Live-Cell Coimaging of the Genomic RNAs and Gag Proteins of Two Lentiviruses

Iris Kemler, Anne Meehan, and Eric M. Poeschla

Department of Molecular Medicine and Division of Infectious Diseases, Mayo Clinic College of Medicine, Rochester, Minnesota

Received 17 February 2010/Accepted 7 April 2010

Human immunodeficiency virus type 1 (HIV-1) Gag and genomic RNA determinants required for encapsidation are well established, but where and when encapsidation occurs in the cell is unknown. We constructed MS2 phage coat protein labeling systems to track spatial dynamics of primate and nonprimate lentiviral genomic RNAs (HIV-1 and feline immunodeficiency virus [FIV]) vis-à-vis their Gag proteins in live cells. Genomic RNAs of both lentiviral genera were observed to traffic into the cytoplasm, and this was Rev dependent. In transit, FIV Gag and genomic RNA accumulated independently of each other at the nuclear envelope, and focal colocalizations of genomic RNA with an intact packaging signal (ψ) and Gag were observed to extend outward from the cytoplasmic face. In contrast, although HIV-1 genomic RNA was detected at the nuclear envelope, HIV-1 Gag was not. For both lentiviruses, genomic RNAs were seen at the plasma membrane if and only if Gag was present and ψ was intact. In addition, HIV-1 and FIV genomes accumulated with Gag in late endosomal foci, again, only ψ dependently. Thus, lentiviral genomic RNAs require specific Gag binding to accumulate at the plasma membrane, packaged genomes counteract with Gag into the endosomal pathway, and plasma membrane RNA incorporation by Gag does not trigger committed lentiviral particle egress from the cell. Based on the FIV results, we hypothesize that the Gag-genome association may initiate at the nuclear envelope.

During the assembly of a retrovirus, unspliced genomic RNA must traffic from the nucleus to the cytoplasm and subsequently to the plasma membrane, where budding occurs. Formation of infectious virions requires packaging (encapsidation) of the genomic RNA by the viral Gag polyprotein, which is mediated by the nucleocapsid (NC) domain of Gag. An RNA dimer is consistently encapsidated, and each monomer can and generally does template reverse transcription in any one replication cycle. The outcome is a genetic recombination rate without parallel in other biological systems. Despite its importance in viral assembly, the intracellular localization and the timing of genomic RNA encapsidation in relation to other assembly steps are not known. Gag protein trafficking in the retroviral producer cell has been analyzed extensively, but experimental limitations have left the post-nuclear trafficking of the genomic RNA poorly understood in comparison. How, when, and where Gag encounters and binds to this RNA and whether genomes that are translated are preferentially encapsidated remain central questions.

Retroviral genomic RNAs must also bypass the normal cellular checkpoint against export of unspliced mRNAs from the nucleus. Different retroviruses have evolved different solutions. Nuclear export of lentiviral genomic RNAs is induced by binding of the viral Rev protein to an RNA secondary structure, the Rev responsive element (RRE). Rev serves as adaptor to the nuclear export receptor Crm1, which mediates the movement of the ribonucleoprotein complex through the nuclear pore into the cytoplasm. The genomic RNA of the simpler type D retrovirus Mason Pfizer monkey virus (MPMV) has a constitutive transport element (CTE) that directly interacts with the export factor NXF1. Moore et al. recently used recombination-based assays to show that human immunodeficiency virus type 1 (HIV-1) dimerization precedes encapsidation but does not occur until the genome exits the nucleus. In addition, HIV-1 Gag packages viral genomes with equivalent efficiencies whether genome nuclear export is mediated by Crm1 or is artificially exported by NXF1.

There are two basic fates for genomic RNAs after delivery into the cytoplasm: translation and encapsidation. The majority are translated to produce the Gag and Gag-Pol polyproteins, and a few are packaged into assembling virions. Cotranslational encapsidation in cis is a possibility, and evidence for this has been reported for HIV-2. However, the effectiveness of split-component HIV-1, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and equine anemia virus (EIAV) vector systems and the evidence provided by circulating recombinant forms for substantial recombination between HIV-1 genomes in vivo suggest that this is not likely to be critical for lentiviruses. Specific recognition of a retroviral genome versus cellular or spliced viral RNAs is mediated by encapsidation (ψ) sequences, which are generally in the 5’ end of the RNA. For HIV-1, four RNA stem-loops, SL1 to SL4, are involved. FIV has a bipartite encapsidation determinant, with one element in the 5’ leader and the other in the proximal 230 nucleotides (nt) of gag. When the gag element is deleted, FIV genome packaging into virions is completely abolished.

Gag is translated on free polysomes and targeted to the
plasma membrane, where it interacts with components of the endosomal sorting complex (ESCRT-I, -II, and -III) (4, 35, 40). Imaging at the plasma membrane with total internal reflection fluorescence microscopy (TIR-FM) indicates that assembly of individual HIV-1 particles is rapid and is completed in 5 to 10 min (24, 25, 27). The subcellular location at which Gag incorporates the viral RNA, either en route to or at the plasma membrane, remains unknown, however, with the TIR-FM-based work as well as a large corpus of earlier studies compatible so far with either scenario. A pericentriolar region was reported to be the assembly site for type D retroviruses (50, 51). Subsequently, HIV-1 genomic RNA and Gag were also reported to colocalize in a region at or adjacent to the centriole (45). HIV-1 genomic RNA was suggested in another study to accumulate at the microtubule organizing center (MTOC), in this case only after knockdown of the RNA trafficking protein hnRNPA2 (heterogeneous nuclear ribonucleoprotein A2) (31). The MTOC, however, has also been shown to accumulate incoming HIV-1 particles (37). The Gag protein of the avian retrovirus Rous sarcoma virus is particularly interesting because it appears to shuttle between cytoplasm and nucleus, suggesting that the nucleus could be a site of encapsidation (48, 49).

