Gag-Pol Supplied in trans Is Efficiently Packaged and Supports Viral Function in Human Immunodeficiency Virus Type 1

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Received 18 January 2001/Accepted 12 April 2001

The intracellular trafficking and subsequent incorporation of Gag-Pol into human immunodeficiency virus type 1 (HIV-1) remains poorly defined. Gag-Pol is encoded by the same mRNA as Gag and is generated by ribosomal frameshifting. The multimerization of Gag and Gag-Pol is an essential step in the formation of infectious viral particles. In this study, we examined whether the interaction between Gag and Gag-Pol is initiated during protein translation in order to facilitate the trafficking and subsequent packaging of Gag-Pol into the virion. A conditional cotransfection system was developed in which virion formation required the coexpression of two HIV-1-based plasmids, one that produces both Gag and Gag-Pol and one that only produces Gag-Pol. The Gag-Pol proteins were either immunotagged with a His epitope or functionally tagged with a mutation (K65R) in reverse transcriptase that is associated with drug resistance. Gag-Pol packaging was assessed to determine whether the Gag-Pol incorporated into the virion was preferentially packaged from the plasmid that expressed both Gag and Gag-Pol or whether it could be packaged from either plasmid. Our data show that translation of Gag and Gag-Pol from the same mRNA is not critical for virion packaging of the Gag-Pol polypeptide or for viral function.

Although retroviral assembly can occur independently of genomic RNA packaging (as shown by HIV-1 packaging signal [Psi] deletion mutants that generate empty particles [for a review, see reference 1]), in these cases assembly may have been promoted by the host cell’s nucleic acids (10). It is generally believed that Gag-Pol is incorporated into the virion via interactions with Gag (19, 31, 33), and there is evidence to suggest that the multimerization of Gag and Gag-Pol occurs prior to their association with the cell membrane. For HIV-1 assembly to occur, Gag must undergo myristoylation (14), a step that is critical for the transport of Gag to the plasma membrane. However, both nonmyristoylated Gag and nonmyristoylated Gag-Pol can be packaged into progeny virions when coexpressed with myristoylated Gag (9, 28, 30). Velocity sedimentation analysis of Gag-only particles has shown that both myristoylated and nonmyristoylated Gag proteins can assemble into multimeric assembly intermediates, but myristoylation is required to complete virus-like particle formation (24, 27). Furthermore, Lee et al. (22, 23) have reported the formation of Gag and Gag-Pol precursor complexes in the cytoplasm of HIV-1-infected CD4+ T cells.

Experiments using Moloney murine leukemia virus have shown that the Gag portion of Gag-Pol is not required for the packaging of Pol proteins. However, a lower level of reverse transcriptase (RT) activity was observed in the resulting virions than in wild-type virions (3), suggesting a deficient packaging of Pol. In HIV-1, the provision of Gag in trans to Gag-Pol results in the formation of virus-like particles containing both Gag and Gag-Pol (28, 30). Taken together, these investigations show that the packaging of Gag-Pol does not require Gag and Gag-Pol to be synthesized from the same mRNA. However, these studies do not establish if the multimerization of Gag and Gag-Pol preferentially occurs during protein translation as a means of facilitating the transport and subsequent packaging.
of Gag-Pol into progeny virions. In this study, we have assessed whether the incorporation of Gag-Pol that is supplied in trans is as efficient as the incorporation of Gag-Pol that is synthesized from the same mRNA as Gag. Using a conditional co-transfection system designed to distinguish between packaged Gag-Pol proteins expressed either from the same construct that expresses Gag or from a separate construct, we observed that Gag and Gag-Pol multimerization during translation from a single mRNA molecule is not critical for virion packaging of the Gag-Pol polyprotein. Furthermore, we found that an interaction between Gag and Gag-Pol proteins generated from the same mRNA is not necessary to generate functional virions.

