In some retroviruses, such as Rous sarcoma virus and prototype foamy virus, Gag proteins are known to shuttle between the nucleus and the cytoplasm and are implicated in nuclear export of the viral genomic unspliced RNA (gRNA) for subsequent encapsidation. A similar function has been proposed for human immunodeficiency virus type 1 (HIV-1) Gag based on the identification of nuclear localization and export signals. However, the ability of HIV-1 Gag to transit through the nucleus has never been confirmed. In addition, the lentiviral Rev protein promotes efficient nuclear gRNA export, and previous reports indicate a cytoplasmic interaction between Gag and gRNA. Therefore, functional effects of HIV-1 Gag on gRNA and its usage were explored.

Expression of gag in the absence of Rev was not able to increase cytoplasmic gRNA levels of subgenomic, proviral, or lentiviral vector constructs, and gene expression from genomic reporter plasmids could not be induced by Gag provided in trans. Furthermore, Gag lacking the reported nuclear localization and export signals was still able to mediate an efficient packaging process. Although small amounts of Gag were detectable in the nuclei of transfected cells, a Crm1-dependent nuclear export signal in Gag could not be confirmed. Thus, our study does not provide any evidence for a nuclear function of HIV-1 Gag. The encapsidation process of HIV-1 therefore clearly differs from that of Rous sarcoma virus and prototype foamy virus.

Encapsidation of the retroviral genome occurs at the assembly site of the viral proteins Gag/Gag-Pol, leading to budding of new viral particles containing two copies of the genomic viral RNA (gRNA). Precise information about temporal and spatial aspects of the interaction between gRNA and Gag are, however, lacking for most, if not all, retroviruses. Since the integrated provirus is transcribed in the nucleus, unspliced gRNA molecules have to be actively exported to the cytoplasm (14, 19). Binding of the genomic transcripts by Gag translated from the unspliced RNA is mediated by a specific interaction between a secondary structure in the gRNA called encapsidation signal (Ψ) and an RNA binding domain in Gag composed of either one or two zinc fingers in the nucleocapsid (NC) domain of all orthoretroviruses (17).

Association between Gag and gRNA of Rous sarcoma virus (RSV) seems to occur in the nucleus (RSV Gag is synthesized as a Gag-protease fusion but is referred to as Gag here). Initial export of the gRNA is mediated by cis-acting direct repeat (DR) elements leading to the synthesis of Gag (37, 47, 48, 68). RSV Gag has been shown to contain two nuclear localization signals (NLS) and one nuclear export signal (NES). By forming complexes with different importins and the nuclear export factor Crm1, Gag shuttles constitutively between the nucleus and the cytoplasm (12, 26, 58, 59). Gag mutants that bypass the nuclear compartment encapsidated reduced levels of gRNA, although particle formation was not impaired (13, 58). Furthermore, association of Gag with RNAs bearing the encapsidation signal led to dissociation of importins and stimulated binding of the nuclear export factor Crm1 (26). Binding of RSV Gag to its gRNA in the nucleus therefore promotes the formation of an export-competent Gag-gRNA complex. These observations led to a model in which Gag is first translated from gRNA exported from the nucleus by its DR elements. After import into the nucleus, Gag associates with the nuclear gRNA and exports gRNA by a Crm1-dependent pathway for packaging at the plasma membrane (50). Recently, Gag of the prototype foamy virus (PFV) has also been shown to be a Crm1-dependent nuclear-cytoplasmic shuttle protein and an encapsidation mechanism similar to that of RSV was suggested (57).

In the case of HIV, nuclear export of the unspliced RNA is mediated by the viral protein Rev. Rev interacts with incompletely spliced and unspliced HIV RNAs containing a Rev-responsive element (RRE) and transports these transcripts via the cellular Crm1-mediated export pathway from the nucleus to the cytoplasm (19, 23, 25). Subsequently, Gag is translated from the gRNA.

Similar to RSV and PFV, a nuclear role of HIV-1 Gag was suggested by reports on karyophilic properties of HIV-1 Gag. Virion-associated capsid (CA), matrix (MA), and MA-containing Gag cleavage products were shown to enter the nucleus shortly after fusion of the viral and cellular membranes (21, 62, 69). After transfection of proviral constructs, wild-type Gag is localized mainly in the cytoplasm and at the plasma membrane, whereas an NC deletion mutant of Gag was detected inside the nucleus in a subpopulation of cells (24). Furthermore, in cells transfected with gag expression plasmids (27, 52) or proviral constructs (38, 40, 53), small amounts of wild-type Gag were also detectable inside the nucleus. A direct role of uncleaved Gag in the export of gRNA from the nucleus to the cytoplasm was proposed when the cellular protein VAN was identified as a binding partner of MA and Gag. Endogenous VAN colocalized with MA and Gag, and in over 25% of the cells, both proteins were present in the nucleus (27). Additionally, abnormal trafficking of HIV-1 gRNA was shown to affect the cellular localization of Gag (4). Silent mutations in the genomic tran-
script inhibiting its hnRNP A2-dependent trafficking caused a redistribution of gRNA into the nucleus. Concomitantly, Gag was sequestered in the nuclear periphery and in a small subset of cells also inside the nucleus (4). In addition, two NLS and a Crm1-dependent NES were identified in HIV-1 Gag (10, 18, 28). Mutation of the NES in a proviral construct was reported to result in a packaging defect based on nuclear sequestration of the gRNA. The NES mutant of Gag also induced nuclear accumulation of wildtype gRNA (18). Based on these observations, a model emerges in which Rev mediates the initial export of the gRNA to the cytoplasm leading to the production of Gag proteins. After nuclear import mediated by its NLS, Gag associates with the gRNA and facilitates a Crm1-dependent export of the Gag-gRNA complex to the cytoplasm, thereby enabling translation and encapsidation of the gRNA (41) (Fig. 1).

However, there are also a number of observations that are difficult to reconcile with this model. In particular, there is evidence for the existence of a single pool of HIV-1 gRNAs for translation and encapsidation (11). Dimerization of the gRNAs occurs in the cytoplasm prior to encapsidation (45, 46). Gag is localized primarily in the cytoplasm and at the plasma membrane (e.g., see reference 53) and is not able to rescue the infectious titer of lentiviral particles. After nuclear import mediated by its NLS, Gag associates with the gRNA and facilitates a Crm1-dependent export of the Gag-gRNA complex to the cytoplasm, thereby enabling translation and encapsidation of the gRNA (41) (Fig. 1).