Until recently, methods to detect lentiviral RNAs were largely limited to in situ hybridization in fixed cells, with Gag identified by antibody labeling (25, 26). To visualize lentiviral genomic RNAs in live cells in the present study, we applied a method that has been used by cell biologists to track individual RNAs in living cells, first in *Saccharomyces cerevisiae* and then in mammalian cells (3, 18, 19). Green fluorescent protein (GFP) or yellow fluorescent protein (YFP) is fused to the RNA-binding coat protein of the single-stranded RNA phase MS2 (3). The RNA of interest is engineered to contain one or more copies, generally tandem repeats, of the specific phase RNA segment that binds this protein. The MS2 system has also been applied to analysis of retroviral RNA trafficking (1), and Moore et al. (39) were able to use it to assess genomic RNA dimer composition within individual, budded virions. When Jouvenet et al. used an MS2 system with TIR-FM to monitor the plane of the plasma membrane (27), they could detect small numbers of HIV-1 Gag molecules associated with MS2-labeled genomic mRNA; these complexes exhibited progressively restricted lateral movement in the plasma membrane, whereupon further nucleation of virion assembly and budding appeared to occur. Here, we used the MS2 approach in confocal imaging with complementary in situ RNA hybridizations and particle composition analyses. We pursued a comparative approach and constructed two independent MS2 systems, one for a primate lentivirus (HIV-1) and one for a nonprimate lentivirus (FIV).

**MATERIALS AND METHODS**

**Cell culture, transfections, and plasmid constructions.** Cells were cultured in Dulbecco’s modified Eagles medium with 10% fetal calf serum. DNA transfection was carried out with calcium phosphate coprecipitation (293T, HeLa, and CEFK cells) or FuGene 6 (Roche Applied Science) for COS-7 cells.

Correct plasmid constructions were all confirmed by sequencing. To generate MS2-Cherry, we replaced GFP between BamH1 and ClaI in pMS2-GFP with an amplicon generated using template pRSSET-B-mCherry (52) with sense primer 5′-BamMS2-Cherry (5′-ATATGATGATCCGACAACTACGGTACAGGAACTCCG-3′) and antisense primer 3′-ClaNLS-Cherry (5′-ATATATCGATTATATACCTTCTCTTTTTTTGAGCGTTGACAGCTGTCCACGCACTCC-3′; restriction sites are underlined). For pCRev, an FIV 34TFFI0 Rev cDNA was synthesized by reverse transcription-PCR (RT-PCR) with primers 5′-TACCTGGAAATGTGCAAAAGTATTTCGACG-3′ and 5′-AATCTAGATACCTGCAATTTATTC-3′ and inserted into pCI (Invitrogen) between XhoI and XbaI. pMS2-YPF and pMS2-2GF were kindly provided by R. Singer (18).

pFIV-Δ24 was generated from a previously described FIV vector system (32, 43, 44) by inserting 24- of the 19-nt MS2 stem-loops (18) by blunt end ligation of a BamHI-BglII fragment into the AvrII site of FIV transfer vector pC26 (Fig. 1). The presence and correct orientation of all of the stem-loops were verified by sequencing. FIVΔ24 was created by replacing a BglII-SphI fragment containing the human cytomegalovirus (CMV) immediate-early gene promoter, R, repeat, U5, leader sequences, and 388 bp of Gag with the corresponding BglII-SphI fragment from pGNNF-W60 (28), resulting in the net deletion of all FIV Gag sequences.

In FIVΔ-24-CTE and FIVΔ-24-CTE, the RRE was replaced by four copies of the constitutive transport element from MPMV (5). First, one copy of a CTE was made by annealing and extending the following oligonucleotides: 5′-XhoI-Nhe-CTE-3′ (5′-ATATCTCGAGTCATGCTCCACCTTGCCTGAAATGATGCTGACGACCGCATGAAGGAGGACTGACATTCTTCTAACCTAGTCTAACCTAGTAG-3′) and 3′-SphI-CTE (5′-ATATGTCGACACATATCCCTCGGAGGCTGCGCCTGTCTTAGGTTGGAGTGATACATTTG-3′); restriction sites are underlined. Then a XhoI fragment from pBS-SphI was cloned into the BamHI-BglII fragment of the RRE (Fig. 1). For pHIVgagΔ-24, a BglII fragment into pHR-BglII fragment into pHR-GC lacZ (57) between the encapsidation signal and the glycine at position two in Gag with an alanine.

A Tat cDNA amplicon was generated with the primers 5′-ATATCTCGAGTCATGCTCCACCTTGCCTGAAATGATGCTGACGACCGCATGAAGGAGGACTGACATTCTTCTAACCTAGTCTAACCTAGTAG-3′ and 3′-SphI-CTE (5′-ATATGTCGACACATATCCCTCGGAGGCTGCGCCTGTCTTAGGTTGGAGTGATACATTTG-3′); restriction sites are underlined. Then a XhoI fragment from pBS-SphI was cloned into the BamHI-BglII fragment of the RRE (Fig. 1). For pHIVgagΔ-24, a BglII fragment into pHR-BglII fragment into pHR-GC lacZ (57) between the encapsidation signal and the glycine at position two in Gag with an alanine.

**Virion production and Western blotting.** Viral particles were purified as described in Kemler et al. (28). Forty-eight hours after 293T cells were transfected, medium was centrifuged at 828 g for 10 min in a Sorvall tabletop centrifuge, and supernatant was filtered through a 0.45-μm-pore-size filter. The virus was
then pelleted through a 20% sucrose cushion for 2 h at 112,700 × g in an SW28 rotor. The viral pellet was resuspended in TNE buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA). Concentrated virus was lysed in 5× radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitors (Complete Mini; Roche). Cells were lysed in 1× RIPA buffer. Eight micrograms of cell lysate and equal amounts of viral lysates were separated on a reducing 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membranes were blocked in 0.2% I-block (Tropix)–0.1% Tween and then probed with a monoclonal anti-GFP antibody (1:5,000; BD Biosciences), a polyclonal anti-dsRed antibody (1:16,000; BD Biosciences), or with a monoclonal antibody (MAb) to HIV-1 p24 (AG3.0; NIH AIDS Research and Reference Reagent Program). For secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit antibody (1:1,000; Calbiochem) was used. Bands were visualized with Lumi-Light detection reagent (Roche).