MATERIALS AND METHODS

Construction of DNA plasmids. The HIV-1 DNA constructs used in this study were derived from either the full-length wild-type HIV-1 plasmid HXB2-BH10 (32) or a luciferase reporter plasmid pNL4-3-LucR-E- (12). pNL4-3-LucR-E- was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health, from N. Landau. For all experiments we used two fundamental HIV constructs, one that expresses both Gag and Gag-Pol but with a functional deletion in rev (G:GP) and one that expresses only Gag-Pol due to a frameshift mutation that allows continuous expression of Gag-Pol and bypasses the Gag termination codon (GP). GP:G was created by deleting the majority of the second half of exon 2 of rev in HXB2-BH10 as previously described (29). GP was constructed in HXB2-BH10 as previously described (29) to alter the heptanucleotide sequence that is responsible for −1 ribosomal frameshifting, by direct base changes and the addition of an extra nucleotide (5′-TTTTTTA-3′). The histidine (His) epitope tag was introduced into the 3′ end of integrase (IN) in the HXB2-BH10-based G:GP and GP plasmids. An MluI site was introduced into IN to replace the existing BspMI site located near the 3′ end of IN (5′-TGGCAG-3′-ACGCGT-3′) with PCR stitch mutagenesis, a technique that introduces mutations via the PCR primers as previously described (16).

Subsequent digestion with HindIII and ligation with a double-stranded DNA adapter complementary to the MluI cleavage site and containing the His epitope (5′-CAT CAC CAT CAC CAT CAC-3′) generated the G:GPtrim and GPtrim plasmids. The KS6R mutation is a lysine-to-arginine change at amino acid 65 in HIV-1 RT that is associated with resistance to the antiretroviral drug (−)−B,2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) (15). This mutation (AAA→AGA) was introduced into the HXB2-BH10-based G:GP and the GP plasmids by PCR stitch mutagenesis to generate G:GPks6r and GPks6r. A functional deletion in rev was introduced into pNL4-3-LucR-E- by digestion with MluI, which recognizes the second exon of rev, followed by S1 nuclease treatment according to the manufacturer’s instructions (Boehringer Mannheim) to remove 25 bp and generate Luc.Rev-. The frameshift mutation and/or the K6SR mutation were introduced by subcloning from the G:GP, G:GPks6r, GP, and GPks6r into Luc.Rev- or pNL4-3-Luc.R-E-, generating Luc.G:GP, Luc.G: GPks6r, Luc.GP, and Luc.GPks6r. All PCRs were conducted using the high-fidelity DNA polymerase enzyme Pfu as specified by the manufacturer (Gibco BRL). All constructs were sequenced to confirm the presence of the desired mutations.

Cotransfection and virus production. The G:GP and GP plasmids were co-transfected at a ratio of 20 µg of G:GP to 1 µg of GP in order to generate equal levels of Gag-Pol proteins derived from all constructs. The Luc.G:GP and Luc.GP plasmids were also cotransfected at a ratio of 20 to 1, and 5 µg of pSVSV-G (a kind gift from J. C. Burns, University of California, San Diego) was included to provide a viral envelope that would enable infection (5). The calcium phosphate co precipitation method was used for the transient transfection of 293T cells. Supernatants were collected at 36 h posttransfection and centrifuged for 30 min at 3,000 rpm (Beckman model GS-6) to remove cellular debris. Virions were purified and concentrated by ultracentrifugation (Beckman model L-90, SW41 rotor) at 35,000 rpm for 1 h at 4°C through a 20% sucrose cushion. For protein isolation, viral pellets were resuspended in 50 µl of 2× Tris-buffered saline lysis buffer containing 1% Nonidet P-40, 20 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, and 1 µM leupeptin. For use in the reverse transcription assay and the natural endogenous reverse transcription (NERT) assay, viral pellets were resuspended in Tris-EDTA. Virus production from transfections was quantified by measuring p24 antigen levels in the pelleted virion stocks and the cell culture supernatants (HIV-1 p24 assay; Abbott Laboratories).