In order to resolve these controversial data leading to the different models, we directly analyzed the effect of Gag on cytoplasmic gRNA levels and the utilization of the gRNA for translation and encapsidation.

FIG 1 Two models of the primary interaction between Gag and gRNA of HIV-1. After the translation of multiply spliced transcripts, the viral protein Rev is imported into the nucleus and binds to the RRE in singly spliced (not shown) and unspliced gRNAs. Rev facilitates nuclear export (step 1) and translation of the gRNA. In the widely accepted model of the packaging process, Gag binds to the encapsidation signal (3’ end) of the cytoplasm (2’) and encapsidation of gRNA dimers into Gag particles subsequently takes place at the plasma membrane (3’). However, after the identification of NLS and NES in Gag, an alternative model has been proposed in which Gag is imported into the nucleus (2’), where it associates with the encapsidation signal (3’) to allow Gag-mediated nuclear gRNA export (4’) for packaging at the plasma membrane (5’).
293T cells were transfected using the calcium phosphate coprecipitation method (60) or by polyethylenimine (3). The total amount of transfected DNA was adjusted to 15 μg for calcium phosphate transfections and to 10 μg for polyethylenimine transfections with calf thymus carrier DNA (Invitrogen). The transfected amount of the subgenomic, proviral, and lentiviral vector constructs was 5 μg DNA for calcium phosphate transfections and 0.5 μg DNA for polyethylenimine transfections because the latter approach allowed higher transfection efficiencies (data not shown). Transfections of 50,000 HeLa cells in a 24-well plate format by calcium phosphate coprecipitation were done with a total DNA amount of 3 μg.

One day after transfection, the cell culture medium was renewed. Plasmids pCMV-GLuc-1 and pEGFP-C1 were cotransfected, and expression of the luciferase gene and gfp was analyzed to assess the efficiency of transfection.

**LMB treatment, immunofluorescence, and microscopy.** HeLa cells were seeded into 24-well plates on glass coverslips and incubated with 13 nM leptomycin B (LMB; Sigma) in DMEM with 1% fetal calf serum 2 days after transfection. After LMB treatment, the cells were fixed in 3.5% paraformaldehyde in phosphate-buffered saline (PBS; Invitrogen), permeabilized in 0.2% Triton X-100 in PBS, and blocked with 1% bovine serum albumin (BSA) in PBS. Detection of proteins was achieved by incubation with the primary mouse antibody 183-H12-5C against p24CA (NIH AIDS Research and Reference Reagent Program) and the primary rabbit antibody against IκBα (C-21; Santa Cruz Biotechnology), followed by the secondary antibodies Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 555-labeled goat anti-mouse IgG (Invitrogen). DNA was stained with To-Pro-3 (Invitrogen). Cells were examined under a DM IRE2-TCS SP2 confocal laser scanning microscope (Leica) using excitation wavelengths of 488, 543, and 633 nm.

**Western blot analyses.** Nuclear and cytoplasmic fractions were isolated essentially as described previously (6, 7, 36). Two days after transfection, HEK 293T cells were detached and washed in cold PBS (Invitrogen). Plasma membranes were lysed by resuspension of the pelleted cells in 175 μl of cold RLN buffer (50 mM Tris-Cl [pH 8.0], 140 mM NaCl, 1.5 mM MgCl₂, 0.5% [vol/vol] Nonidet P-40, 1,000 U/ml RNase inhibitor, 1 mM dithiothreitol) and incubation for 5 min on ice. The nuclear fraction was carefully separated from the cytoplasm after a short (2-min) low-speed centrifugation at 300 x g and 4°C. An additional high-speed centrifugation step (3 min at 4°C and 13,000 x g) of the cytoplasmic fraction was used to pellet any remaining nuclear contaminations (36). The remaining cytoplasmic fraction was adjusted to 500 μl with BLP lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 40 mM Naf, 5 mM EDTA, 5 mM EGTA, 1% [vol/vol] Nonidet P-40, 0.1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS). The nuclear pellet was washed in 900 μl cold 

---

**FIG 2** Vector and expression constructs. Shown are the subgenomic, proviral, and lentiviral vector constructs and the gag and gag/gag-pol expression constructs used in this study. Long terminal repeat (LTR) and promoter regions are indicated as wide light gray boxes. Slim dark gray bars represent open reading frames of viral proteins, and white bars represent inactivated open reading frames. Hatched bars show codon-optimized sequences. Asterisks demonstrate the insertion of mutations (frameshift mutations or insertion of stop codons), and a Δ marks deleted sequences. The encapsidation signal Ψ and the RRE are represented by black dots on the unspliced transcript. The length and position of all elements are drawn to scale.
PBS (Invitrogen), centrifuged at 300 X g for 2 min at 4°C, and resuspended in 500 μl BLP lysis buffer. After centrifugation through a QiShredder column (Qiagen), the flow-through was collected as the nuclear fraction. Similar protein amounts were subjected to SDS-PAGE as determined by Bradford assay (Bio-Rad). Whole-cell lysates were prepared using the stringent BLP lysis buffer. After separation by PAGE and transfer to nitrocellulose membranes, proteins were detected with the following antibodies: mouse anti-Gag/p24CA (183-H12-5C; NIH AIDS Research and Reference Reagent Program), rabbit anti-MEK-1 (C-18; Santa Cruz Biotechnology), mouse anti-PDI (C-2; Santa Cruz Biotechnology), rabbit anti-α-tubulin (600-401-880; Rockland), and mouse anti-GFP (B-2; Santa Cruz Biotechnology). Appropriate secondary antibodies coupled with horseradish peroxidase (Dako) were used in combination with an ECL substrate (Alpha Innotech) to stain the blots.

**Encapsulation assay and quantitative reverse transcriptase PCR (RT-qPCR).** RNA copy numbers in the cytoplasm and in virus particles harvested from the supernatant of transfected HEK 293T cells were determined essentially as described previously (6, 7). In brief, the cytoplasmic fraction was isolated as described above (see Western blot analyses). The RNA was extracted using the RNeasy Mini kit (Qiagen). Virus particles in the supernatant of transfected cells were pelleted through a 30% sucrose cushion and resuspended in 150 μl PBS (Invitrogen). RNA was isolated from 140 μl with the QIAamp Viral RNA Mini kit (Qiagen). Cytoplasmic and virus particle RNA samples were stored at −70°C until RT-qPCR analysis. For the detection of extracellular p24CA levels, 10 μl of the re-suspended virus particle solution were analyzed by ELISA (see below) after storage of the samples at −20°C.

