinated loss of titre with incremental deletions in the 5′UTR and corresponding reductions in the packaging efficiency suggest that truncations of the 5′UTR continue

Fig. 1. (on facing page) Between 90 and 120 bp of the 5′ UTR of FIV are required for efficient packaging of the FIV genomic RNA in the presence of gag. (A) Schematic representation of FIV transfer vectors MB1–9 containing various amounts of the 5′UTR in the presence of 333 bp of gag. These vectors were constructed using several stages of cloning (details available from the authors on request). The relative titres obtained as c.f.u. ml^{-1} virus stock on transduction of the transfer vectors into the target cells are indicated in the table. The value of each titre represents the mean of three independent experiments performed in duplicate ± SD. The -fold reduction refers to the relative level of titre observed with each vector compared with TR394. No hygromycin-resistant (Hyg^r) colonies were observed for any of the transfer vectors or packaging and env expression constructs when transfected alone. >, essentially 100 % reduction of viral titres. (B) RNA slot-blot and Western blot analyses of virus particles and cellular RNAs isolated from Cos cells transfected with the various transfer vectors along with the packaging (MB22) and vesicular stomatitis virus G protein (VSV-G) env (MD.G.) expression vectors from one representative experiment. The RNAs were DNase-treated, diluted and subjected to slot-blot analysis. Panel (I) represents a 1 : 2 dilution of 0·5 μg cellular RNA (0·5, 0·25 and 0·125 μg) probed with a fragment of the 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 75 % of 9 ml media containing virus particles, also probed with a fragment of the hygromycin gene. The table refers to the -fold reduction in packaging efficiency relative to TR394 after normalization for RNA loading and transfection efficiency (see Schmidt et al., 2003, for details). Panel (IV) shows Western blot analysis of equivalent amounts of the remaining 25 % 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; *, not determined.

to affect vector RNA packaging, which in turn affects vector RNA propagation proportionally.

To determine whether FIV gag sequences are important for the encapsidation process, we generated MTB, a construct that contains the entire 5′UTR in the absence of gag (Fig. 2A). Next, sequential amounts of the gag sequence were added to MTB after the 270 bp of the 5′UTR, from 30 bp of gag in MB10 to 210 bp in MB14 (Fig. 2A). Tests of MTB and MB10–14 in the in vivo packaging assay revealed that slightly lower levels of particles were produced in cultures expressing MTB, MB10 and MB13 (Fig. 2A, panel IV). However, these levels were comparable with those expressed by the control vector, TR394. Slot-blot analysis of RNA isolated from purified virions revealed that there was a 3·6-fold loss of packaging in the absence of any gag sequences in MTB compared with TR394 (Fig. 2B, panel III), when normalized to the amount of RNA loaded and the intracellular steady-state levels of transfer vector RNAs expressed (Fig. 2B, panels I and II). This loss remained essentially the same (3·12-fold compared with TR394) in the presence of 30 bp of gag (MB10, Fig. 2B, panel III). It was not until 90 bp of the gag sequence was present (MB11) that the packaging efficiency became comparable (less than 2-fold) with TR394 (Fig. 2B, panel III), and remained essentially the same for vectors MB12–14. The lower level of RNA packaged in MTB and MB10 was not due to lower transfection efficiency of these vectors, since MB13 and TR394 were expressed at about the same level or even lower and yet were packaged efficiently (Fig. 2B, panels II and III).