Western blot analysis. Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) gel and transferred to a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech). Western blot analysis was carried out as previously described (29), using an α-R antibody (NEN) to detect p66 RT, an α-His antibody (Qiagen) to detect the His-tagged Gag-Pol polyproteins, and pooled sera from HIV-infected patients to detect total HIV-1 viral proteins. An ECL (enhanced chemiluminescence) detection kit (Amersham Pharmacia Biotech) was used to visualize the proteins for assessment by laser densitometry.

Cell-free RT assay. The cell-free RT assay was adapted from a method described by Boyer et al. (2). For each sample, 0.2 µg of a 20-µg/ml stock of M13-47 sequencing primer (New England Biolabs) was annealed to 0.25 µg of single-stranded M13mp18 DNA (New England Biolabs) by heating to 95°C for 5 min and then slowly cooling to room temperature. In a final volume of 60 µl, each sample contained 10 µl of Nonidet P-40, pelleted virus (5 ng of p24), 25 mM Tris-Cl (pH 8.0), 75 mM KCl, 8.0 mM MgCl2, 2 mM dithiothreitol, 100 µg of bovine serum albumin per ml, 10 mM CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 10 µM each dATP, dGTP, and dCTP, 5 µM dTTP, 0.5 µCi of [α-32P]dTTP (NEN), and 3′-TCT-triphosphate (Moravek Biochemicals), which was routinely used at a concentration of 2.5, 5, or 15 µM. The samples were incubated for 1 h at 37°C, and then 8 µl of each reaction was spotted onto a DE3 filter paper, which was washed six times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate and then microanalyzed on a microortex counter (Wallac).

NERT assay. The NERT assay was carried out as previously described (17). Briefly, sucrose-purified virus (typically 100 pg of p24) was supplemented with MgCl2 and DNase I and incubated at 37°C for 90 min in the presence and absence of deoxynucleoside triphosphates. Reverse transcription was terminated by the addition of EDTA and protease K followed by boiling for 10 min. Stopped reactions were assayed for negative-strand stop-polymerase DNA by PCR. Standard curves were generated by amplifying serial dilutions of an HIV-1 proviral plasmid, and all PCRs were performed in the linear range of the assay.

Single-round infection analyses with the pNL4-3-Luc.R-E constructs. Cleared viral supernatants containing 10 ng of p24 were adjusted to 200 µl with culture medium and incubated with 106 MT-2 cells for 2 h. The virus was then removed by washing the infected cells once with phosphate-buffered saline without calcium chloride and magnesium chloride and resuspending them in 1 ml of culture medium. The luciferase activity in the cells was determined 48 h postinfection using a luciferase assay kit (Promega). In experiments conducted in the presence of 3TC (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health), the MT-2 cells were incubated with 50 nM 3TC for 4 h prior to infection, 2 h during infection, and 48 h postinfection. The mean 50% inhibitory concentration of 3TC, which was shown to inhibit several different strains of HIV-1 in primary cells, ranges between 2.5 nM and 0.67 µM (11). Trypan blue staining was used to monitor cytotoxic effects of 3TC, and no differences were observed between control and 3TC-treated cultures.

RESULTS AND DISCUSSION

G:GP and GP were cotransfected to determine whether the interaction between Gag and Gag-Pol is initiated during protein translation. If Gag and Gag-Pol interact during their synthesis from a single mRNA molecule to facilitate Gag-Pol packaging, the progeny virions would be expected to contain primarily Gag-Pol from the plasmid synthesizing both Gag and Gag-Pol. Conversely, the progeny virions will contain similar levels of Gag-Pol proteins from both plasmids if the Gag/Gag-Pol interaction can occur efficiently when Gag-Pol is supplied in trans.