Indirect immunofluorescence and live-cell imaging. HeLa cells were transfected with 1 μg of pFIVgag-dsRed, fixed with 4% paraformaldehyde for 10 min, washed with phosphate-buffered saline (PBS), quenched with 50 mM NH₄Cl for 10 min, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min, washed, and blocked with 1% bovine serum albumin (BSA)–PBS for 15 min. Cells were then incubated with monoclonal antibodies against CD63, Lamp2, EEA1 (1:100; BD Biosciences), or DDX6 (1:150; Bethyl Laboratories) for 1 h at 37°C. After cells were washed with PBS, they were blocked again and then incubated with an anti-mouse Alexa Fluor 488-conjugated antibody (1:1,000; Molecular Probes) for 30 min. Slides were analyzed on a Zeiss LSM 510 laser scanning confocal microscope using a 100× (oil) objective. MAb PAK3-2C1 (Custom Monoclonal Antibodies International, Sacramento, CA) was used for indirect immunofluorescence for wild-type Gag. For live-cell imaging, HeLa cells or COS-7 cells were seeded in Lab-Tek glass-bottom chamber slides (Nunc) and observed 20 or 48 h posttransfection on a Zeiss LSM 510 confocal laser scanning microscope equipped with an Axiovert 200 M inverted platform using a Plan-Apochromat 100× (1.4 numerical aperture) oil immersion objective. CFP was excited with an Argon laser at 458 nm, and emission was detected with a band-pass filter of 475 to 525 nm. mCherry was excited with a helium-neon laser at 543 nm, and emission was detected with a band-pass filter of 585 to 615 nm. YFP was excited with an Argon laser at 514 nm, and emission was detected with a band-pass filter of 530 to 600 nm.

In situ RNA hybridization. Cells were seeded on glass coverslips, and in situ RNA hybridizations were performed as described by Chartrand et al. (7). The 50-nt-long oligonucleotide used was complementary to a sequence between the MS2 stem-loops (5′-TtTCTAGGCAAtTAGGTACCTtAGGATCTAAtGAACC...).
CGGAAATACGCA-3′) and contained five amino-modified C6 deoxyribosyl-thymines (dT6; indicated by lowercase Ts) which were subsequently coupled to Cy3 fluorophore (Amersham), according to the manufacturer’s protocol. An oligonucleotide complementary to a sequence in the FIV pol gene (5′-CAGCC TTGGTGGTAGACcACACCACAAAtCTCCTTCCGTCCCGCAtT-3′) was used. The oligonucleotides were purified by high-performance liquid chromatography (HPLC). Subsequent to the in situ RNA hybridization immunofluorescence was performed with MAb 414 (Covance) recognizing the nuclear pore complex and the anti-mouse Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Cells were imaged on a Zeiss LSM 510 confocal laser scanning microscope.

**RESULTS**

**Labeling FIV genomic RNA with MS2-GFP does not inhibit packaging into particles.** To investigate trafficking of the genomic RNA and Gag in living cells, we devised systems to fluorescently label both molecules. For the genomic RNA, we adapted the methods of Singer and colleagues (3, 18). GFP, YFP, or mCherry was fused to the RNA-binding coat protein of the RNA phage MS2 (indicated as MS2-XFP, where XFP is YFP, GFP, or mCherry) (Fig. 1A). A nuclear localization sequence (NLS) confines the MS2-XFP protein to the nucleus unless it associates with a cytoplasmically trafficked mRNA. To enable specific binding of MS2-XFPs to full-length HIV-1 or FIV lentiviral transfer vector RNAs, 24 tandem copies of the MS2 phage coat protein RNA binding element, a 19-nt stem-and-loop structure, were inserted downstream of the viral major splice donor (Fig. 1B and E). This positioning allows for exclusive visualization of unspliced genomic RNA.

Of the two lentiviruses, we began with FIV, using FIVψ-24 (Fig. 1B). To simultaneously track Gag, we fused either CFP, dsRed, or mCherry to the Gag C terminus. The resulting expression constructs, pFIVgag-CPF, pFIVgag-RFP, and pFIVgag-mCherry, function purely to supply Gag and Rev in trans and lack all genomic RNA encapsidation signals as well as all cis-acting elements needed for reverse transcription and integration (Fig. 1D). To determine whether Gag-XFP fusion proteins were incorporated into virions, we expressed them alone or together with unlabeled Gag-Pol (pFP93) (Fig. 1C).

As shown in Fig. 2A for FIV Gag-RFP, a single 90-kDa band corresponding to the predicted size of the intact fusion protein was detected in cell lysates (data not shown) and in purified virions (lane 3). If wild-type Gag-Pol was coexpressed with Gag-RFP, protease cleavage occurred, resulting in detection of the 90-kDa precursor and smaller RFP-containing bands (Fig. 2A, lane 4). To determine whether genomic RNAs with bound MS2-GFP molecules are incorporated into viral particles, we coexpressed Gag-RFP, the FIVψ-24 genomic RNA, and MS2-GFP in 293T cells. Immunoblotting of purified virions with an anti-GFP antibody showed that the 45-kDa MS2-GFP protein was incorporated into particles (Fig. 2B, lanes 2 and 3). In addition, MS2-GFP incorporation was increased when the amount of FIVψ-24 transfer vector was doubled (lane 2 versus lane 3), indicating a quantitative effect of RNA input on encapsidation of MS2-GFP. Importantly, MS2-GFP incorporation into virions was dependent on the viral RNA’s having the encapsidation elements. No MS2-GFP incorporation was detected in virions produced with a packaging signal mutant (28) transfer vector, FIVΔψ-24 (Fig. 2B lanes 4 and 5; the region deleted from FIVψ-24 is shown in Fig. 1B). This was consistently the case despite equivalent budding of virions (lanes 7 to 10) and equivalent intracellular expression of MS2-GFP (data not shown). Thus, a Gag-XFP fusion protein is capable of specifically packaging viral RNA with bound MS2-XFP. Furthermore, these data show that the C-terminal positioning of a fluorescent protein (thus, in proximity to NC), does not compromise RNA packaging properties of Gag.