Once again, the packaging efficiency correlated well with the transduction efficiency in the presence of the entire 5′UTR (Fig. 2B, panel III), when normalized to the amount of virus particles produced (Fig. 2B, panel IV), revealing that the first 90 bp of FIV gag are sufficient for efficient RNA packaging with no effect of the first 333 bp of gag on vector RNA propagation beyond packaging. Thus, the core
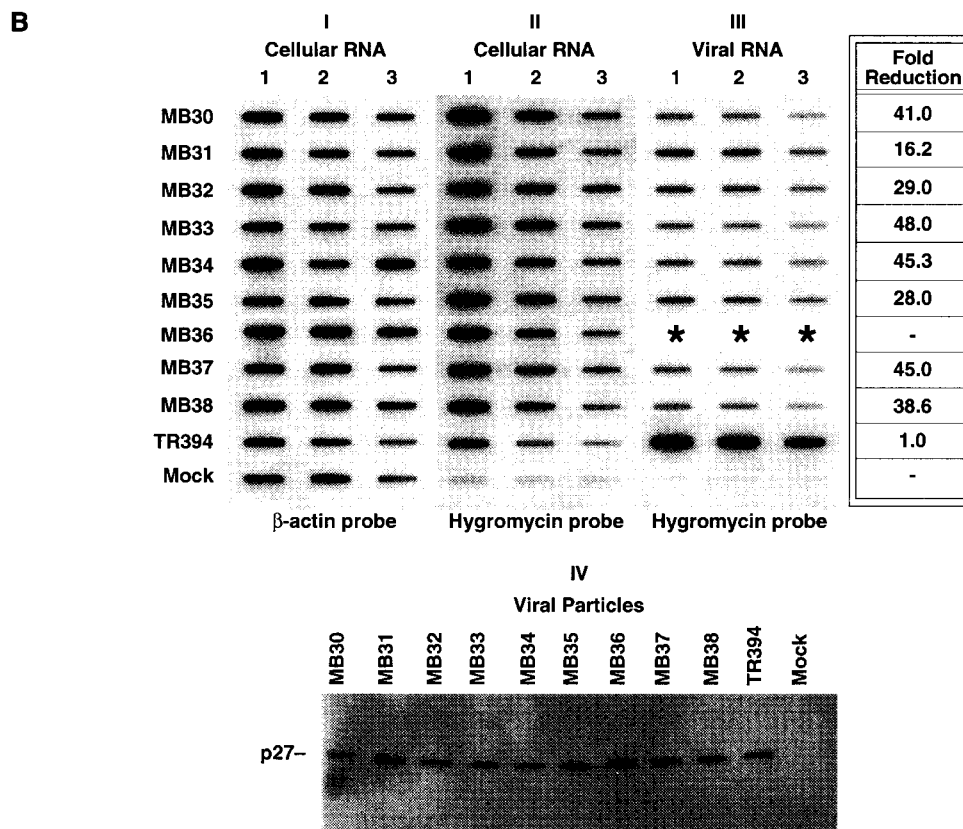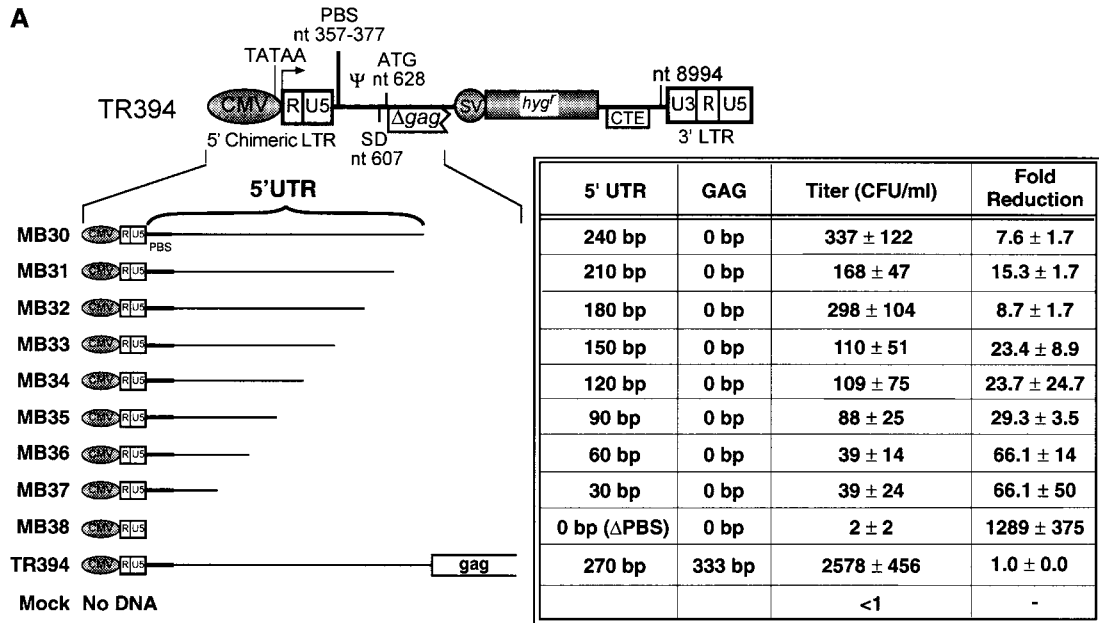
FIV Ψ appears to be multipartite and requires sequence elements in both the 5′UTR and gag simultaneously for efficient encapsidation.