Development of a conditional cotransfection system. The conditional cotransfection system allows viral particle formation only when both cotransfected plasmids are simultaneously expressed in the same cell. This system takes advantage of the fact that both Gag and Rev are required for viral particle formation. As Rev is required for the transport of genomic RNA from the nucleus to the cytoplasm (for a review, see...
reference 18), transfection of a Rev-deficient HIV-1 plasmid (G:GP) alone results in viral mRNA being trapped in the nucleus. Similarly, viral particles will not be formed when GP is transfected alone, as Gag is required for virion production. Accordingly, when either G:GP or GP was transfected into 293T cells, no pelletable HIV-1 proteins were detected (data not shown). As the natural ratio of Gag to Gag-Pol synthesis is 20:1, the two plasmids were cotransfected at a ratio of 20 G:GP to 1 GP (based on DNA concentration) so that equivalent amounts of Gag-Pol proteins would be derived from all constructs. This enables a direct comparison of packaged Gag-Pol synthesized from either G:GP or GP. The HIV-1 constructs used in these analyses are depicted in Fig. 1A.

The Gag-Pol proteins were tagged with a His epitope that was inserted into the C-terminal region of IN (Fig. 1B) to allow semiquantitative assessment of Gag-Pol incorporation by Western blot analysis. Two types of viral particles, G:GP\textsubscript{His}/GP and G:GP/GP\textsubscript{His}, were obtained by cotransfection. If Gag and Gag-Pol interact during translation to facilitate Gag-Pol packaging, the G:GP\textsubscript{His}/GP virion should incorporate greater levels of His-tagged Gag-Pol than G:GP/GP\textsubscript{His}. As the His tag may alter Gag-Pol packaging by changing the conformation of the Gag-Pol protein, an alternative labeling system in which Gag-Pol proteins were functionally tagged with a K65R mutation in RT was also used (Fig. 1B). This mutation mediates resistance to 3TC, enabling wild-type Gag-Pol and K65R Gag-Pol to be differentiated in the presence of the drug. The K65R mutation was chosen because it maintains a drug-resistant phenotype when used in a cell-free RT assay (15). Four types of virus were produced through cotransfection: the drug-sensitive control G:GP/GP, the drug-resistant control G:GP\textsubscript{K65R}/GP\textsubscript{K65R}, and the two test cases G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP. If Gag and Gag-Pol interact during translation, Gag and Gag-Pol in progeny virions will be preferentially derived from the G:GP plasmid that expresses both proteins, so that one would expect G:GP/GP\textsubscript{K65R} to be drug sensitive and G:GP\textsubscript{K65R}/GP to be drug resistant. If Gag-Pol can be packaged with equivalent efficiency when translated with Gag or when translated alone, both types of virion should be similarly susceptible to 3TC-triphosphate.

Gag and Gag-Pol do not interact during translation from the same mRNA to facilitate Gag-Pol packaging. Epitope tagging of the Gag-Pol proteins was used to ascertain the amounts of packaged Gag-Pol derived from either the G:GP or the GP plasmid. G:GP\textsubscript{His}/GP and G:GP/GP\textsubscript{His} viral particles obtained by cotransfection of 293T cells were concentrated by ultracentrifugation and resuspended in protein lysis buffer. A protein dilution series of each virus was resolved by SDS-PAGE for Western blotting, and the resultant blots were hybridized successively with \textalpha\textsubscript{-}RT, \textalpha\textsubscript{-}His, and pooled sera from HIV-1-infected patients. RT levels provided a comparative measure of the total amount of HIV-1 Gag-Pol polyprotein in each virus stock, and the concentration of \textalpha\textsubscript{-}His reactive protein reflects the amount of Gag-Pol packaged from a particular plasmid (Fig. 2). For the representative experiment depicted in Fig. 2, the His signals (standardized to equivalent levels of RT) were 993, 421, and 309 (densitometry units) for the three dilutions of G:GP\textsubscript{His}/GP and 906, 478, and 270 for the three dilutions of G:GP\textsubscript{K65R}/GP, indicating similar efficiencies of Gag-Pol packaging from the two plasmids. Hybridization with the pooled patient sera indicated that there were no obvious differences in
FIG. 2. Western blot analysis of virion lysates produced from cotransfections in 293T cells to produce two virion types, G:GP/GP and G:GP/GPK65R. Virion lysates were analyzed by SDS-PAGE with twofold dilutions of each lysate alongside the mock and the wild-type HXB2-BH10 control. RT (p66) is indicative of the overall level of Gag-Pol protein. His-tagged IN reflects the level of one particular Gag-Pol population in each virus type.

total HIV-1 protein expression levels or patterns between the two viruses (data not shown).