**Subcellular distribution of FIV Gag in live cells.** We then investigated the distribution of FIV Gag-XFPs in living cells. In HeLa (Fig. 3A) and COS-7 cells (Fig. 3B, C, and D), Gag-CFP had a dispersed and nonhomogenous cytoplasmic distribution, and it was readily seen to accumulate at the plasma membrane in HeLa and COS-7 cells (arrows). This subcellular distribution is similar to the reported distribution for an HIV-1 Gag-CFP fusion protein (14, 26). The same results were obtained with Gag-mCherry (Fig. 3D), demon-

---

**FIG. 2.** Encapsidation of MS2-GFP-labeled genomes. Encapsidation of MS2-GFP requires the viral RNA encapsidation signal and is proportional to genomic RNA input. (A) 293T cells were transfected with 10 μg of pFIVgag-dsRed, 10 μg of pCT5efs (proviral clone, expressing wild-type Gag-Pol), or 5 μg of each. Purified virions were lysed and separated on a 10% SDS-PAGE gel and probed with a mouse anti-GFP antibody. (B) 293T cells were transfected with the indicated amounts of DNA (μg). Purified virions were lysed and separated on a 10% SDS-PAGE gel and probed with a mouse anti-GFP antibody (lanes 1 to 5) or a rabbit anti-dsRed antibody (lanes 6 to 10).
strating that the XFP fused to Gag does not influence its trafficking. Importantly, the subcellular distribution of fluorescently protein-labeled Gag was equivalent to the distribution of unmodified wild-type FIV Gag (Fig. 3E). High-resolution images were obtained and clearly identified Gag at the plasma membrane (Fig. 3D, enlarged lower image). Discrete plasma membrane accumulations were more prominent in COS-7 (Fig. 3B, C, and D) or 293T cells (see Fig. S4 in the supplemental material) than in HeLa cells. However, if the amount of transfected pFIVgag-CFP was doubled in HeLa cells, abundant Gag-CFP was also readily seen at the plasma membrane in these cells (Fig. 3A, inset).

Strikingly, FIV Gag was also seen consistently at the nuclear envelope in all cell types (Fig. 3B, open arrowheads and enlargement). This finding is analyzed further below. Note also that, as shown in Fig. 3C, producing Gag from the RNA that is encapsidated rather than producing it in trans did not influence the Gag-CFP pattern. Here, Gag-CFP was expressed within a full-length FIV proviral DNA in which only pol is replaced by CFP (see Fig. S1 in the supplemental material); data in subsequent figures show that genomes with an intact [psi(−)] RNA distribution of the viral genomic RNA changed drastically when FIV Rev was coexpressed. In clear contrast, Gag-RFP did not localize to EEA1-containing early endosomes (Fig. 3H). These distributions are consistent with those recorded by other investigators for HIV-1 Gag (41, 42, 53).

MS2-XFP labeling in live cells reveals FIV genomic RNA accumulation at the nuclear envelope. We next investigated cellular trafficking of FIV genomic RNA using the MS2 system (Fig. 4). When expressed alone, MS2-YFP was confined to the nucleus in a diffusely homogeneous distribution, as expected because of its NLS (Fig. 4A). An FIV Rev expression plasmid was constructed and validated biochemically (see Fig. S2 in the supplemental material). MS2-YFP remained unchanged in distribution when coexpressed with either FIV Rev (Fig. 4B) or a transfer vector, FIVψ (Fig. 4C). In contrast, coexpression of MS2-YFP and an FIV transfer vector RNA having 24 tandem copies of the phage coat protein-binding element (FIVψ-24) resulted in a clearly different, nonhomogeneous (although still exclusively nuclear) distribution, generally with a speckled appearance consistent with focal intranuclear cotrafficking of genomic RNA and MS2-YFP (Fig. 4D).

The distribution of the viral genomic RNA changed drastically when FIV Rev was coexpressed (Fig. 4E). Labeled RNA was exported from the nucleus and migrated to the cytoplasm,
where it distributed throughout the cytoplasmic space in a speckled, punctate pattern. Notably, the genomic RNA was seen to accumulate around the nucleus in a rim-like pattern, suggesting a nuclear envelope location (e.g., Fig. 4E). Colocalization with the nucleoporin Nup153 confirmed this (Fig. 4K and L). Cells consistent with a range of temporal progressions in the Rev-dependent RNA export pathway were observed. For example, in many cells the majority of the MS2-YFP protein had been exported from the nucleus with trafficking RNA, and the remaining intranuclear MS2-YFP was highly altered as well, acquiring a speckled appearance. A similar RNA distribution was observed when the source of Rev was the FIV packaging plasmid pFP93, which expresses both Rev and unlabeled Gag-Pol (Fig. 4F). In COS-7 cells MS2-mCherry-labeled viral genomic RNA was also exported from the nucleus and accumulated in the cytoplasm in a speckled, punctate pattern (Fig. 4H), showing that the trafficking of the RNA was independent of the cell type or the XFP fused to the MS2 protein. In addition, note that the distribution of MS2-YFP-labeled RNA lacking the packaging signal (FIVΔψ-24) was the same as the distribution of wild-type RNA (Fig. 4G). This RNA migrated to the cytoplasm and also accumulated around the nuclear envelope.

Nuclear export of FIV genomic RNA was also induced when the RRE was replaced by four copies of the constitutive transport element (CTE) from Mason Pfizer monkey virus (MPMV) (5). The MS2 label was distributed throughout the cytoplasm and also accumulated around the nuclear envelope (Fig. 4I), indicating that the latter property was not Rev specific but common to both Rev- and CTE-mediated viral RNA export pathways. As with Rev/RRE export, the distribution of a CTE-dependent RNA lacking the packaging signal was similar to one containing it (Fig. 4J).

