Human Immunodeficiency Virus Type 1 Gag Polyprotein Modulates Its Own Translation

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The full-length viral RNA of human immunodeficiency virus type 1 (HIV-1) functions both as the mRNA for the viral structural proteins Gag and Gag/Pol and as the genomic RNA packaged within viral particles. The packaging signal which Gag recognizes to initiate genome encapsidation is in the 5' untranslated region (UTR) of the HIV-1 RNA, which is also the location of translation initiation complex formation. Hence, it is likely that there is competition between the translation and packaging processes. We studied the ability of Gag to regulate translation of its own mRNA. Gag had a bimodal effect on translation from the HIV-1 5' UTR, stimulating translation at low concentrations and inhibiting translation at high concentrations in vitro and in vivo. The inhibition was dependent upon the ability of Gag to bind the packaging signal through its nucleocapsid domain. The stimulatory activity was shown to depend on the matrix domain of Gag. These results suggest that Gag controls the equilibrium between translation and packaging, ensuring production of enough molecules of Gag to make viral particles before encapsidating its genome.

Human immunodeficiency virus type 1 (HIV-1), the major etiological agent of AIDS, is a complex retrovirus. Its life cycle involves reverse transcription of viral RNA into a double-stranded DNA provirus that is integrated into the chromosome of its host cell. Upon activation of proviral transcription, a number of spliced RNAs are produced that encode the regulatory proteins Tat and Rev, which allow the efficient transcription and nuclear export of singly spliced (Env-coding) and unspliced HIV-1 RNA. The full-length viral RNA functions as both mRNA encoding the Gag and Gag/Pol polyproteins and genomic RNA to be packaged into new virions.

Packaging of retroviral RNA depends on the presence of the packaging signal (Ψ), a complex RNA structure that, in HIV-1, is present only on the full-length unspliced RNA, and its specific recognition by the Gag polyprotein (2, 16). Binding of Gag to Ψ is associated with dimerization of the viral RNA and probable multimerization of Gag along the RNA in a sequence-independent manner. The Gag-RNA ribonucleoprotein is transported to the cell membrane and acquires an Env glycoprotein-embedded lipid envelope. The immature virus particle buds from the host cell prior to maturation by cleavage of Gag and Gag/Pol.

The full-length HIV-1 RNA is the only mRNA encoding Gag, and Gag is responsible for encapsidation of this RNA species to produce new viral particles. In the case of simple retroviruses such as murine leukemia virus, published evidence is consistent with two separate pools of the full-length RNA, one sequestered for translation and the other for packaging (20). However, it has been shown that in HIV-1 and HIV-2 there is only one pool of unspliced RNA, from which an RNA molecule may be translated, packaged, or both translated and

then packaged (13, 30). Since it is unlikely that ribosomes would be able to translate an RNA fully coated with Gag, there is likely to be competition between translation and packaging of the HIV-1 RNA.

Competition has been observed in Rous sarcoma virus, where expression of Gag protein in quail cells inhibits expression of luciferase when driven by the Rous sarcoma virus leader (37) in transient transfections. It has thus been proposed that Gag protein binding to the 5' untranslated region (UTR) of retroviral RNA inhibits translation of the full-length RNA, allowing packaging to occur (5, 7, 37, 39, 40). The Gag product of the *Idefix* retrotransposon of *Drosophila melanogaster* has also been shown to inhibit translation from its own 5' UTR (23). HIV-1 Gag has been reported to inhibit translation in vitro in a nonspecific manner through sequestration of elongation factor 1 α (7) and initiation factor 5B (40) via its matrix domain.

Optimization of viral output requires control of the equilibrium between packaging and translation. In this study we have investigated the role of HIV-1 Gag protein in specifically regulating translation from the HIV-1 5' UTR. We have identified a novel bimodal effect of Gag, by which the virus may coordinate the equilibrium between translation and packaging of the HIV-1 RNA.