The gRNA levels in cytoplasmic and particle-associated RNA samples were determined by the Quantitect Probe RT-PCR kit (Qiagen) as described previously (6–8). The specificity of the amplification was confirmed by omitting the RT and agarose gel and melting curve analysis after the amplification process. The test sensitivity for wild-type gag mRNA was more than 10^-6-fold higher than that for the gag mRNA of codon-optimized Hgp^opt (data not shown). The cellular fractionation procedure had been validated previously (6, 7). Nuclear proteins were not detectable in the cytoplasmic fraction, and an unspliced nuclear pre-mRNA was concentrated 16- to 31-fold higher in the nuclear fraction than in the cytoplasmic fraction.

**FACS analysis.** Two days after transfection with the proviral construct NL4.3Ren-MAGFP-Luc, HEK 293T cells were detached from the flask, resuspended in fluorescence-activated cell sorter (FACS) buffer (0.5% [wt/vol] BSA and 0.01% [wt/vol] NaN3 in PBS [Invitrogen]) after low-speed centrifugation and incubated for 10 min with 7-aminoactinomycin D (BD Pharmingen) to stain dead cells. The percentage of GFP-positive living cells was determined using a FACSCalibur flow cytometer (BD Biosciences).

**Titer examination.** HEK 293T cells were transfected with a proviral or lentiviral vector construct, pcTat, pHit/G, or pcMV-Gluc-1, and with or without pcRev and Hgp^opt. As a negative control, pHit/G was omitted from some transfections. Two days later, TZM-bl cells or HEK 293T cells expressing beta-galactosidase-forming units per ml of supernatant. GFP-positive HEK 293 cells were counted under a fluorescence microscope after 3 days of infection with lentiviral vector-containing supernatants to quantify GFP-forming units per ml of supernatant.

**ELISAs.** To analyze intracellular Gag levels, cell lysates were incubated in 96-well plates over night at 4°C in coating buffer (100 mM Na2CO3, pH 9.5). After blocking with skim milk powder in PBS—0.05% Tween 20, the samples were incubated with the p24CA-specific primary antibody 183-H12-5C (NIH AIDS Research and Reference Reagent Program) and a secondary rabbit anti-mouse antibody coupled to horseradish peroxidase (Dako). The optical density at 450 nm was measured in a Sunrise microplate ELISA reader (Tecan) after incubation with 3,3',5',5'-tetramethylbenzidine (Dako) and subsequent stopping of the reaction with 0.5 M H2SO4. Quantification of the samples was achieved by analyzing a serial dilution of recombinant HIV-1 SF2 Gag (NIH AIDS Research and Reference Reagent Program) in parallel. The lower detection limit was 20 ng of Gag/ml.

After ultracentrifugation of virus particle-containing supernatants, extracellular p24CA/Gag levels were quantified by the HIV-1 DIY p24 sandwich ELISA kit 2 (Aalto Bio Reagents).

**RESULTS**

Influence of Gag on expression of subgenomic lentiviral reporter constructs. To explore whether HIV-1 Gag can mediate export of gRNA from the nucleus to the cytoplasm the subgenomic expression plasmid UTRg*pRRE was constructed. The unspliced RNA of this expression plasmid contains the core encapsidation signal (starting with stem-loop 1, also called the dimerization initiation site), followed by wild-type coding sequences for Gag and Gag-Pol (Fig. 2). The RRE was added 3' to the pol sequence and comprises HIV-1 splice acceptor site 7. Since we wanted to compare the effects of Gag and Rev independently of each other, a frameshift mutation was introduced near the 5' end of gag in the MA domain (indicated by the asterisk in g*p) to prevent the production of full-length Gag. Rev-independent expression of gag/gag-pol was achieved by the codon-optimized expression plasmid Hgp^opt. Since transcription from this plasmid generates an RNA lacking Rev binding sites, Gag/Gag-Pol levels are not influenced by the presence or absence of Rev (6, 7) (Fig. 3D). After cotransfection of UTRg*pRRE with or without Hgp^opt or a rev expression plasmid (pcRev), the transfected HEK 293T cells were fractionated and the cytoplasmic RNA was isolated. The unspliced transcript was quantified by RT-PCR specific for a gag sequence at the beginning of CA. Due to codon optimization, the Hgp^opt RNA is not detected in this setting (data not shown). Control PCRs performed in the absence of RT excluded contamination of the cytoplasmic RNA with the transfected UTRg*pRRE DNA. Cytoplasmic RNA levels are presented relative to the RNA levels obtained after transfection of UTRg*pRRE without pcRev and Hgp^opt (Fig. 3A). Rev clearly increased the amount of cytoplasmic unspliced RNA by a factor of 3.5, while cotransfection of Hgp^opt did not change the cytoplasmic RNA levels. Providing Gag/Gag-Pol and Rev simultaneously did not increase cytoplasmic gRNA levels of UTRg*pRRE above the levels obtained by Rev alone (Fig. 3A). Gradually reducing the amount of UTRg*pRRE DNA to 0.01 μg did not reveal an effect of Gag on cytoplasmic UTRg*pRRE gRNA levels at any dose (Fig. 3B).