To study the relative contribution of sequences within the 5′UTR and gag towards packaging, we generated MB30–38, which contained the same successive 5′UTR deletions made in MB1–9, but in the absence of gag (Fig. 3A). Tests of MB30 revealed a drastic 41-fold reduction in packaging efficiency compared with TR394, after normalization for RNA loading and intracellular steady-state levels of transfer vector RNAs (Fig. 3B, panels I and II). MB30 lacked the last 30 bp of the 5′UTR in addition to gag sequences. This loss represented an approximately 11-fold greater reduction in packaging efficiency compared with MTB, the vector that also lacked gag sequences, but contained the entire 270 bp of the 5′UTR (Fig. 2). The greater reduction in packaging efficiency, therefore, can be attributed to the loss of the last 30 bp of the 5′UTR in the absence of gag sequences, since only a 1·3-fold reduction in RNA packaging was observed with this deletion in the absence of gag, while deletions as much as 4-fold larger than this reduced packaging by only 2-fold in the presence of gag (see MB1–5, Fig. 1). Thus, the last 30 bp of the 5′UTR are critical for the functioning of the FIV packaging determinant located at the beginning of the 5′UTR in the absence of gag sequences.

Further deletions in the 5′UTR in the absence of gag sequences continued to affect packaging severely (16–48-fold) irrespective of the amount of 5′UTR present (210 bp in MB31 to none in MB38, Fig. 3B, panel III). The drastic reduction in packaging efficiency in MB30–34 was observed despite the actual presence of the 5′UTR cis-acting sequences important for packaging mapped earlier, the first 90–120 bp of the 5′UTR (Fig. 1). Thus, these data suggests that deletions at the 3′ end of the 5′UTR, in the absence of gag sequences, may have inadvertently affected formation or stability of a putative higher-order structure of the packaging
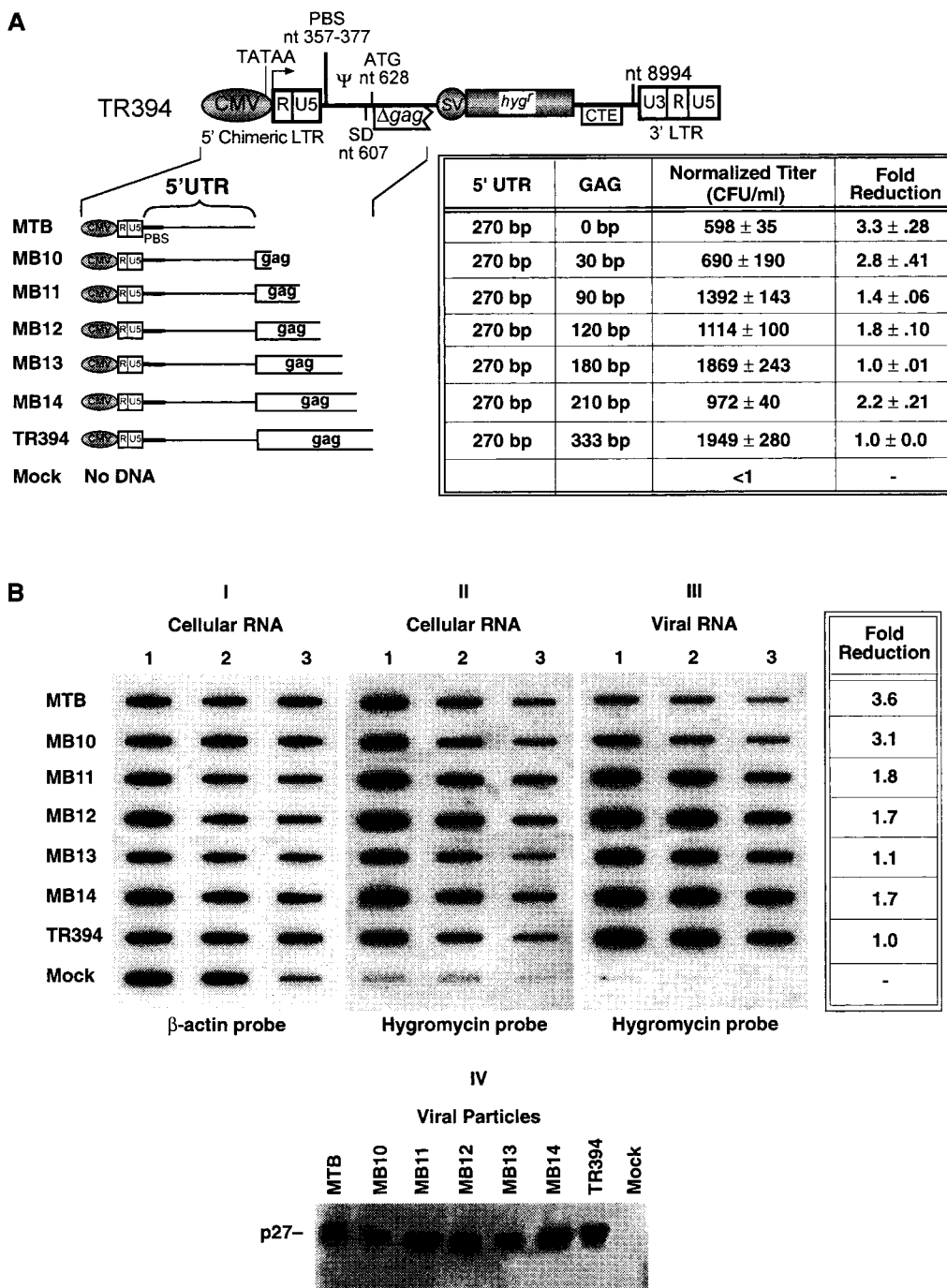
Fig. 2. The first 90 bp in *gag* are critical for packaging of the FIV genome in the presence of the entire 5′UTR. (A) Schematic representation of the FIV transfer vectors MTB (containing none of the *gag* sequence) and MB10–14 containing various amounts of the *gag* sequence. These vectors were constructed using several stages of cloning (details available from the authors on request). The normalized titres obtained as c.f.u. ml^{-1} virus stock on transduction of the transfer vectors into the target cells are indicated in the table (see Schmidt *et al.*, 2003, for details). The value of each titre represents the mean of three independent experiments performed in duplicate $\pm$ SD. The -fold reduction refers to the relative level of titre observed with each vector compared with TR394. (B) RNA slot-blot and Western blot analyses of virus particles and cellular RNAs isolated from Cos cells transfected with the various transfer vectors (see Fig. 1) from one representative experiment. Panels (I) and (II) are slot-blots of cellular RNAs, while panel (III) is a slot-blot of virion RNA isolated from transiently transfected Cos cells. The table refers to the -fold reduction in packaging efficiency relative to TR394 after normalization for RNA loading and transfection efficiency (see Schmidt *et al.*, 2003, for details). The RNAs were DNase-treated, diluted and subjected to slot-blot analysis (see Fig. 1). Panel (IV) shows Western blot analysis of purified virions harvested from each of the transfected cultures, as described in Browning *et al.* (2001). Mock, cells transfected without DNA.