MATERIALS AND METHODS

Construction of plasmids for RNA synthesis. The 5' UTR (nucleotides [nt] 1 to 336) of HIV-1 HXB2 (14) was amplified by PCR using the SalI/HIV-1 (TAG CTAGTCGACGGTCTCTCTGGTT) and HIV-1/BamHI (CTCGGATCCATCT CTCTCCTTCTAGC) primers and subcloned into pJHRV10-605 (3) digested with SalI and BamHI to give pJHIV-1. The NS' open reading frame and 3' UTR are nucleotides 47 to 889 of influenza A virus segment 8 (accession no. CY003692). pJHIV-1 Δ TAR was also constructed using SalI/HIV-1 and HIV-1/BamHI primers to amplify the mutant 5' UTR from KSCA Δ TAR. KSCA Δ TAR was created as follows. The AmpR (GCGGTTAGCTCCTTCGG TCC) and TAR upstream (GATCTGTTTCAACCAGAGAGACCC) primers and TAR downstream (GCTCTCTTTCGAAGTAGGGAACCC) and Gag367-348 (CCCCGCTTAATACTGACGC) primers were used to amplify DNA se-

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quences on either side of a deletion in the *trans*-activation response element (TAR) stem-loop (nt 14 to 45) from HVPΔEC (34). The upstream fragment was digested with SfiI and BstBI, and the downstream fragment was digested with BssHII and BstBI. These were then ligated into KSCA (ClaI-ApaI of the HIV-1 provirus, encompassing the 5' flanking region, 5' long terminal repeat and Gag, in Bluescript) digested with SfiI and BssHII. KSCA had previously been mutated to eliminate BssHII sites in the vector (non-HIV) sequence. pJHIV-1ΔΨ was made by PCR amplification of nt 1 to 223 of the HIV-1 5' UTR using SalIHIV-1 and BamHIIΔPsi (CTCGGATCCATCTCTGGTTCCCTTTCGC) primers and subcloned into pJHIV-1 that had been digested with SalI and BamHI. pJCAA₁₉, which consists of a 5' UTR of 19 repeats of CAA (32) upstream of NS', was a gift from J. Batley and R. Jackson. pXLJHRV10-611 (3), which carries the *Xenopus laevis* cyclin B2 open reading frame upstream of the HRV-2 internal ribosome entry site and NS' open reading frame, was a gift from R. Jackson. The luciferase open reading frame was amplified from pGL-2 by PCR and subcloned into pJHIV-1 which had been digested with BamHI and EcoRI to give pHIVluc or with SalI and EcoRI, to give pJluc. pJGag was constructed by PCR amplification of the HIV-1 Gag open reading frame and subcloning into pJHIV-1 which had been digested with SalI and EcoRI.

Construction of plasmids for protein synthesis. The expression construct for wild-type HIV-1 Gag, pGexGag1, has been described previously (26). pGexGagΔp6 was created by PCR amplification of the matrix-nucleocapsid region of HIV-1 Gag using the 1GagF (26) and 1NCR (CGATGAGTCGACATAGCCTGTCTCTCAGT) primers and subcloning into pGex4T1 (Amersham Biosciences) digested with EcoRI and SalI. pGexGagΔZn1 + 2 was created by introducing a stop codon at amino acid position 391 (nt 1171 to 1173) of Gag by site-directed mutagenesis (QuikChange; Stratagene) using the primers CCAAGAAAGATTGTTTGTAGTGTTCATTTGTGGC and GCCACAATTGAAACACTAAACAATCTTTCTTTGG. pGexGagC28/49S was created by PCR amplification of the Gag open reading frame with the 1GagF and 1GagR primers (26) from a C28/49S HIV-1 provirus, described previously (12), and subcloning into pGex4T1. pGexGagΔMA was created by PCR amplification of the capsid-p6 region of HIV-1 Gag using the CA1F (CGCGGAATTCCTATAGTGCAGACATC) and 1GagR primers and subcloning into pGex4T1. pGexMA was created by PCR amplification of HIV-1 matrix using the 1GagF and MA1R (GATCGTCGACGTAATTTGGCTGACCTG) primers and subcloning into pGex4T1.

Expression and purification of recombinant proteins. Wild-type and mutant pGexGag plasmids were used to transform *Escherichia coli* BL21 (Invitrogen). Five-milliliter overnight cultures were used to seed 250-ml cultures. These were grown at 37°C to an optical density at 600 nm of 0.5 to 0.6 (around 2.5 h) and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Invitrogen) for 3 h. The cultures were harvested and cell pellets frozen at -20°C. Cells were lysed using 0.5 mg/ml lysozyme (Sigma) followed by sonication (Soniprep 150; Sanyo). The lysate was cleared by centrifugation at 10,000 × g. Glutathione S-transferase (GST) fusion proteins were purified using glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions and dialyzed against H100 buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 1 mM dithiothreitol).