To verify that the distribution of the MS2 labeling reveals true RNA localization and not artifact induced by binding of the MS2-YFP protein, we performed RNA in situ hybridization experiments. HeLa cells were transfected with pFIV/H9274 in the presence or absence of pCiRev and then fixed and hybridized with a Cy3-labeled oligonucleotide probe that recognizes the RNA sequence linking MS2 binding loops (Fig. 5). Consistent with the results in living cells, FIVΔψ-24 RNA was confined to the nucleus in the absence of Rev (Fig. 5A). If Rev was coexpressed, FIVΔψ-24 RNA was exported from the nucleus and accumulated in the cytoplasm in a speckled pattern (Fig. 5B and C). Note that if cells were treated with RNase H before the hybridization with the Cy3 oligonucleotide probe,
no signal was detected, demonstrating that the hybridization observed was due to binding of the probe to RNA and not DNA (Fig. 5D). Untransfected cells (Fig. 5F) or cells transfected with FP93 (Fig. 5E), which lacks the stem-loops, resulted in background staining. In contrast to results of the live-cell experiments, we did not observe cells where the nucleus was completely devoid of RNA, which is likely due to limiting amounts of MS2-XFP protein molecules versus genomic RNAs. The MS2-XFP-RNA complexes have a predicted protein-to-RNA stoichiometry of 48:1 (MS2 binds as a dimer). In addition, Fusco et al. showed that on average 33 MS2 proteins are bound to one mRNA (18).

In situ hybridization was also done directly for full-length wild-type FIV genomic RNA. HeLa cells were transfected with a full-length FIV proviral clone, fixed, and hybridized with a Cy3-labeled oligonucleotide probe recognizing a sequence in the pol gene. Corroborating the MS2 experiments, this showed genomic RNA accumulation around the nuclear envelope (Fig. 5G). The RNA colocalized with an antibody to nuclear pore complexes, confirming that nuclear envelope accumulation of MS2-XFP-labeled RNA in living cells (Fig. 4) is not a result of artifact induced by bound MS2-XFP.

Colocalization of FIV Gag-CFP and MS2-XFP-labeled genomic RNAs at the plasma membrane requires Gag and an intact encapsidation signal. Having validated the RNA and Gag systems, we combined them to image FIV RNA and Gag simultaneously in live HeLa cells (Fig. 6). In the absence of a viral genome, MS2-YFP was strictly nuclear, as in Fig. 4A to C, whether or not Gag and/or Rev was also present (data not shown). When Gag-CFP, Rev, and MS2-YFP were coexpressed with FIVψ-24 RNA, colocalization was clear and abundant, as were marked shifts in MS2-YFP location (Fig. 6). The labeled RNA trafficked to the cytoplasm and associated with Gag in discrete cytoplasmic locations (Fig. 6A and B).

Note that substantial colocalization of FIV Gag and MS2-labeled FIV genome RNA occurred at the nuclear envelope (Fig. 6). Focal coaccumulations of the ψ(+)/ψ(-) genomic RNA with Gag were also seen in apposition to the cytoplasmic face of the nuclear envelope, perhaps at nuclear pores, where they extended into the perinuclear cytoplasm (Fig. 6A). Fig. S3 and Movie S1 in the supplemental material show time-lapse imaging of an MS2-Cherry-labeled RNA granule forming with Gag-CFP in the proximity of the nuclear envelope, followed by movement away from the nuclear envelope into perinuclear cytoplasm. In the cell shown in Fig. 6A, the majority of the RNA has been exported from the nucleus, but a spectrum of phenotypes consistent with different stages was observed. For example, Fig. 6B shows a cell with substantial amounts of nuclear MS2-YFP fluorescence and less colocalization of Gag-CFP and RNA in the cytoplasm, representing an earlier stage of RNA export. In contrast, when the genomic RNA lacked the encapsidation signal, cytoplasmic colocalization of the two signals was much less (Fig. 6, compare panels A and B with panels C and D).

In HeLa cells, we did not detect abundant steady-state colocalization of FIV Gag-CFP and MS2-labeled RNA at the plasma membrane (Fig. 6). This is consistent with the relatively less frequently observable plasma membrane accumulation of FIV Gag-CFP in HeLa cells demonstrated in Fig. 3A. However, Gag-XFP and genomic RNA colocalization was very evident at the plasma membrane in COS-7 cells (Fig. 7) and 293T cells (see Fig. S4 in the supplemental material). We compared viral genomic RNAs with and without the full encapsidation signal of the FIV RNA at the plasma membrane was observed, and this colocalized with Gag-CFP (Fig. 7D and E; see also Fig. S4A). In important contrast, FIVΔψ-24 RNA did not traffic to the plasma membrane although Gag-CFP accumulated there (Fig. 7F; see also Fig. S4B). This result was corroborated by quantitation of the different distribution patterns observed of FIV Gag-CFP and genomic RNA with (ψ) or without (Δψ) the packaging signal (Fig. 8). Gag and ψ-intact RNA colocalized at the plasma membrane in 17.1% of cells (Fig. 8C). In contrast, when the viral RNA was ψ(-), Gag still reached the plasma membrane in a similar percentage of cells (14.5%), but colocalization with the RNA was never seen (Fig. 8D). None of the other distribution patterns exhibited a difference between FIVψ-24 and FIVΔψ-24 RNA (Fig. 8A, B, E, F, and G). Thus, plasma membrane trafficking of the RNA genome requires that it contain the packaging signal. This encapsidation signal-dependent trafficking also required the presence of Gag, since it was not observed when Rev protein alone was coexpressed with the RNA (Fig. 7C). Coexpression of MS2-mCherry with the FIV genomic RNA but no Gag or
FIG. 6. Colocalization of FIV Gag-CFP and MS2-YFP-labeled FIV genomic RNA in live cells. HeLa cells were transfected with various combinations of plasmids. Bar, 10 μm. In the absence of a viral RNA, MS2-YFP had the appearance shown in Fig. 4A to C. (A) pFIVgag-CFP, pMS2-YFP, and pFIVψ-24. The nuclear envelope region (box, right panel) is shown enlarged below (scale bar, 5 μm). (B) pFIVgag-CFP, pFP93, pMS2-YFP, and pFIVψ-24. (C and D) pFIVgag-CFP, pFP93, pMS2-YFP, and pFIVΔψ-24. Live-cell confocal imaging was performed 48 h after transfection, and representative images are shown.
Rev resulted in nuclear redistribution of the RNA (Fig. 7B), as seen in HeLa cells (Fig. 4D).