Insertion of frameshift mutation at the 5’ end of the gag open reading frame does not abolish gag expression entirely. N-terminally truncated Gag isoforms can be produced due to translational initiation from ATG142 located at the beginning of the CA domain (9, 44, 54). Such isoforms were detectable after the transfection of UTRg*pRRE in the presence of Rev (Fig. 3C and D). Measurement of CA levels by ELISA demonstrated that Rev enhanced expression from UTRg*pRRE and UTRg*pRRE but not from UTRgp, which lacks the RRE (Fig. 3C). Comparison of UTRg*pRRE and nonmutated UTRg*PpRRE revealed a 37-fold reduction of the amount of Gag expressed from the mutated construct in the presence of Rev (Fig. 3C). The N-terminal deletion of
MA with its myristoylation signal should abolish the binding of Gag to membranes, and the truncated isoforms were indeed not able to form viral particles (data not shown). Since these isoforms are also detectable in HIV-infected cells and seem to promote virus replication (9, 44), they should not interfere with known functions of Gag. Because some of the truncated Gag proteins were expressed only from UTRg*pRRE and not from the codon-optimized expression plasmid Hgpsyn, we were able to study the influence of Gag not only on cytoplasmic UTRg*pRRE RNA but also on protein levels encoded by this RNA. The truncated Gag isoforms (marked with white arrows in Fig. 3D) could be detected only in the presence of Rev. Cotransfection of UTRg*pRRE with Hgpsyn only revealed the expression of the full-length Gag/Gag-Pol isoform pattern. Bands corresponding to the truncated Gag isoforms were not detectable (Fig. 3D). In agreement with Gag’s inability to increase cytoplasmic levels of the unspliced UTRg*pRRE RNA, it is also not able to enhance the levels of the protein encoded by this RNA.

**Influence of Gag on the expression of proviral and lentiviral vector constructs.** Sequences upstream and downstream of the HIV-1 core encapsidation signal influence the efficiency of the packaging process (17, 41). Upstream elements are not included in the UTRg*pRRE construct. It is therefore possible that the ability of Gag to associate with the UTRg*pRRE transcript is impaired. RNA molecules that contain all of the sequence elements known to influence encapsidation were therefore provided by proviral constructs and a lentiviral vector (Fig. 2). All proviral constructs contain inactivating point mutations in rev and a frameshift in env (NL4.3Re) (6). In addition to the frameshift mutation in the MA domain, another mutation was introduced into the proviral construct NL4.3Reg** to minimize the synthesis of truncated Gag proteins. The mutation changes ATG142 to ATC, further reducing the size of the truncated Gag proteins that might be produced by the use of alternative start codons (see below). The proviral construct NL4.3Re-mutCA was generated by introducing two stop codons after ATG142, thereby preventing the production

![FIG 3](http://jvi.asm.org/)

**A** Ability of Gag to mediate gRNA export and translation from a subgenomic reporter construct. (A) After extraction of the cytoplasmic RNA from cells cotransfected with UTRg*pRRE, Hgpsyn ( + Gag), and pcRev (+ Rev) as indicated, the gRNA was quantified by RT-qPCR. Fold stimulation of cytoplasmic gRNA abundance is shown. Mean values and standard deviations of at least three independent transfection experiments are shown. In the presence of Rev and Gag, mean RNA copy numbers after the transfection of 5 µg of UTRg*pRRE were 1.3 × 10⁷/µg of cytoplasmic RNA. (B) The amount of transfected UTRg*pRRE plasmid DNA was reduced stepwise to 0.01 µg. Corresponding cytoplasmic gRNA levels were quantified by RT-qPCR. Logarithmic transformation of copy numbers obtained by RT-qPCR was done before the calculation of mean values. Depicted are mean values and standard deviations of at least three independent transfection experiments. (C) Gag levels in cell lysates of transfected HEK 293T cells were analyzed by a p24CA-specific ELISA. Mean values and standard deviations of two independent transfection experiments are shown. †, both values below the detection threshold. (D) Western blot analysis with an anti-p24CA antibody detected the wild-type Gag protein pattern from the codon-optimized Hgpsyn and truncated Gag isoforms (white arrows) encoded by UTRg*pRRE. Shown are results of two independent cotransfections with pcRev (lanes 2 and 3) and Hgpsyn (lanes 4 and 5). Loading of similar amounts of protein was verified by incubation of the stripped Western blot with an antibody against the endogenous protein α-tubulin. Transfection efficiencies were similar, as demonstrated by a separate Western blot analysis against GFP encoded by the cotransfected pEGFP-C1.
of CA-containing Gag isoforms (data not shown). Use of the lentiviral vector VH, which does not encode any viral protein, also allows direct comparison of the posttranscriptional effects of Gag and Rev (7).

Cytoplasmic gRNA levels were quantified after transfection as described for the subgenomic UTR\textsuperscript{g,pRRE} construct. The unspliced lentiviral vector transcript was quantified by the insertion of a PCR target sequence upstream of the RRE between splice donor 1 and splice acceptor 7 of HIV-1 (7). Mean cytoplasmic gRNA levels per \(rac{\mu g}{H9251}\) of cytoplasmic RNA in the presence of Gag/Gag-Pol and Rev were 9.9 \(\times 10^6\) for NL4.3Reg\textsuperscript{**}, 3.5 \(\times 10^7\) for NL4.3Re-mutCA, and 1.7 \(\times 10^9\) for the lentiviral vector VH. A 7- to 8-fold increase in the cytoplasmic gRNA levels was observed in the presence of Rev for the proviral constructs (Fig. 4A and B). The effect on the lentiviral vector RNA was smaller but still 4-fold (Fig. 4C). The presence of Gag and Rev, cytoplasmic gRNA levels were lower than in the presence of Rev only (Fig. 4A to C). A potential explanation is that efficient packaging is possible under these experimental conditions at the expense of the amount of cytoplasmic gRNA. Thus, the analysis of proviral and vector constructs confirmed that Gag is not able to increase the cytoplasmic levels of different gRNAs containing the entire encapsidation signal.

Synthesis of truncated Gag isoforms from NL4.3Reg\textsuperscript{**} also allowed an analysis of whether Gag provided in trans enhances gene expression from a proviral construct. The truncated proteins detected with a CA antibody by Western blot analysis partially co-migrated with cleavage products of wild-type Gag (white arrows in Fig. 4D). However, after prolonged incubation of the Western blot, it was obvious that addition of Rev allowed the production of truncated Gag isoforms (white arrows in Fig. 4D). In contrast, coadministration of Gag/Gag-Pol did not induce the synthesis of the truncated isoforms from the genomic RNA. To verify the effects of Rev and Gag on the expression from gRNAs, the proviral reporter construct NL4.3Ren-MAGFP-Luc was generated by fus-