In vitro transcription and translation. Wild-type and mutant pJHIV-1 and pJCAA₁₉ plasmids were linearized with EcoRI and used as templates for in vitro transcription. pXLJHRV10-611 was linearized with BamHI and used as a template for in vitro transcription of cyclin RNA. Reaction mixtures contained 5 μg DNA template, 1 mM each ribonucleoside triphosphate, 20 units RNasin (Promega), 100 units T7 RNA polymerase (Ambion), 1× T7 polymerase buffer (Ambion), and a trace amount of [³²P]UTP (Amersham Biosciences) in a final volume of 50 μl. Reaction mixtures were incubated at 37°C for 1 h, 1 μl was removed for quantitation as described previously (10), and the reactions were cleaned up by phenol-chloroform extraction and ethanol precipitation. Each of the RNAs was used to program in vitro translation reactions at 4 mM. Reaction mixtures contained 20 μM amino acids (minus methionine), 4 μCi [³⁵S]methionine (Amersham Biosciences), 50% rabbit reticulocyte lysate (Promega), 10% HeLa cytoplasmic extract (18), and 100 mM KCl in a final volume of 10 μl. Reaction mixtures were incubated at 30°C for 1 h and treated with 10 μl RNase Stop (50 μg/ml RNase A, 10 mM EDTA) for 15 min, and 80 μl 1× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer was added. Samples were boiled prior to loading of 10 μl on a 12% SDS-polyacrylamide gel. Gels were run at 25 mA, stained with Coomassie blue, destained, dried, and exposed to Kodak Biomax film overnight. Films were developed using a Vari-speed 150 (Xograph Imaging Systems) developer and quantified using ImageJ software. Western blotting of translation reactions was carried out using polyclonal antibodies to HIV-1 p17 (ARP431; CFAR, NIBSC [donated by G. Reid]). pHIVluc, pJluc, and pJGag were linearized with NheI and used as

templates for transcription with an mMESSAGE mMACHINE T7 ultra kit (Ambion) according to the manufacturer's instructions. These mRNAs were used in transfection of COS-1 cells.

Gel mobility shift assays. pJHIV-1 was linearized with BamHI and used as a template for in vitro transcription. Reaction mixtures contained 2.5 μg DNA template; 10 units RNasin (Promega); 0.5 mM CTP, GTP, and ATP; 12 μM UTP; 25 units T7 RNA polymerase; 1× buffer (Ambion); and 20 μCi [³²P]UTP (Amersham Biosciences) in a final volume of 25 μl. Reactions were then treated as described above. Gel mobility shift assays were set up containing 1× binding buffer (10 mM HEPES [pH 7.5], 3 mM MgCl₂, 5% glycerol, 1 mM dithiothreitol), 1 μg yeast tRNA (Sigma), 100 mM KCl, 10 units RNasin (Promega), 0.5 nM HIV-1 5' UTR probe, and 0 to 0.2 μM recombinant protein in a final volume of 10 μl. Reaction mixtures were incubated at room temperature for 15 min and then loaded on a 4.5% Tris-borate-EDTA-polyacrylamide gel. Gels were run at 10 mA, fixed, dried, and exposed to Fujifilm at -70°C for 30 min. Films were developed and quantified as described above.

Transfection of COS-1 cells. COS-1 simian epithelioid cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum, penicillin, and streptomycin. Cells were transfected in six-well plates with 2 ng pHIVluc or pJluc mRNA, a range of concentrations of pJGag mRNA, and 2 μg pcDNA-green fluorescent protein plasmid as a transfection control, using Lipofectamine 2000 (Invitrogen). Cells were harvested at 22 h posttransfection and assayed for luciferase activity, using luciferase assay reagent (Promega) and an AutoLumat LB953 luminometer (EG&G Berthold).

RESULTS

Gag has a bimodal effect on translation from the HIV-1 5' UTR. To isolate the activity of Gag on translation of HIV-1 RNA, an in vitro system was set up in which rabbit reticulocyte lysate supplemented with 10% HeLa cytoplasmic extract was programmed with an HIV-1 reporter RNA and recombinant Gag protein. Supplementation of rabbit reticulocyte lysate with HeLa extracts has been shown to allow efficient translation from the HIV-1 5' UTR (38), and we also found this to be the case. The HIV-1 reporter RNA (pJHIV-1) used to program in vitro translation reactions consisted of the highly structured HIV-1 5' UTR (nt 1 to 336) upstream of a truncated influenza virus NS1 open reading frame and 3' UTR (Fig. 1A). Recombinant Gag was expressed and purified as an N-terminal GST-tagged fusion protein from bacteria. Figure 1B shows a schematic of the domains of the GST-Gag polyprotein.