A

5' UTR	GAG	Titer (CFU/ml)	Fold Reduction
240 bp	0 bp	337 ± 122	7.6 ± 1.7
210 bp	0 bp	168 ± 47	15.3 ± 1.7
180 bp	0 bp	298 ± 104	8.7 ± 1.7
150 bp	0 bp	110 ± 51	23.4 ± 8.9
120 bp	0 bp	109 ± 75	23.7 ± 24.7
90 bp	0 bp	88 ± 25	29.3 ± 3.5
60 bp	0 bp	39 ± 14	66.1 ± 14
30 bp	0 bp	39 ± 24	66.1 ± 50
0 bp (ΔPBS)	0 bp	2 ± 2	1289 ± 375
270 bp	333 bp	2578 ± 456	1.0 ± 0.0
		<1	-

B

	Fold Reduction
MB30	41.0
MB31	16.2
MB32	29.0
MB33	48.0
MB34	45.3
MB35	28.0
MB36	-
MB37	45.0
MB38	38.6
TR394	1.0
Mock	-

determinant located within the first 90–120 bp of the 5′UTR, thus reducing its ability to function as an effective Ψ. This may also explain the variable effect of continued deletions on packaging, since each incremental increase in the deletion could have affected the putative structure of the core packaging element at the 5′UTR differently.