FIG 4 Ability of Gag to mediate gRNA export and translation from proviral and vector constructs. (A to C) Genomic RNA levels were quantified by specific RT-qPCRs after extraction of the cytoplasmic RNA from cells transfected with the proviral construct NL4.3Reg\textsuperscript{**} (A) or NL4.3Re-mutCA (B) or the lentiviral vector VH (C). Fold stimulation of cytoplasmic gRNA abundance is shown. Depicted are mean values and standard deviations of at least four independent transfection experiments. (D) The wild-type gag/gag-pol expression pattern and the truncated Gag isoforms (white arrows) encoded by NL4.3Reg\textsuperscript{**} were detected by an anti-p24CA Western blot analysis. A prolonged incubation of the Western blot demonstrates that the presence of Gag/Gag-Pol did not induce a detectable level of the truncated isoforms (white arrows, right). Shown are results of two independent cotransfections with pcRev (lanes 2 and 3) and Hgp\textsuperscript{syn} (lanes 4 and 5). Loading of similar amounts of protein was verified by incubation of the stripped Western blot with an antibody against the endogenous protein \(\alpha\)-tubulin. Transfection efficiencies were similar, as demonstrated by a separate Western blot analysis against GFP encoded by the cotransfected plasmid pEGFP-C1. (E) Living, GFP-positive cells were analyzed by flow cytometry 2 days after the cotransfection of HEK 293T cells with the proviral plasmid NL4.3Ren-MAGFP-Luc and Hgp\textsuperscript{syn} (+ Gag; 0.5 or 0.05 \(\mu g\) of plasmid DNA) and/or pcRev (+ Rev) as indicated. The fold increase in the mean fluorescence intensities (MFI) and standard deviations of at least four independent transfection experiments is shown.
ing the open reading frames of MA and EGFP without an intervening protease cleavage site and followed by stop codons. The firefly luciferase gene was inserted in place of nef (Fig. 2). As expected for the MA-GFP fusion protein, GFP fluorescence was detected only in the cytoplasm, as judged by confocal laser scanning microscopy (data not shown). The mean fluorescence intensities from flow cytometric analyses of living HEK 293T cells transfected with NL4.3Ren-MAGFP-Luc demonstrated that Rev increased GFP levels 27-fold. Cotransfection of Hgpsyn, however, decreased GFP levels by a factor of 2.5. Hence, Gag/Gag-Pol did not stimulate nuclear export of and gene expression from gRNA in this assay either.

**Influence of Rev and Gag on virion infectivity.** Cotransfection of cells with a codon-optimized gag/gag-pol expression plasmid and an expression plasmid encoding a fusogenic surface protein (like VSV-G) results in the formation of virus particles that contain all of the viral proteins necessary for the early steps of viral replication. If nuclear Gag proteins were able to export the gRNA to the cytoplasm for encapsidation at the plasma membrane, expression of VSV-G, gag/gag-pol, and the genomic RNA in the absence of Rev should lead to the production of infectious viral particles containing the gRNA. The infectious titers of proviral and lentiviral vector constructs were therefore analyzed in the absence and presence of Rev and Gag/Gag-Pol (Fig. 5A and B).

TZM-bl indicator cells were infected with cell culture supernatants in serial 10-fold dilutions 2 days after the cotransfection of HEK 293T cells with the proviral construct NL4.3Reg** (A, light gray) or NL4.3Re-mutCA (A, dark gray) or the lentiviral vector V^H (B) in combination with pC Tat and pHit/G and with or without Hgp^NN^ and pCRev. The infectious titer was evaluated after infection of TZM-bl cells as beta-galactosidase-forming units per ml of supernatant or after infection of HEK 293T cells as GFP-forming units per ml of supernatant for the proviral constructs or the lentivector, respectively. The parental provirus NL4.3Re (depicted as Re+Gag+Rev) was analyzed in parallel to NL4.3Reg** and NL4.3Re-mutCA. The dashed line represents background values obtained without pC Tat. (C) The encapsidation efficiency of gRNA was analyzed after the cotransfection of either the proviral construct NL4.3Re with Hgp^NN^ or the proviral construct NL4.3Re-MA with Hgp^NN^AMA with or without coexpression of rev. Re-MA and Hgp^NN^AMA contain a replacement of the whole matrix sequence with the myristoylation signal sequence of c-src. Cytoplasmic and particle-associated gRNA levels were quantified by specific RT-qPCR. Furthermore, the amount of p24CA in the virus particle preparations was analyzed by ELISA. (D) Two days after transfection, cellular lysates and particle preparations were collected and analyzed for intracellular and virion-associated p24CA levels by ELISA. Mean values and standard deviations of at least three independent experiments are shown (A to D).
strated a 3- to 4-fold reduction in infectivity (Fig. 5A). However, omission of Rev and coadministration of Gag/Gag-Pol alone reduced the titers by >2 orders of magnitude (Fig. 5A). The remaining infectivity was only marginally affected by the omission of Gag/Gag-Pol and is most likely due to pseudotransduction by VSV-G-coated cellular vesicles transferring Tat protein or mRNA.

Infectious lentiviral vector particles were produced as described for the proviral constructs, and HEK 293 cells were infected with supernatants harvested 2 days after transfection. To reduce the background due to pseudotransduction, GFP-positive cells were counted 3 days after infection. Without Gag/Gag-Pol or without VSV-G, the amount of GFP-positive cells after infection was equally low (Fig. 5B). The infectious titer of the lentiviral vector in the presence of Gag/Gag-Pol and Rev was $3.2 \times 10^5$ GFP-forming units per ml of supernatant. Omission of Rev reduced the titer 76-fold (Fig. 5B). Gag/Gag-Pol alone is therefore not able to rescue the titer of gag- and rev-deficient proviral or lentiviral vector constructs.

Role of NLS and NES in Gag for gRNA encapsidation. The codon-optimized gag/gag-pol expression plasmid Hgp$^{\text{syn}}$ allows the production of constant high levels of Gag/Gag-Pol, irrespective of whether Rev is present or not. Since Gag is sufficient for virus particle formation, the encapsidation efficiency could be analyzed in the absence and presence of Rev. Therefore, cytoplasmic RNA was isolated 2 days after cotransfection of HEK 293T cells with rev- and env-deficient provirus NL4.3Re and Hgp$^{\text{syn}}$ in combination with or without the rev expression plasmid. Virus particles were purified from the supernatant of the transfected cells by ultracentrifugation through a sucrose cushion, and the particle-associated RNA was isolated. The encapsidation efficiency was defined as the ratio of particle-associated and cytoplasmic gRNA levels. Therefore, the amount of gRNA in the cytoplasm and in the viral particles was examined by quantitative RT-PCR (Fig. 5C). While omission of Rev reduced the amount of gRNA in the cytoplasm less than 10-fold, particle-associated gRNA levels were reduced more than 1,000-fold, thereby confirming our previous results with lentiviral vectors (7) and proviral constructs (6). In the absence of Rev, Gag/Gag-Pol is clearly not able to promote an efficient packaging process. The purity of the cytoplasmic fraction has been analyzed before (6, 7). Nuclear proteins are not detectable in the cytoplasmic fraction, and contamination of the cytoplasmic fraction with the nuclear pre-glyceraldehyde 3-phosphate dehydrogenase (pre-GAPDH) mRNA ranges from only 3 to 7% of the total amount of pre-GAPDH-mRNA in the cell (6, 7).