A range of concentrations of Gag was added to the translation reaction mixtures to simulate conditions in an HIV-infected cell at different times after translation of Gag begins. A representative autoradiograph of SDS-polyacrylamide gel electrophoresis of the translation reactions is shown in Fig. 1C (top left panel). Predictably, above a certain concentration of Gag (0.2 μM), translation of the HIV-1 reporter was inhibited compared to the control (no Gag), and higher [Gag] led to increased inhibition. No translation products could be detected at a Gag concentration of above 0.8 μM. However, at lower [Gag] the addition of Gag to the reaction mixture enhanced translation from the HIV-1 5' UTR, up to a maximum stimulation of 100% at 0.1 μM Gag. This result, although surprising, was reproducible with different protein and RNA preparations. The mean levels of translation stimulation and inhibition at different concentrations of Gag, and standard errors of the means, are shown graphically in Fig. 1D. A Western blot of the translation reactions using anti-HIV-1 p17 antibody (Fig. 1C, top right panel) shows the increasing level of Gag protein in the reactions. The full-length protein and near-full-length C-terminally truncated species are characteristic of GST-Gag

FIG. 1. A. Schematic of pJHIV-1 mRNA, including HIV-1 5' UTR secondary structures. B. Schematic of GST-Gag polyprotein with GST and the domains of Gag indicated: MA, capsid (CA), p2, NC, p1, and p6. C. Translation of pJHIV-1 RNA in the presence of 0, 0.05, 0.075, 0.1, 0.2, 0.4, and 0.8 μ M GST-Gag (top panels); 0, 0.05, 0.1, 0.2, 0.4, and 0.8 μ M GST-GagΔp6 (middle panels); and 0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μ M GST (lower panels). Autoradiographs are shown on the left; Western blots probed with anti-HIV-1 p17 antibody are shown on the right. D. Graphical representation of translation from pJHIV-1 in the presence of different levels of Gag protein. Error bars represent standard errors of the means; $n > 3$. E. 32 P-labeled pJHIV-1 RNA (lane 1, input) was extracted from translation reaction mixtures that contained 0, 0.1, 0.2, 0.4 and μ M Gag (lanes 2 to 5).

(4, 22), as is the smaller truncated product. In order to show that these truncations do not affect the activity of Gag on translation, GST-Gag lacking the p6 domain (GagΔp6) was used in the translation assay (Fig. 1C, middle panels). This protein behaved the same as GST-Gag (as has been reported for studies on particle assembly [6]) in stimulating and inhibiting translation from the HIV-1 5' UTR, and the Western blot shows that this protein is 90% homogeneous. Addition of GST

alone at these and higher concentrations (up to 3.2 μ M) had no effect on translation (Fig. 1C, bottom panels).

To confirm that addition of Gag to the translation reactions did not affect the stability of the pJHIV-1 mRNA, the mRNA was labeled using [32 P]UTP and used to program translation reactions in the absence or presence of increasing concentrations of Gag. After the 1-h reaction, the mRNA was extracted with phenol-chloroform, run on an agarose gel, and autoradio-

FIG. 2. A. Schematics of Gag, Gag Δ Zn1 + 2, and GagC28/49S proteins, with deletions and mutations shown. B. Gel mobility shift assays showing the HIV-1 5' UTR probe alone (lanes 1, 5, and 9) or with 0.05, 0.1, or 0.2 μ M GST-Gag (lanes 2 to 4), GST-Gag Δ Zn1 + 2 (lanes 6 to 8), or GST-GagC28/49S (lanes 10 to 12). The positions of monomeric and dimeric HIV-1 5' UTRs are indicated, and the positions of RNA-protein complexes are shown by arrowheads. C. Translation of pJHIV-1 RNA in the presence of 0, 0.1, 0.2, 0.4, and 4 μ M GST-Gag, GST-Gag Δ Zn1 + 2, or GST-GagC28/49S. Autoradiographs, in which the position of NS' is indicated, are shown in the upper panels; Western blots probed with anti-HIV-1 p17 are shown in the lower panels. D. Graphical representation of translation results. Diamonds, GST-Gag; squares, GST-Gag Δ Zn1 + 2; triangles, GST-GagC28/49S. Error bars represent standard errors of the means; $n > 3$. Asterisks indicate a significant difference from wild-type GST-Gag ($P < 0.005$).

graphed. Figure 1E shows the mRNA prior to the reactions (input) and mRNA extracted from the translation reactions. Addition of Gag to the translation reactions did not affect the integrity of the mRNA.