Tracking the HIV-1 genomic RNA: targeting of the plasma membrane and endosomal vesicles requires Gag and an intact encapsidation sequence. To gain comparative insight, we also applied the MS2 system to HIV-1. The 24 stem-loops were similarly inserted in an HIV-1 vector, and a packaging-deficient version was created by deleting the four adjacent stem-loops (SL1 to SL4) upstream of the 5′ portion of the gag gene and 40 bp of the gag gene (Fig. 1E). To visualize HIV-1 Gag, we fused CFP to its C terminus (Fig. 1F).

Similar to FIV Gag-CFP, HIV-1 Gag-CFP is incorporated into viral particles (data not shown). Also similar to FIV, MS2-GFP incorporation was dependent on an intact HIV-1 encapsidation element (see Fig. S5, lanes 3 and 4, in the supplemental material). An HIV-1 RNA lacking the 24 MS2 stem-loops (HIV-lacZ) did not enable MS2-GFP particle incorporation (see Fig. S5, lane 2). To investigate the subcellular distribution of Gag-CFP in live cells, we transfected HeLa or COS-7 cells with pHIVgag-CFP together with plasmids encoding HIV-1 Tat and Rev (Fig. 9A and C) or unlabeled HIV-1 Gag-Pol, Tat, and Rev (Fig. 9B and D). HIV-1 Gag-CFP showed a punctate cytoplasmic distribution and strong accumulation at the plasma membrane in both HeLa and COS-7 cells (Fig. 9A to D). This is in contrast to FIV Gag-CFP, which showed less intense signal at the plasma membrane in HeLa cells (compare to Fig. 3A). In COS-7 cells HIV-1 Gag-CFP accumulated along the plasma membrane and in an intracellular compartment (Fig. 9C and D). These intracellular HIV-1 Gag-CFP puncta may reflect Gag-CFP endocytosed from the plasma membrane (26).

We next investigated the distribution of HIV-1 genomic RNA. In both HeLa and COS-7 cells, MS2-mCherry protein labeled the HIV-1 RNA and demonstrated efficient export from the nucleus and migration to the cytoplasm (Fig. 9E to G). In HeLa cells (Fig. 9E to G) the HIV-1 RNA showed a dispersed cytoplasmatic distribution.
In COS-7 cells, the HIV-1 genomic RNA was distributed throughout the cytoplasmic space in the presence of Tat and Rev (Fig. 9f). However, when unlabeled Gag-Pol was additionally provided, many cells accumulated RNA in the cytoplasm within relatively large focal punctate structures (Fig. 9f; compare with i). Importantly, trafficking of HIV-1 genome RNA to these cytoplasmic structures required both Gag and an intact encapsidation signal since RNAs lacking the encapsidation signal were distributed diffusely in the cytoplasm and never accumulated in such puncta (Fig. 9k; compare with j). Note that the COS-7 cell intracellular puncta with HIV-1 RNA (Fig. 9f) resemble closely the intracellular compartment in which HIV-1 Gag-CFP accumulated in these cells (Fig. 9c and d). We therefore investigated if the genomic RNA and CFP-labeled Gag co-localized. Indeed, when COS-7 cells were cotransfected with pHIVgag-CFP, pCMVΔR8.9, pMS2-mCherry, and packaging signal-intact HIVψ-24 RNA, many cells showed accumulation of Gag-CFP and the RNA in the same punctate cytoplasmic structures (Fig. 10a). In contrast, this colocalization was not observed with HIV-1 RNA with a deletion of ψ (Fig. 10c; here the Gag puncta still form, but without the RNA). Quantification of these differences is shown in Fig. 10d. Like FIV, the intracellular HIV-1 Gag puncta co-localize with the late endosomal markers CD63 and Lamp2; however, they did not colocalize with the P body marker DDX6 (RCK/p54) (data not shown). Taken together, these specific ψ(+) genomic RNA internalizations show that Gag and genomic RNA are internalized together from the plasma membrane into endosomes, and they show that the MS2 system can track the genomic RNA faithfully in the cell, even after the plasma membrane anchoring event. They show that it is not just Gag but also nascent virions with encapsidated genomes that become internalized. Both HIV-1 Gag-CFP and the labeled RNA could be seen coaccumulated at the plasma membrane (Fig. 10b, arrowhead). However, in contrast to findings in FIV, we could not detect coaccumulation of HIV-1 Gag and RNA at the nuclear envelope, suggesting either that this does not occur or that its kinetics are too rapid to allow visualization.

To further understand the molecular requirements, we tested Gag mutants that cannot be myristoylated (mutant G2A, which we introduced into both FIV and HIV-1 Gag proteins) and therefore are unable to accumulate at the plasma membrane (20). As shown in Fig. 11b, myristoylation-deficient FIV GagG2A-CFP and FIVψ-24 genomic RNA did colocalize around the nucleus, as with the normally myristoylated Gag. However, FIV GagG2A-CFP did not accumulate at the plasma membrane, and it was not able to bring the FIVψ-24 RNA to the plasma membrane. HIV-1 GagG2A-CFP did not accumulate at the plasma membrane or in cytoplasmic puncta, and the HIV-1 genomic RNA was distributed diffusely in the cytoplasm (compare Fig. 11c and d). These results are further evidence that lentiviral genomic RNAs are trafficked to (or secured at) the plasma membrane by Gag and that Gag and the genomic RNA recycle together.