The previously reported nuclear localization and export signals of Gag are all located in the MA domain. The major role of MA within uncleaved Gag is binding to membranes by its N-terminal myristate anchor, a stretch of highly basic amino acids, and its ability to associate with the membrane compound phosphatidylinositol 4,5-bisphosphate (48). However, it is known that MA can be replaced with heterologous myristoylation signals. For example, replacement of MA with the myristoylation signal of the protein v-Src allows efficient assembly and budding of Gag particles from transfected cells (30). Therefore, it was possible to analyze the ability of Gag to encapsidate gRNA in the absence of all known NLS and NES in Gag. The sequence encoding the whole MA domain (including half of the protease site between MA and CA) in the proviral construct NL4.3Re and in the Rev-independent gag/gag-pol expression plasmid Hgp$^{\text{syn}}$ was replaced with a sequence encoding the myristoylation signal of the cellular protooncoprotein c-Src (Re$\Delta$MA and Hgp$^{\text{syn}}$ΔMA in Fig. 5C and D). Characterization of these mutants with a CA-specific antibody by ELISA after the transfection of HEK 293T cells demonstrated that the mutant Gag proteins were still able to form extracellular Gag/Gag-Pol particles that can be isolated from the cellular supernatant by ultracentrifugation through a sucrose cushion (Fig. 5D). Furthermore, the Rev dependence of the proviral construct was preserved (data not shown) and Gag/Gag-Pol levels encoded by the codon-optimized MA substitution mutant of Hgp$^{\text{syn}}$ were not influenced by the presence or absence of Rev (Fig. 5D). However, comparison of intracellular and extracellular Gag/Gag-Pol levels for the codon-optimized plasmids showed that the intracellular levels of the MA substitution mutant were decreased while extracellular Gag/Gag-Pol particle levels were increased (Fig. 5D). The proviral mutant displayed lower intracellular Gag/Gag-Pol levels but an extracellular amount of Gag/Gag-Pol similar to that of parental NL4.3Re (Fig. 5D). Both results suggest that the budding efficiency was higher for the MA substitution constructs. Since budding and encapsidation are intimately linked, these results need to be considered when analyzing the encapsidation efficiency of different Gag mutants.

Examination of cytoplasmic and particle-associated gRNA levels after cotransfection of the proviral and gag/gag-pol expression plasmids containing the MA substitution mirrored the data obtained with the nonmutated counterparts (Fig. 5C). In the absence of Rev, the amount of particle-associated gRNA was strongly reduced, demonstrating that the mutant Gag was not able to promote packaging without Rev (Fig. 5C). Even more important is the observation that in the absence of Rev, large amounts of gRNA could be detected in the particles. A highly efficient process of encapsidation by Gag that lacks all known NLS and NES was therefore possible (Fig. 5C). A closer examination of the results obtained in the presence of Rev indicated that the mutant Gag particles displayed a modest packaging defect in comparison with their wild-type counterparts. Whereas the cytoplasmic gRNA levels were comparable, the particle-associated level was approximately 3-fold lower for the MA substitution mutant of Gag. Furthermore, an approximately 3-fold larger amount of virus particles was detected in the supernatant (Fig. 5C). The reason for the decreased rate of packaging could be the increased budding efficiency of the MA substitution mutant form of Gag (Fig. 5D). It is also known that sequences at the beginning of the matrix gene that are deleted in this proviral construct positively influence the packaging process (17, 41). Nevertheless, the packaging defect in the absence of Rev is far stronger in both systems and the large amount of gRNA in the particles of the MA substitution mutants clearly indicates that packaging is possible without all of the proposed NLS and NES in Gag (Fig. 5C).

Nuclear localization and Crm1-dependent nuclear export of Gag. Since these results did not provide any evidence for a functional effect of nuclear Gag, it was important to analyze whether under our experimental conditions Gag is, on the one hand, detectable inside the nucleus, as confirmed recently (38), and, on the other hand, exported to the cytoplasm by a CRM-1-dependent NES (18). Therefore, HeLa cells were transfected either with the env and nef deletion-containing proviral construct HXB2enfΔNefCAT or Hgp$^{\text{syn}}$ cotransfected with the subgenomic gag/gag-pol expression plasmid UTRgpRRE and the rev expression plasmid. Two days after transfection, cells were fixed and stained for Gag. Analysis of the cells by confocal laser scanning
microscopy demonstrated a strong cytoplasmic Gag signal (Fig. 6A). In a subpopulation of cells, weak nuclear Gag staining was detectable after the contrast of the images was increased postimaging (Fig. 6A). The weak staining seems to be specific, because surrounding untransfected cells did not show a similar nuclear Gag signal.

In order to confirm the presence of uncleaved Gag in the nucleus, we also analyzed nuclear and cytoplasmic fractions by Western blot assay. HEK 293T cells were transfected with either the proviral construct HXB2ΔenvΔnefCAT or the Rev-independent, codon-optimized gag/gag-pol expression plasmid HgpSYN or cotransfected with UTRgpRRE and pcRev. After 48 h, nuclear and cytoplasmic fractions were obtained and subjected to Western blotting with a CA-specific antibody. As expected, most of the Gag was present in the cytoplasmic fraction (Fig. 6B). However, faint bands representing the full-length Gag protein could also be detected in all of the nuclear fractions (Fig. 6B). The cytoskeleton protein α-tubulin, the soluble cytoplasmic protein MEK-1, and the endoplasmic reticulum protein PDI were present only in the cytoplasmic fractions, thereby demonstrating that contamination of the nuclear fraction with the cytoplasmic fraction was below the level of detection (Fig. 6B). It is important to note that small cleavage products of Gag containing CA could possibly diffuse into the nucleus and cause the weak nuclear signal seen in the confocal analyses. However, preferential accumulation of these small proteins was not observed in the nuclear fractions. These results demonstrate that a small amount of uncleaved Gag seems to be imported into the nuclei of transfected, gag-expressing cells.