Inhibition of translation depends on the RNA-binding activity of Gag. A number of mutants of GST-Gag were made and tested in the *in vitro* translation system to determine their

effect on translation from the HIV-1 5' UTR. Figure 2A shows two mutant Gag proteins, in addition to wild-type Gag, designed such that their RNA-binding activity would be disrupted. The nucleocapsid (NC) domain of Gag contains two CCHC zinc fingers, as well as basic residues at its N terminus and between the two zinc fingers, which have all been shown to contribute to both sequence-specific and sequence-indepen-

dent binding to RNA (8, 9, 11, 12, 31). Gag Δ Zn1 + 2 was mutated to contain a stop codon immediately upstream of the first zinc finger domain of NC, giving rise to a protein lacking both zinc fingers and the p6 domain of Gag. GagC28/49S contains point mutations of the first cysteine residue of each zinc finger (C28 and C49 of NC) to serine. These mutations have been shown to inhibit HIV-1 RNA packaging by Gag by 95% and to abrogate HIV-1 replication (12).

To check that the mutations introduced did reduce protein binding to RNA, the ability of wild-type and mutant GST-Gag proteins to shift a 32 P-labeled HIV-1 5' UTR probe was compared using gel mobility shift assays (Fig. 2B). The HIV-1 probe (containing the high-affinity binding site for Gag, Ψ) runs as both monomeric and dimeric forms since the RNA contains a dimerization initiation site (36) and the RNA was not heat denatured prior to the binding reactions. Wild-type Gag (Fig. 2B, lanes 1 to 4) shifted an increasing proportion of the monomeric RNA at 0.05 and 0.1 μ M and was able to shift all of the monomeric and dimeric RNA at 0.2 μ M into a stable complex under these assay conditions. Gag Δ Zn1 + 2 (lanes 5 to 8) was able to shift only a very small proportion (7%) of the monomeric RNA at 0.2 μ M, indicating that this protein is severely disabled in terms of its ability to bind the HIV-1 5' UTR. Although GagC28/49S (lanes 9 to 12) was able to form a clearly visible complex with the monomeric RNA at 0.2 μ M, only 15% of the probe was shifted, confirming the deficient RNA-binding activity of the protein.

Figure 2C shows the results of translation assays in which 0.1, 0.2, or 0.4 μ M wild-type or mutant GST-Gag protein was added to the reaction mixtures. Autoradiographs are shown in the upper panels and Western blots are shown in the lower panels. The left panels show the bimodal effect on translation of wild-type Gag (60% stimulation at 0.1 μ M, 40% inhibition at 0.2 μ M, and 80% inhibition at 0.4 μ M). The zinc finger deletion mutant of Gag (middle panels) stimulated translation by approximately 50% at 0.1 and 0.2 μ M and by around 20% at 0.4 μ M. The GagC28/49S mutant (right panels) stimulated translation by around 60% at 0.1 μ M and 40% at 0.2 and 0.4 μ M. These results are represented graphically in Fig. 2D. The activity of the mutant proteins is significantly different from that of wild-type Gag at 0.2 and 0.4 μ M ($P < 0.005$ by Student's t test). The Western blots show increasing levels of wild-type and mutant Gag proteins in the translation assays. The concentration of mutant proteins corresponded to the total concentration of all three full-length and near-full-length proteins in the wild-type GST-Gag preparation. The lack of RNA-binding activity of the mutant proteins thus correlated with a lack of inhibition of translation from the HIV-1 5' UTR compared to wild-type Gag. The stimulatory effect of Gag on translation was unaffected by the mutations, showing that this does not depend on its RNA-binding activity.

Inhibition of translation depends on intact Ψ sequences in the HIV-1 5' UTR. The requirement of specific HIV-1 sequences in the mRNA for Gag activity was tested using a number of different RNAs. Figure 3A (left) shows a schematic of the HIV-1 5' UTR with the TAR and packaging signal (Ψ) structures indicated. Reporter RNAs containing deletions of the TAR stem-loop (Δ TAR) and Ψ ($\Delta\Psi$) and used to program *in vitro* translation reactions in the presence of 0.1, 0.2, and 0.4 μ M wild-type Gag.

FIG. 3. A. Schematic of the secondary structure of the HIV-1 5' UTR, with the TAR and packaging signal (Ψ) indicated. B. GST-Gag (0, 0.1, 0.2, or 0.4 μ M) was added to translation reaction mixtures programmed with pJHIV-1 Δ TAR, pJHIV-1 $\Delta\Psi$, pJ(CAA)₁₉, and cyclin and pJHIV-1 RNAs. The RNA is shown above each panel. The positions of NS' and cyclin products are shown to the right of the autoradiographs. C. Graphical representation of translation results. Diamonds, HIV-1-NS'; open squares, Δ TAR-NS'; triangles, $\Delta\Psi$ -NS'; gray squares, (CAA)₁₉; circles, cyclin. Error bars represent standard errors of the means; $n > 2$. Asterisks indicate a significant difference from HIV-1-NS' ($P < 0.005$).