**DISCUSSION**

Genome encapsidation is a critical event in HIV-1 assembly and an unexploited therapeutic target. It intersects with important current problems such as species-specific retroviral restriction (e.g., APOBEC3 protein incorporation into particles) and drug-resistant HIV-1 genome recombination. The molecular binding determinants in the genomic RNA and Gag are precisely known from quantification of virion and intracellular RNAs after deletions of ψ regions or their transfers to test RNAs. High-resolution structures of the complexes are even established (12, 15). However, because of methodological con...
constraints, the actual cell-biological process of lentiviral encapsidation—the transit of the RNA genome from nucleus to assembled particle—is less understood. Available methods have allowed Gag localization and trafficking to be analyzed extensively, recently culminating in the important delineation of host protein networks that participate in budding (40) and also achievement of increasing clarity on main routes of particle egress (26). In contrast, informative imaging of lentiviral genomic RNA trafficking has been more limited.

Our study applies the MS2 system to evaluate the cell-wide distribution and trafficking of the genomic RNAs of two different lentiviruses, FIV and HIV-1. Labeling with the combined MS2-XFP/Gag-XFP system preserves known main features of assembly, including budding and specific packaging of MS2-XFP-labeled genomic RNA into particles (Fig. 2; see also Fig. S5 in the supplemental material). We also verified that intracellular localizations of MS2-XFP-labeled genomes are the same as unlabeled genomes (Fig. 5). We find that plasma membrane accumulation of either lentiviral genomic RNA requires packaging-signal-specific interaction with its respective Gag protein, persuasively excluding a main alternative model in which the genome concentrates independently at the plasma membrane. RNA is visible at the plasma membrane if and only if the packaging signal is intact and if Gag is present. Thus, Gag clearly anchors RNA at the plasma membrane. Gag may also deliver it there, but this has not been clearly established by our study or other studies and represents a main future question for the field. This conclusion on Gag anchoring agrees with that of Jouvenet et al., who used MS2 tracking and fluorescent Gag proteins with TIR-FM to monitor individual HIV-1 assembly events in the plane of the plasma membrane (27). In that study, plasma membrane docking of RNA puncta required Gag and manifested as a single step in MS2 label intensity, with Gag accretion thereafter requiring the capacity of Gag to multimerize. TIR-FM studies by Ivanchenko et al. also suggested that Gag molecules are recruited to the assembling particle from the cytosol (24). Our results show also that when Gag is internalized into the endosomal pathway from the plasma membrane, encapsidated genomic RNA traffics with it (Fig. 6 and 10). Thus, it is important to interpret future analyses of genomic RNA trafficking with the understanding that intracellular RNA accumulations may represent not only recently synthesized genomes in the process of translation or encapsidation but also those that have been reinternalized after already

![Image of intracellular localization of HIV Gag-CFP or MS2-mCherry-labeled HIV-1 genomic RNA in HeLa and COS-7 cells.](http://jvi.asm.org/)

**FIG. 9.** Intracellular localization of HIV Gag-CFP or MS2-mCherry-labeled HIV-1 genomic RNA in HeLa and COS-7 cells. HeLa cells (A, B, and E to H) or COS-7 cells (C, D, and I to L) were transfected with various combinations of plasmids: pHIVgag-CFP, pCITat, and Rev (pLP2) (A and C); pHIVgag-CFP and pCMVΔR8.9 (B and D); pMS2-mCherry, pHIVψ-24, pCITat, and Rev (pLP2) (E and I); pMS2-mCherry, pHIVΔψ-24, and pCMVΔR8.9 (F and J); pMS2-mCherry, pHIVΔψ-24, and pCMVΔR8.9 (G and K); pMS2-Cherry (H and L). Live-cell confocal imaging was performed 20 h after transfection. Arrows in panels C, D, and J point to intracellular puncta, and arrows in panels F and G indicate accumulation of HIV-1 genomic RNA at the nuclear envelope. Representative images are shown. Bar, 10 μm.
cycling through the entire assembly pathway. Endosomal reuptake from the plasma membrane such as we observed may provide an alternative explanation for the findings of Basyuk et al., who proposed that MLV utilizes a vesicular assembly pathway in which endosomal or lysosomal membranes recruit MLV genomes from the cytosol and transport them to the plasma membrane along microtubules (2).

Neither our study nor TIR-FM experiments have so far been

FIG. 10. HIV Gag-CFP and MS2-mCherry-labeled HIV-1 genomic RNA colocalize in intracellular puncta in COS-7 cells. COS-7 cells were transfected with various combinations of plasmids. (A and B) pHIVgag-CFP, pCMVΔR8.9, pMS2-mCherry, and pHIVΔψ-24. Arrows in panel A point to intracellular puncta, and arrowheads in panel B point to plasma membrane accumulation. (C) pHIVgag-CFP, pCMVΔR8.9, pMS2-mCherry, and pHIVΔψ-24. Live-cell confocal imaging was performed 20 h after transfection. Scale bar, 10 μm. (D) Quantitation of colocalization of HIV-1 Gag-CFP and MS2-mCherry-labeled HIV-1 genomic RNA in intracellular puncta in COS-7 cells. A total of 381 puncta in 34 cells were evaluated for Δψ(+) RNA, and 324 puncta in 29 cells were evaluated for Δψ(-) RNA. Puncta were scored for containing both Gag and RNA, exclusively Gag (RNA absent), or exclusively RNA (Gag absent). The average of two independent experiments is shown.
able to determine where the initial Gag-genomic RNA association occurs in the cell or whether this can be reversible. Nevertheless, several of our findings with regard to the nucleocytoplasmic trafficking phase of the genomic RNA, particularly that of FIV, suggest novel features of assembly. Whether or not there are fundamental differences between these two lentviruses or whether certain processes are simply more detectable for FIV, suggest novel features of assembly. Whether or not these lendviruses or whether certain processes are simply more detectable for FIV with the present system is not clear. FIV and HIV-1 genomic RNAs were each seen to traffic from the nucleus to the cytoplasm (Fig. 4E and 9E). This was Rev dependent but Gag independent. RNA genomes of both lentiviruses were also found to localize packaging-signal-independently and Gag-independently at the nuclear envelope. This was more prominent for FIV genomic RNA, which accumulated conspicuously in a rim around the nucleus in the presence or absence of an intact encapsidation signal (Fig. 4F and G and Fig. 6). The same was detectable for HIV-1 genomic RNA, which accumulated less than FIV genomic RNA (Fig. 9E to G). FIV genomes were observed to track to the outer nuclear envelope whether the RNA was exported via the Rev-Crm1 pathway or via the MPMV CTE-NXF pathway (Fig. 4). Moore et al. showed recently that HIV-1 Gag is able to package genomic RNA from either export pathway, irrespective of the transport pathway used by the Gag-encoding mRNA (39).