G:GP/GP, G:GP\textsubscript{K65R}/GP\textsubscript{K65R}, G:GP/GP\textsubscript{K65R} and G:GP/GPK65R/GP virions were also generated by cotransfection of 293T cells. The four virion samples were normalized on the basis of p24 levels, and a cell-free RT assay was used to determine RT activity in the presence and absence of 3TC-triphosphate, a competitive inhibitor of dCTP incorporation into the nascent DNA chain. Initially three concentrations (2.5, 5, and 15 $\mu$M) of 3TC-triphosphate were selected that gave clearly distinguishable differences between the drug-sensitive control virion G:GP/GP and the drug-resistant control virion G:GP\textsubscript{K65R}/GP\textsubscript{K65R}. Averaged data from three assays of virions produced from three separate transfections are shown in Fig. 3. The levels of 3TC-triphosphate inhibition of RT activity in the two test cases (G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP) were very similar to each other. While not readily distinguishable from the drug-susceptible control, the level of inhibition of RT activity for G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP was clearly distinct from that of the drug-resistant control. 3TC-triphosphate concentrations between 0.5 and 16 $\mu$M were used to address the possibility that lower drug concentrations may better determine a preference for Gag-Pol packaging in a population of both wild-type and drug-resistant Gag-Pol polproteins. RT enzymes from G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP displayed very similar levels of 3TC-triphosphate susceptibility. A virus dilution series demonstrated that our results were not influenced by saturating concentrations of RT enzyme (data not shown). Overall, the RT assay results showed that both drug-sensitive and drug-resistant Gag-Pol proteins were packaged into both G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP.

Consequently, no preference involving Gag-Pol encapsidation could be distinguished by either Western blot analysis with His-tagged Gag-Pol or RT assays with functionally labeled Gag-Pol. These results are in agreement with those of Park and Morrow (28) and Smith et al. (30), who demonstrated that Gag-Pol could be incorporated into the virion when supplied in trans to Gag. Moreover, our data show that the Gag-Pol polypeptide is efficiently packaged into the virion irrespective of being translated with Gag or translated alone.