Translation of the Δ TAR-NS' RNA (Fig. 3B, top left panel, and C) responded to Gag with the same trend as RNA containing the full wild-type HIV-1 5' UTR (Fig. 2C, top left panel). The level of translation stimulation from this RNA (approximately 20%), while clearly visible, was lower than that from the wild-type RNA. While this may suggest that the missing sequences are required for the full stimulatory effect of

Gag, it more likely reflects the higher basal level of translation of these RNAs compared to the wild-type HIV-1 5' UTR. A substantial amount of RNA secondary structure known to be inhibitory to translation (24, 38) was removed in this construct, resulting in more efficient translation of NS' in the absence of Gag (data not shown). Hence, a stimulatory mechanism which overcame an inhibitory effect of secondary structure would be less easily detected.

Deletion of Ψ from the HIV-1 5' UTR (Fig. 3B, top right panel, and C) resulted in a 60% stimulation of translation at 0.1 μ M Gag, and stimulation remained at 50% at 0.4 μ M Gag, a concentration at which translation from the full-length HIV-1 5' UTR is inhibited by 80%. This result shows that Ψ is likely to be involved in the inhibition of translation by Gag.

As a control, a reporter RNA consisting of CAA repeats upstream of NS' was tested in this assay (Fig. 3B, bottom left panel, and C). Translation from this unstructured 5' UTR is efficient, and the presence of Gag has little effect on this. There is no effect of Gag on translation at 0.1 and 0.2 μ M and approximately 20% inhibition at 0.4 μ M. To mimic conditions within the cell where multiple RNAs are being translated simultaneously, a different RNA (cyclin) was cotranslated with the HIV-1-NS' RNA at the same RNA concentrations, in the same reactions (Fig. 3B, bottom right panel, and C). Translation of the cyclin RNA was affected very little by Gag, showing 20% inhibition at 0.4 μ M Gag, whereas translation from the HIV-1 5' UTR showed a bimodal effect of Gag. Statistical analysis comparing different RNAs with the wild-type HIV-1-NS' RNA at each concentration of GST-Gag showed significant differences ($P < 0.005$) (Fig. 3C).

Short 5' UTRs give high basal levels of translation, and the stimulatory activity of Gag may be detected only where translation is inefficient due to long or structured 5' UTRs. Accordingly, Gag may be acting to stimulate translation of the unspliced HIV-1 RNA to overcome its inherently low rate of translation initiation.

Stimulation of translation depends on the matrix region of Gag. We next investigated the stimulatory activity of Gag. A Gag mutant in which the matrix (MA) domain was deleted (Gag Δ MA) was made (Fig. 4A). This protein was able to bind to a HIV-1 5' UTR probe in a gel mobility shift assay almost as well as full-length Gag (compare lanes 1 to 4 Fig. 4B and 2B); all of the probe was shifted into a stable complex by the Gag Δ MA protein at 0.2 μ M. As predicted by its HIV-1 5' UTR binding ability, Gag Δ MA was able to inhibit translation from this 5' UTR (Fig. 4C, left panel, and D), by 25% at 0.1 μ M and by 80% at 0.4 μ M. However, this protein was unable to stimulate translation, suggesting that the matrix domain of Gag may be involved in its ability to stimulate HIV-1 translation.

The matrix domain alone (Fig. 4A) was unable to bind the HIV-1 5' UTR in the gel mobility shift assay (Fig. 4B, right panel) and did not inhibit translation from the HIV-1 5' UTR at these concentrations. However, when added to the *in vitro* translation assay (Fig. 4C, right panel, and D), it gave a small stimulation of translation (15% and 40% at 0.1 and 0.4 μ M, respectively). The results seen with the MA domain, which does not bind the HIV-1 5' UTR, suggest that the stimulation may not depend directly on binding of Gag to the 5' UTR.

FIG. 4. A. Schematics of the matrix-deleted Gag protein (Gag Δ MA) and MA. B. Gel mobility shift assays showing HIV-1 5' UTR probe alone (lanes 1 and 5) or with 0.05, 0.1, and 0.2 μ M Gag Δ MA (lanes 2 to 4) or MA (lanes 6 to 8). The positions of monomeric and dimeric HIV-1 5' UTR are indicated, and the positions of RNA-protein complexes are shown by arrowheads. C. Translation of pJHIV-1 RNA in the presence of 0, 0.1, and 0.4 μ M Gag Δ MA or MA. The position of the NS' product is shown to the right of the autoradiographs. D. Graphical representation of the translation results. Diamonds, GST-Gag; squares, GST-Gag Δ MA; triangles, GST-MA. Error bars represent standard errors of the means; $n > 3$. Asterisks indicate a significant difference from wild-type GST-Gag ($P < 0.01$).