Once again, reduction in genomic RNA incorporation into nascent virions observed in MB30–38 was not completely abrogated and a constant low level of packaging was detectable, even in the absence of the entire 5′UTR and *gag* sequences (MB38, Fig. 3B). The presence of low levels of packaging, even in the absence of the entire 5′UTR and *gag* sequences, suggests that there are additional packaging determinants outside these sequences. We can exclude

Fig. 3. *(on previous page)* The FIV packaging signal is composed of two discontinuous core elements required together for efficient RNA packaging. (A) Schematic representation of the FIV transfer vectors MB30–38 containing various amounts of 5'UTR in the absence of *gag* sequences. These vectors were constructed using several stages of cloning (details available from the authors on request). The relative titres obtained as c.f.u. ml^{-1} virus stock on transduction of the transfer vectors into the target cells are shown in the table. The value of each titre represents the mean of three independent experiments performed in duplicate $\pm$ SD. The -fold reduction refers to the relative level of titre observed with each vector compared with TR394. (B) RNA slot-blot and Western blot analyses of virus particles and cellular RNAs isolated from Cos cells transfected with the various transfer constructs (see Fig. 1) from one representative experiment. Panels (I) and (II) are slot-blots of cellular RNAs, while panel (III) is a slot-blot of virion RNA isolated from transiently transfected Cos cells. The table refers to the fold reduction in packaging efficiency relative to TR394 after normalization for RNA loading and transfection efficiency (see Schmidt *et al.*, 2003, for details). The RNAs were DNase-treated, diluted and subjected to slot-blot analysis (see Fig. 1). Panel (IV) shows Western blot analysis of purified virions harvested from each of the transfected cultures, as described in Browning *et al.* (2001). Mock, cells transfected without DNA; *, not determined.

non-FIV sequences present on the vectors such as the human cytomegalovirus (hCMV) promoter, the SV-Hyg^r cassette and the constitutive transport element (CTE) as contributing to non-specific FIV RNA packaging, since the presence of these sequences on several other transfer vectors in the absence of any FIV sequences has not led to any FIV-specific RNA packaging by the structural proteins expressed by MB22 (Browning *et al.*, 2001). The only other FIV-specific sequences found in our vectors were the FIV LTR and ∼129 bp immediately upstream of the FIV 3'LTR. Therefore, these sequences could potentially harbour additional packaging determinants for genomic RNA encapsidation. A similar involvement of sequences of secondary importance to RNA packaging has been observed in other retroviruses, such as murine leukaemia virus (Yu *et al.*, 2000), Rous sarcoma virus (Sorge *et al.*, 1983) and HIV-1 (Kim *et al.*, 1994; McBride & Panganiban, 1996, 1997; McBride *et al.*, 1997; Harrich *et al.*, 2000).

Tests of MB30 in the *in vivo* transduction assay revealed that, despite the presence of almost the entire 5'UTR, the lack of *gag* sequences and the last 30 bp of the 5'UTR reduced the transduction efficiency by nearly 8-fold compared with TR394 (Fig. 3A). This pattern of progressive loss in titre continued with each incremental deletion in the 5'UTR until essentially no Hyg^r colonies were observed with MB38, the vector that contains no 5'UTR, including the PBS (Fig. 3A). The continued drop in transduction efficiency was observed despite the fact that equal amounts of virions were produced in each of the transfected cultures (Fig. 3B, panel IV). The essentially steady drop in viral titres also did not correlate with the reduction in the packaging efficiency, which oscillated between 16- and 48-fold depending on the deletion. Additionally, the drop in viral titres was accompanied by large standard deviations not observed with the 5'UTR deletion series in the absence of *gag* (Fig. 1). The large standard deviations of viral titres along with the oscillation in packaging efficiency observed, once again, suggested that the absence of *gag* and the last 30 bp of the 5'UTR variably affected the formation of some structural element(s) at the 5' end of the UTR important for vector RNA packaging and propagation.

In short, the present study reveals that, similar to the Ψ of complex retroviruses, the Ψ of FIV is multipartite, consisting of at least two discontinuous core elements, one residing within the first 90–120 bp of the 5'UTR upstream of the major SD (Fig. 1), while the other resides in the first 90 bp of the *gag* ORF (Fig. 2). The two core elements are equally important and simultaneously required for packaging, since deletion of either element alone reduces packaging to a similar low level (Figs 1 and 2), while deletion of both elements together reduces packaging drastically (Fig. 3). We suspect that these two regions may form a higher-order structure(s). Our preliminary computer analysis of the 5' end of the FIV genome has revealed a complex secondary RNA structure. Whether this structure will be important for FIV RNA packaging remains to be determined.

ACKNOWLEDGEMENTS

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