The presence of nuclear Gag raises the question of whether Gag is able to shuttle between the nucleus and the cytoplasm by virtue of its NLS and NES. To examine this, we inhibited the Crm1-mediated export pathway by LMB, which covalently binds to Crm1, thereby preventing the export of NES-containing proteins (33, 34, 67). HeLa cells were transfected with gag/gag-pol expression plasmids and incubated with LMB 40 to 44 h later. After different incubation times, the cells were fixed and analyzed by immunofluorescence and confocal laser scanning microscopy. Without LMB, Gag was localized predominantly in the cytoplasm and at the plasma membrane of transfected cells for all of the constructs analyzed (Fig. 7). The endogenous protein IkBα was diffusely distributed throughout the cells, with a cytoplasmic predominance in some of the cells. IkBα is known to be a nuclear-cytoplasmic shuttle protein that is exported from the nucleus by Crm1. Incubation with LMB for 4.5 h efficiently accumulated endogenous IkBα in the nuclei (Fig. 7). Only cells with dominant nuclear staining for IkBα reveal efficient inhibition of Crm1-mediated export and were therefore examined for Gag localization. In contrast to the endogenous IkBα control, localization of Gag did not change under LMB treatment (Fig. 7). Very similar results were obtained after the transfection of subgenomic gag/gag-pol or gag expression plasmids (UTRgpRRE, HgpSYN, or Gag2995) or after the transfection of a proviral construct (HXB2ΔenvΔnefCAT). Since it is possible that Gag contains stronger NES or weaker NLS than IkBα and therefore accumulates more slowly in the nucleus, the incubation time was extended to 12.5 h. Under these experimental conditions, the number of adherent, living cells was strongly reduced because of the cytotoxicity of LMB (data not shown). However, even prolonged LMB treatment did not lead to enhanced nuclear Gag staining in HgpSYN-transfected cells (Fig. 7, bottom right).

**DISCUSSION**

In the present study, we analyzed a potential role of HIV-1 Gag in nuclear gRNA export, translation, and packaging in the absence of Rev. Deletion of Rev or RRE prevents not only Rev-mediated gRNA export but also gag/gag-pol expression. To analyze the distinct influences of Rev and Gag/Gag-Pol, we took advantage of a codon-optimized gag/gag-pol expression plasmid to be able to provide large and constant amounts of Gag/Gag-Pol, irrespective...

**FIG 6** Detection of nucleus-localized Gag. (A) HeLa cells transfected with the indicated gag/gag-pol expression plasmids were analyzed by confocal immunofluorescence microscopy. To demonstrate nuclear Gag signals, the contrast of anti-Gag immunofluorescence images was increased postimaging (Adobe Photoshop). White arrowheads mark examples of nuclei of untransfected cells that do not show nuclear Gag fluorescence after a contrast increase. White scale bars represent 10 μm. (B) Transfected HEK 293T cells were fractionated into nuclear (N) and cytoplasmic (C) fractions for anti-p24CA Western blot analysis. The stripped blot was incubated with an antibody against the cytoplasmic protein α-tubulin, the soluble cytoplasmic protein MEK-1 (Cytoplasm), and therefore accumulates in the nuclei (Fig. 7). Only cells with dominant nuclear staining for IkBα reveal efficient inhibition of Crm1-mediated export and were therefore examined for Gag localization. In contrast to the endogenous IkBα control, localization of Gag did not change under LMB treatment (Fig. 7). Very similar results were obtained after the transfection of subgenomic gag/gag-pol or gag expression plasmids (UTRgpRRE, HgpSYN, or Gag2995) or after the transfection of a proviral construct (HXB2ΔenvΔnefCAT). Since it is possible that Gag contains stronger NES or weaker NLS than IkBα and therefore accumulates more slowly in the nucleus, the incubation time was extended to 12.5 h. Under these experimental conditions, the number of adherent, living cells was strongly reduced because of the cytotoxicity of LMB (data not shown). However, even prolonged LMB treatment did not lead to enhanced nuclear Gag staining in HgpSYN-transfected cells (Fig. 7, bottom right).
of whether Rev was present or not. The results thus aid in resolving controversial data on a nuclear transit of HIV-1 Gag (41, 51).

 Trafficking of HIV-1 Gag through the cell is complex and only incompletely understood (5, 48, 56). A transient nuclear phase of unsealed HIV-1 Gag late in the replication cycle seemed possible when NLS and NES were identified in Gag but has not been confirmed (18, 28, 41, 51). The presence of small amounts of nucleus-localized Gag could be demonstrated by immunofluorescence coupled with laser scanning confocal microscopy and Western blotting of nuclear and cytoplasmic fractions of transfected cells (Fig. 6). Weak nuclear Gag signals were also detectable in different studies analyzing wild-type proteins (27, 40, 52, 53) and protein or virus mutants (4, 24). A Western blot analysis of cellular fractions very similar to that shown here was published recently (compare Fig. 6 and Fig. S3 in the supplemental material of Lehmann et al. [38]) confirming the presence of Gag in the nucleus. Although we could detect uncleaved Gag in the nucleus, the previously reported Crm1-dependent NES in the MA domain of Gag (18) could not be verified in our experiments (Fig. 7). It is important to note that very similar experimental settings were used, i.e., transient transfections of subgenomic gag expression plasmids into HeLa cells, followed by immunofluorescence stainings. Nevertheless, the experimental outcome was strikingly different. In cells that showed efficient inhibition of the cellular Crm1-dependent export pathway by accumulation of an internal control protein IkBα, successful inhibition of the Crm1 pathway was confirmed by the accumulation of IkBα in the nuclei of the cells. The cytoplasmic Gag staining pattern did not change after LMB incubation. Very similar images were obtained after the transfection of gag (Gag<sup>opt</sup>) and gag/gag-pol (UTRgpRRE, Hgpsyn) expression plasmids or a proviral (HXB2ΔenvΔnefCAT) construct. Scale bars, 10 μm.