Gag has a bimodal effect on translation from the HIV-1 5' UTR *in vivo*. In order to specifically test the effect of Gag on translation from the HIV-1 5' UTR in cells, we set up transient-transfection studies in COS-1 cells. A reporter mRNA consisting of luciferase under the control of the HIV-1 5' UTR or an unstructured 5' UTR was cotransfected with mRNA expressing HIV-1 Gag. The results of three independent experiments are summarized in Fig. 5. Cotransfection of increasing concentrations of Gag mRNA led to an increase in translation of the pHIVluc reporter mRNA. The stimulation of translation peaked at 100% at 31 ng of Gag mRNA and then decreased as the concentration of Gag continued to increase, suggesting that the higher levels of Gag were becoming inhib-

FIG. 5. Translation of pHIVluc RNA (black bars) and pJluc RNA (gray bars) when cotransfected into COS-1 cells with increasing amounts of pJGag RNA (4 to 250 ng). Error bars represent standard errors of the means; $n > 3$. Asterisks indicate a significant difference from results with 0 ng Gag RNA ($P < 0.005$).

itory to translation. Translation of the control mRNA pJluc was not affected by Gag. Increasing the concentration of Gag mRNA above 2 μ g led to inhibition of translation from the pHIVluc mRNA to below basal levels (data not shown); however, these levels of Gag mRNA had a cytotoxic effect on the cells, so we were unable to determine the specificity of the inhibition.

DISCUSSION

We have investigated the role of Gag protein in translation from the HIV-1 5' UTR to understand how the balance between translation and packaging of the unspliced full-length HIV-1 RNA is regulated. We have shown that Gag has a bimodal effect on translation that depends on its concentration: stimulation at low concentration and inhibition at high concentration.

The inhibition of translation from the HIV-1 5' UTR is dependent on the ability of Gag, through the zinc fingers of its nucleocapsid domain, to bind to the packaging signal of the HIV-1 RNA. This is likely to precipitate formation of an initial packaging complex, eventually leading to coating of the RNA with Gag molecules. Notably, at 0.8 μ M Gag (at which translation of pJHIV-1 RNA is almost completely inhibited in our system), the ratio of Gag molecules to RNA molecules is 200:1. Estimates vary; however, it is suggested that the NC region may footprint around six to eight nucleotides (11). Thus, 200 molecules of Gag would be predicted to coat our 1,200-nt reporter RNA. This suggests that the inhibition we observe is associated with near-complete coating of the reporter RNA.

The major mRNP protein YB-1 (p50) has been reported to stimulate translation of cellular mRNAs at low concentrations, although the mechanism is as yet unknown, and to inhibit translation at high concentrations (25, 29). Nonspecific binding of YB-1 to the RNA backbone displaces eukaryotic translation initiation factor 4G (eIF4G) to inhibit mRNA translation (27). YB-1 also inhibits its own translation at lower concentrations through binding of an element in the 3' UTR of the YB-1 mRNA. This inhibition is antagonized by the binding of poly(A)-binding protein to an overlapping binding site and

displacement of YB-1 from the 3' UTR (35). One possibility is that the specific interaction between HIV-1 Gag and Ψ inhibits translation from the HIV-1 5' UTR by displacing eIF4G from the mRNA.

The inhibition of HIV-1 translation by Gag has been proposed as a possible mechanism of switching translation to packaging (5, 7, 39, 40). In contrast, stimulation of translation by Gag at lower concentrations was neither predicted nor expected. However, in the context of viral replication, this makes perfect sense. Retroviral RNAs, in common with mRNAs from other virus families such as *Picornaviridae* (1) and *Togaviridae* (15) but in contrast to most eukaryotic messages, concentrate many *cis*-acting signals in their 5' UTRs. In the case of HIV-1, these structures generate a major obstacle to efficient translation (24). At early stages when the unspliced HIV-1 RNA appears in the cytoplasm and translation begins, [Gag] is low and stimulates more Gag production to ensure the synthesis of enough Gag molecules to package viral RNA into new particles. Immediate inhibition of translation by nascent Gag molecules emerging from the ribosome would likely result in very few new virions being produced.