Gag and Gag-Pol interaction during translation from the same mRNA is not required for endogenous reverse transcription or viral infectivity. To determine whether or not an interaction between Gag and Gag-Pol at the time of translation is important for correct protein folding and the proper molecular arrangement of proteins within the virion, functional competence of the K65R labeled viruses was assessed by NERT and viral infectivity assays. The NERT assay measures the endogenous RT activity within intact virions, which is thought to reflect the viruses’ ability to successfully initiate infection (35, 36). The NERT assays (Fig. 4A) show that the drug-sensitive (G:GP/GP) and the drug-resistant (G:GP\textsubscript{K65R}/GP\textsubscript{K65R}) controls are affected by 3TC-triphosphate as anticipated, with G:GP\textsubscript{K65R}/GP\textsubscript{K65R} being two- to threefold more resistant to 3TC-triphosphate than G:GP/GP (Fig. 4A). However, the two test cases, G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP, displayed similar levels of NERT activity; both were approximately 1.3-fold more resistant than the drug-sensitive control and 0.5-fold less resistant than the drug-resistant control (Fig. 4A). Viral infectivity was assessed from the infection of MT-2 cells in the presence and absence of 3TC via the luciferase reporter gene. The controls were clearly separable on the basis of drug response, with the Luc.G:GP/GP\textsubscript{K65R} and G:GP\textsubscript{K65R}/GP\textsubscript{K65R} viruses being 2- to 2.5-fold more susceptible to 3TC than the Luc.G:GP\textsubscript{K65R}/Luc.G:GP\textsubscript{K65R} virions. However, the two test samples, Luc.G:GP\textsubscript{K65R}/Luc.G:GP\textsubscript{K65R} and Luc.G:GP\textsubscript{K65R}/Luc.G:GP\textsubscript{K65R}, consistently generated similar levels of luciferase activity, with both approximately 1.3-fold more resistant than the drug-sensitive control and 0.5-fold less resistant than the drug-resistant control (Fig. 4B). The results of both the NERT and the viral infectivity assays were consistent with the Gag-Pol packaging data described above, which established that Gag-Pol was efficiently packaged when supplied in trans. Moreover, the NERT and viral infectivity assays show that an interaction between Gag and Gag-Pol during translation is not essential for the function of Gag-Pol proteins in virions. The packaging of the retroviral enzymes in...
the form of a precursor protein is in itself suggestive of a role for Gag-Pol in coordinating the placement of the viral enzymes within the mature virion. Functional RT and IN proteins can be packaged when supplied in trans as Vpr fusion proteins to generate infectious virions (25, 34). However, Wu et al. (34) found that the levels of infection achieved when the proteins were supplied individually as Vpr-RT and Vpr-IN did not reach those of virions complemented by RT-IN supplied in fusion, Vpr-RT-IN, which in turn were not as infectious as wild-type virions. Thus, it remains likely that the expression of the Pol proteins in the form of a precursor is important both for the control of expression in required ratios and the coordinated arrangement of viral enzymes in the virion.

The precise steps and timing of virion assembly and maturation are not clearly understood. Together with the 1,500 or so Gag molecules present in an HIV-1 virion, 70 Gag-Pol proteins must also be incorporated. While our results indicate that the multimerization of Gag and Gag-Pol is not reliant on Gag-Pol being synthesized from the same mRNA as Gag, they do not, however, rule out the possibility that the Gag and Gag-Pol interaction occurs during translation between protein molecules on adjacent separate polysomes. Consequently, Gag-Pol may still be incorporated into a viral assembly complex formed in the context of the polysome. While Gag drives viral assembly and Gag alone will form virus-like particles, expression of Gag-Pol in the absence of Gag generates processed Gag and Pol proteins; however, no progeny virions are formed for study in systems equivalent to those of Gag-only particles (30). Kaye and Lever (21) have reported that Gag-Pol expressed alone in a T-cell line will produce pelletable Gag-Pol and negligible amounts of viral genomic RNA, but there is no further evidence to suggest that virus-like particles are formed in the absence of Gag. The difficulty involved in separating the functions of the shared regions of Gag and Gag-Pol provides a further obstacle in addressing the process of Gag-Pol assembly.

The cotransfection and Gag-Pol labeling system described here enable independent examination of Gag-Pol packaging, as alterations to the Gag-Pol-only expression vector (GP) that affect Gag-Pol packaging can be monitored by changes in the Gag-Pol packaging profiles. This system could also be used to investigate Gag-Pol interactions not only with Gag but also with RNA and host cellular factors involved in viral assembly.

ACKNOWLEDGMENTS

We thank John Mills for critical review of the manuscript. We thank Paul Boyer and Stephen Hughes for helpful advice on developing the cell-free RT assay. We also thank Shaham Campbell, Miranda Shehu-Xhilaga, and Katherine Kedzierska for constructive advice.

This study was funded by grants from the National Health and Medical Research Council (NHMRC) and the Macfarlane Burnet Centre (MBC) Research Fund. Melissa Hill is a recipient of a Burnt Centenary postdoctoral fellowship. Johnson Mak is the recipient of a NHMRC Peter Doherty postdoctoral fellowship. Suzanne Crowe, Bill Hooker, and David Harrich are supported by the Australian National Centre in HIV Virology Research (NCHVR).

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