FIG 7 Crm1-dependent nuclear export. HeLa cells were transfected with the indicated gag and gag/gag-pol expression plasmids. Two days after transfection, cells were incubated with 13 nM LMB, thereby inhibiting the cellular Crm1-dependent export pathway. After 4.5 to 12.5 h, the cells were fixed and stained for Gag and the endogenous shuttle protein IkBα. Successful inhibition of the Crm1 pathway was confirmed by the accumulation of IkBα in the nuclei of the cells. The cytoplasmic Gag staining pattern did not change after LMB incubation. Very similar images were obtained after the transfection of gag (Gag<sup>opt</sup>) and gag/gag-pol (UTRgpRRE, Hgpsyn) expression plasmids or a proviral (HXB2ΔenvΔnefCAT) construct. Scale bars, 10 μm.
Our observation that Gag does not enhance the export of nuclear gRNA for subsequent translation and encapsapsulation is consistent with the RNA packaging model (Fig. 1) in which HIV-1 Gag and the gRNA first interact in the cytoplasm. This model is further supported by a large body of evidence. Analysis of gRNA stability in the cytoplasm and in virus particles showed that HIV-1, in contrast to murine leukemia virus does not subdivide its gRNA into two separate pools for packaging and translation (11). However, nuclear initiation of packaging in combination with cytoplasmic translation would create two groups of gRNA molecules. This is not compatible with the published data. How binding of gRNA by Gag for encapsidation and ribosomes for translation is temporally and/or spatially regulated in the case of HIV is not clear, but our data indicate that nuclear Gag-gRNA binding is not involved.

Independent of the detection system, a clear colocalization of Gag and gRNA in the nucleus has not been observed either (1, 4, 31, 38, 40, 53). Using RNA in situ hybridization and antibody staining of Gag in fixed cells to analyze the trafficking of gRNA and Gag at different time points after transfection, Poole and coworkers stated that nuclear colocalization was never observed in their experiments (53). Furthermore, the low abundance of nuclear Gag seems to prevent a biochemical analysis by, for example, immunoprecipitation approaches (35).

During encapsidation, HIV incorporates two gRNA molecules per viral particle. Dimerization of two gRNAs was shown to occur in the cytoplasm of infected cells and seems to precede the initiation of packaging (45, 46). The exact role of dimerization in packaging by Gag is not known for HIV, but these data imply that Gag interacts with dimerized RNA molecules in the cytoplasm of the cell (41).

The previously reported NLS and NES are localized throughout the MA domain of Gag (18, 28, 51). However, we and others could show that large amounts of gRNA were packaged by Gag molecules lacking most or even all of the MA domain (Fig. 5). A Gag deletion mutant containing only the 15 N-terminal amino acids as a myristoylation signal and 12 C-terminal amino acids spanning the protease cleavage site between MA and CA packaged gRNA efficiently (66). Additionally, replacement of MA with other membrane binding domains like the pleckstrin homology domains of the cellular protein AKT or phospholipase CδI did not reduce the levels of gRNA incorporated into viral particles (61). Furthermore, deletion of amino acids 8 to 126 of MA still enabled delayed viral replication when C-terminally truncated Env was provided in parallel (55). Replication of a similar provirus enabled delayed viral replication when C-terminally truncated Env was provided in parallel (55). These data clearly demonstrate that MA is dispensable and consequently not essential for the HIV encapsidation process in case association of Gag with membranes is enabled by heterologous membrane binding domains or myristoylation signals.

Conclusive evidence that Gag is not able to allow an efficient gRNA packaging process in the absence of Rev was obtained during studies exploring the minimal requirements for the generation of infectious lentiviral vector particles. The gRNA of lentiviral vector constructs does not encode any viral protein, and Gag/Gag-Pol and other viral proteins have to be provided in trans from separate expression plasmids. Additional deletion of the RRE from the vector sequences reduced the infectious titer 10- to 100-fold (2, 15, 42). In a complementary approach, the RRE of the gag/gag-pol expression plasmid was replaced with constitutive transport elements or codon-optimized gag/gag-pol expression plasmids were used. Despite the presence of large amounts of Gag/Gag-Pol, in the absence of Rev, the lentiviral vector titer was strongly reduced (6, 7, 32, 63). Detailed analyses demonstrated an impaired packaging process as the reason for the observed drop in titer (6, 7). However, these studies focused on the influence of Rev on the encapsidation process. An examination of the role of Gag in the trafficking of gRNA from the nucleus to the plasma membrane had not been performed. The results obtained in this study confirm the influence of Rev on export, translation, packaging, and titer. However, despite intensive investigations, we did not obtain any evidence for a role of nuclear Gag of HIV-1 in gRNA export and encapsidation. This indicates that HIV-1 Gag comes into play after Rev mediates the export of the genomic RNA into the cytoplasm. Therefore, the encapsidation process of HIV-1 clearly differs from that of RSV and PFV.

ACKNOWLEDGMENTS
We thank Klaus Sure for his excellent technical support and Vladimir Temchura and Matthias Tenbusch for critical discussions. Furthermore, we thank Walther Mothes for his intellectual input, particularly regarding the MA deletion mutants. Plasmids were kindly provided by M. Malin, J. Hauber, B. Cullen, W. Haselte, J. Münch, and R. Wagner. TZM-bl cells were obtained from the EVA Centre for AIDS Reagents, NIBSC, United Kingdom, and were donated by J. C. Kappes, X. Wu, and Tranzyme Inc. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 SF2 Gag recombinant protein; p96ZM631gag-opt from Yingying Li, Feng Gao, and Beatrice H. Hahn; and HIV-1 p24 hybridoma (183-H12-5C) from Bruce Chesebro and Hardy Chen.

This work was funded by a grant from the DFG (Ue45/11-1). B.G. was and B.H. is supported by a fellowship from the DFG graduate school (1045/2).

We have no conflicts of interest to declare.

B.G., T.G., and K.U. conceived and designed the experiments. B.G., B.H., I.O., and B.T. performed the experiments. B.G., B.H., M.B., S.B., T.G., and K.U. analyzed and interpreted the data. B.G., B.H., and K.U. wrote the paper. M.B., S.B., and B.T. contributed reagents, materials, and/or analysis tools. All of us read and approved the final manuscript.

REFERENCES