Stimulation of translation seems to depend upon the matrix region of Gag. This was also a surprise, since HIV-1 MA has been reported to inhibit translation (7, 40). We tested a range of MA concentrations and also saw significant inhibition of translation of both luciferase mRNA and our HIV-1 reporter RNA, at concentrations of 4 to 8 μ M (data not shown). This is 10 to 80 times higher than the [MA] and [Gag] used in our assays in which we see translation stimulation. The stimulation is more evident on long structured 5' UTRs, where translation in the absence of Gag is less than efficient. Surprisingly, MA alone, which is unable to bind to the HIV-1 5' UTR (although it has been shown to bind to HIV-1 RNA in the *pol* open reading frame [33]), produces a slight stimulation of translation (Fig. 4). This suggests that the role of MA does not require it to bind to RNA. The stimulation is weaker than that of full-length Gag, which could be due to the MA domain not attaining the same structure as when it is part of the Gag polypeptide. Alternatively, this could be explained if the matrix domain is required for stimulation of translation but is not sufficient for full activity by itself.

One possibility is that Gag interacts with a translation initiation factor or other *trans*-acting factor through its MA domain to promote translation. In fact, MA (alone and as part of Gag) has been shown to interact with eIF5B (40), the factor required for joining of 40S and 60S ribosomal subunits (28). Although this was shown to inhibit translation at high concentrations (40), at lower concentrations it is possible that MA could stimulate eIF5B activity or recruit it to the HIV-1 5' UTR via the NC domain of Gag. Interestingly, the developmental regulator Vasa, a 3' UTR-binding protein, has been shown to activate translation of *gurken* mRNA in *Drosophila* embryos through binding of eIF5B (19). Those authors suggest that this may occur through either displacement of eIF5B inhibitor proteins or recruitment of eIF5B to the mRNA. Given the propensity for mammalian viruses to modulate the translation apparatus of their host cell, it is not implausible for HIV-1 to control its translation at the last checkpoint of initiation.

An interaction between MA and the translation elongation factor EF1 α which resulted in translation inhibition has also

FIG. 6. Model of HIV-1 translation modulated by Gag. Initially, in the absence of Gag, there is low-level translation initiation and Gag starts to be produced. Gag stimulates translation initiation, resulting in greater Gag production. Increasing Gag levels result in oligomerization of Gag and subsequent coating of the HIV-1 5' UTR. Translation is inhibited, and viral RNA is sequestered for dimerization and encapsidation. Ψ represents the packaging signal (high-affinity Gag binding site).

been reported (7), although again a much higher concentration of MA was used than in the experiments described here. However, given that the same reporter open reading frame (NS') was used for most of the experiments described in this study and yet we saw different effects of Gag on translation from different 5' UTRs, it is unlikely that the effects of Gag shown

here are mediated at the stage of elongation; rather, they are likely mediated at initiation of translation.

Another possibility is that Gag is recruiting a factor which destabilizes the powerful structural inhibition to translation afforded by the 5' UTR. In spleen necrosis virus the structured leader is destabilized by the action of RNA helicase A (17), and this enzyme has also been implicated in the HIV Rev/RRE interaction (21).

The in vitro results are strengthened by the in vivo effects that were observed. In order to demonstrate the novel phenomenon of translation stimulation by HIV-1 Gag, HIV-1 reporter mRNA and Gag mRNA were cotransfected into mammalian cells. Translation from the HIV-1 5' UTR was increased with increasing Gag concentrations and was then suppressed at higher concentrations back to basal levels. While it has already been reported that overexpression of retroviral Gag protein from an expression plasmid can inhibit reporter gene expression in cells (23, 37), the mRNA transfections have shown the stimulation of translation of an HIV-1 reporter RNA by low concentrations of Gag.

In summary, we have shown that Gag regulates translation from its own 5' UTR in a bimodal manner. Stimulation is mediated through the matrix domain of Gag, and further work will be undertaken to elucidate the mechanism of this effect. Inhibition is mediated by an interaction between the nucleocapsid domain of Gag and its binding site Ψ in the HIV-1 5' UTR. Our model (Fig. 6) of Gag activity is that of a switch mechanism in which Gag initially acts as a positive feedback stimulus to its own production until total coating of the HIV-1 5' UTR blocks further translation and commits that RNA to encapsidation. This mechanism would help to overcome the intrinsic inefficiency of translation of the unspliced RNA and would likely allow enough molecules of Gag to be synthesized for viral particle production prior to a switch from translation to packaging of full-length viral RNAs.

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