Intriguingly, FIV Gag accumulated in the same nuclear envelope location as the RNA (Fig. 6), suggesting a possible locus for encapsidation. We were, in particular, able to observe discrete foci of colocalized FIV Gag and genomic RNA extending

![Figure 11](http://jvi.asm.org/)
from the cytoplasmic face of the nuclear membrane, suggestive of nucleopore association (Fig. 6). This consistent focal decoration suggests that Gag might bind RNA as the latter exits the nuclear pore. It is thus possible that Gag escorts the genome during its entire cytoplasmic journey. Such a model would be consistent with the recent evidence that initial dimerization events and selection of HIV-1 copackaged RNAs occur after nuclear exit (39). In a recent TIR-FM study, an excess of unlabeled Gag was used along with the XFP-labeled HIV-1 Gag (27). It cannot be excluded that unvisualized Gag molecules bind to the genomic RNA in the cytoplasm or even at the nuclear envelope and direct it to the plasma membrane, where visualization of labeled Gag becomes detectable as subsequent particle assembly occurs. The study did suggest that if Gag transports the genome to the plasma membrane, this is done by a few Gag molecules that are below the limit of detection at present. The outer nuclear envelope targeting we observed was packaging signal independent and occurred when either molecule was expressed alone, which implies that the main process being observed is not the trapping or sequestering of Gag by genomic RNA as it emerges from nuclear pores but, rather, independently directed processes driving Gag and the genome to this location. Since both \( \psi^+ \) and \( \psi^- \) genome RNAs are Rev escorted, it is possible that Rev-chaperoned export also enhances Gag acquisition. The DEAD-box RNA helicases DDX3 and DDX1 were suggested to facilitate translocation of the RNA/Rev complex through the nuclear pore (16, 56). DDX1 is a cellular cofactor of Rev, and DDX3 interacts with Crm1. Since DDX3 localizes to the outer nuclear membrane and since FIV genomic RNA also accumulates around the nucleus, it is possible that the DEAD box proteins or other RNA helicases are involved in remodeling genomic RNA structure and in facilitating nuclear export.

In accord with the published results of numerous other laboratories, we could not detect HIV-1 Gag at the nuclear envelope, and this was the main apparent difference between the two lentiviruses. However, it was also more difficult to identify HIV-1 Gag and genomic RNA coaccumulation at the plasma membrane in any cell type or to see the HIV-1 genomic RNA at the nuclear envelope. It is possible that these differences in detectable localization between the two lentiviruses are mainly kinetic in origin, such that the FIV studies reveal general lentiviral properties obscured for HIV-1 by more efficient postsynthesis (46). Indeed, TIR-FM studies indicate that the process of virion assembly and budding at the plasma membrane occurs on a time scale of only a few minutes (24, 25, 27). Rapid transiting, preventing detection of transient HIV-1 RNA locations in steady state, has been reported (31). In addition, HIV-1 Tat induces the expression of DDX3 in HeLa cells (56), which in turn could result in faster removal of the RNA from the nuclear envelope. An additional factor to be considered is that primate cells were used for these studies, and slower FIV kinetics or local accumulations might reflect missing feline cellular factors.

Mutation of the myristoylation signal of Gag (Gag\(_{G2A}\)-CFP) blocks plasma membrane association of the RNA as well, which supports the primary role of Gag in anchoring the RNA there (Fig. 11). However, Gag\(_{G2A}\)-CFP still colocalized with FIV\(\psi\)-24 at the nuclear envelope. If the genome traffics independently of Gag to the plasma membrane and is trapped there by membrane-associated Gag, the failure of \( \psi^- \) RNAs to be seen at all at the plasma membrane would be the result of transience due to lack of Gag capture. A more complex alternative cannot be excluded, namely, that deletion of the encapsidation sequence prevents not just NC binding but also binding of a cellular RNA-trafficking protein that pretargets the RNA to the site of Gag assembly or guides the preformed RNA/Gag complex to the plasma membrane. Nevertheless, Gag proteins of both lentiviruses clearly access the plasma membrane without the genomic RNA. A possible candidate for a cellular trafficking chaperone is the double-stranded RNA binding protein Staufen1, which has been implicated in regulating HIV-1 genomic RNA encapsidation (8, 9).

We favor a model according to which lentiviral Gag-RNA binding occurs before plasma membrane particle assembly, and Gag transports the RNA to the assembly site rather than one in which these two components traffic independently and meet there. It is also clear from our studies that Gag and the encapsidated genomic RNA colocalize from the plasma membrane, with Gag and an intact \( \psi^- \) being necessary for the RNA to traffic in this manner. These experiments thus establish that incorporation of the genomic RNA does not somehow channel the forming particle into an egress-only pathway. Based on the FIV findings with both static and time-lapse imaging, we also propose that lentiviral Gag-genomic RNA interactions could initiate at the nuclear pore, with Gag transporting the genomic RNA through its entire cytoplasmic journey.

**ACKNOWLEDGMENTS**

We thank Robert Singer (Albert Einstein College of Medicine) for pMS2-GFP, pMS2-YFP, and pSL-MS2-24x, Roger Tsien (University of California, San Diego, CA) for pRSET-B-mCherry, Chris Grant (Custom Monoclonal Antibodies International, Sacramento, CA) for the Nup153-GFP plasmid. The monoclonal antibody to HIV-1 p24 (AG3.0) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. We thank Jim Tarara (Mayo Clinic Flow Cytometry and Imaging Core) for helpful discussions, provision of reagents, and technical assistance.

This work was supported by NIH grant AI47536.

**REFERENCES**


8. Chatel-Chaix, L., L. Abrahamyan, C. Frechini, A. J. Mouland, and L. Des-


