Foamy virus Pol protein expressed as a Gag-Pol fusion retains enzymatic activities allowing infectious virus production

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Foamy viruses (FV) synthesize Pol from a spliced pol mRNA independently of Gag, unlike orthoretroviruses that synthesize Pol as a Gag-Pol protein which coassembles with Gag. We found that the prototype FV (PFV) mutants expressing Gag and Pol only as a Gag-Pol protein without the spliced Pol contain protease activity equivalent to that of WT Pol. Regardless of the presence or absence of the spliced Pol, the PFV Gag-Pol proteins can assemble into virus-like particles (VLPs), in contrast to the orthoretroviral Gag-Pol proteins that cannot form VLPs. However, the PFV Gag-Pol VLPs have aberrant morphologies and are not infectious. In the absence of the spliced Pol, coexpression of a PFV Gag-Pol protein with Gag can produce infectious virions. Our results suggest that enzymes encoded in PFV pol (protease, reverse transcriptase, and integrase) are enzymatically active if they are synthesized as part of a Gag-Pol protein.
The foamy virus (FV) genome encodes the three major viral proteins Gag, Pol, and Env that are common to all other retroviruses, in addition to two regulatory proteins, Bet and Tas. However, FV are classified as one of two subfamilies of the Retroviridae, the Spumaretrovirinae, whose viral replication pathway is distinct from that of Orthoretrovirinae. Major differences include the mode of Pol expression and its encapsidation into virions. Orthoretroviruses, such as human immunodeficiency virus (HIV-1) and murine leukemia virus (MLV), synthesize Pol as a Gag-Pol fusion protein. Gag-Pol is produced by translational readthrough which occurs by either ribosomal frame shifting or suppression of a stop codon at the C-terminus of Gag. Readthrough occurs at a frequency of approximately 5-10% relative to gag translation [reviewed in (9)]. The Gag-Pol protein coassembles with Gag through Gag assembly domains (23,30). In HIV-1, when the Gag to Gag-Pol ratio (normally about 20:1) was altered to increase the amount of Gag-Pol relative to Gag, viral assembly was disrupted, possibly due to steric hindrance during the assembly process caused by an excess of Gag-Pol (10,29). Expression of orthoretroviral Gag-Pol protein alone does not lead to the production of viral particles (7,14,22,35). In contrast, FV Pol is expressed from a singly spliced mRNA (38) and Pol expression is regulated at the transcriptional level, using a suboptimal 3’ splice site for the pol gene (15). Because FV Pol is synthesized independently of Gag, the mechanism of FV Pol incorporation into virions is different from that of orthoretroviruses. Cis-acting sequences in the genomic RNA are required for FV Pol packaging (12,24). Previously, we proposed that protein-protein interactions between Gag and Pol are involved in Pol packaging and a Gag/Pol complex binds to genomic RNA for encapsidation into virions (17).
Compared to orthoretroviruses, in FV, there are limited proteolytic cleavages of both Gag and Pol. FV Gag is not cleaved into separate matrix (MA), capsid (CA), and nucleocapsid (NC) proteins that are found in mature orthoretroviruses. FV Gag is partially cleaved once near the C-terminus to release a p3 peptide (Fig. 1A). This cleavage is required for efficient viral infectivity (6,41). Another distinct feature of FV Gag is that it does not contain the conserved cysteine and histidine motifs that are present in orthoretroviral NC, but instead contains two or three glycine/arginine-rich (GR) boxes upstream of the p3 cleavage site near the C-terminus of Gag. The first two GR boxes are involved in essential steps of viral replication, including genomic RNA packaging, Pol packaging, reverse transcription, and particle morphology (17,20,31). GR box 3 is absent in some FV isolates and, as yet, has no known function (34,36), which suggests that GR3 might be dispensable. FV Pol is synthesized as a precursor polypeptide containing protease (PR), reverse transcriptase (RT), and integrase (IN), and Pol is cleaved only once by PR between RT and IN, producing an IN and a PR-RT fusion protein (26) (Fig. 1A). Examination of mutants lacking the cleavage site between RT and IN showed that virus replication is dependent on this cleavage (27).

In the present study, we created several prototype FV (PFV) mutants encoding Gag-Pol fusion proteins. These fusion proteins were expressed alone or together with the Gag protein, to mimic the mode of orthoretroviral assembly. We examined the expression and packaging of viral proteins into virions and the enzymatic activities of Pol during viral assembly and replication. While we were writing this manuscript, another group published an article describing similar PFV Gag-Pol fusion proteins (33).
MATERIALS AND METHODS

Construction of recombinant DNAs. The prototype foamy virus (PFV) used in this study is a chimpanzee FV isolated from a human-derived cell culture, which was previously designated human FV (HFV). PFV Gag-Pol fusions were generated in the context of a full-length proviral clone containing a cytomegalovirus (CMV) immediate early promoter, pcPFV (31). A deletion of 1 nucleotide (nt) just downstream of the p3 cleavage site was used to make an in-frame Gag-Pol fusion protein [Fig. 1A and B, Gag-Pol (G-P)]. Besides this 1 nt deletion, we also made additional modifications which led to changes in the amino acid (aa) sequences of the viruses we created. In all of the in-frame Gag-Pol fusion constructs, seventeen aa were removed from the C-terminus of Gag, because the coding region for this portion of Gag overlaps with the coding region for the N-terminus of Pol (Fig. 1B). The region encoding seven aa downstream of the p3 cleavage site was also altered from Gln-Ser-Ala-Thr-Ser-Thr to Arg-Val-Pro-Arg-Pro-Pro-Gln in all of the Gag-Pol fusion constructs. The deletion of GR box 3 in ∆GR3 G-P mutant starts at nucleotide position 2811 of the pcPFV and ends at position 2905. The aa sequences around the p3 cleavage site were mutated from Val-Asn-Thr-Val-Thr to Val-Gln-Tyr-Arg-Asp in both mp3 G-P and ∆GR3/mp3 G-P mutants (Fig. 1B). An adenine nucleotide at the position 2459 was changed cytosine in order to eliminate the 3’splice site of the pol gene in a ∆3’ss mp3 G-P mutant. All of the site-directed mutations in the PFV genome were generated by two rounds of PCR using four oligonucleotides. Briefly, the two outer oligonucleotides were designed to anneal to the gag or pol gene and contain unique restriction sites at each end. The two inner mutagenic
oligonucleotides (in either forward or reverse orientation) were designed to be complementary to the gag sequences near the p3 cleavage site except for desired mutations. Each mutant construct was sequenced to confirm the presence of correct mutations. Primer sequences will be supplied upon request.

**Cell cultures and transfections.** 293T, HT1080 (human fibrosarcoma cells), and FAB cells (39) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% bovine growth serum and 1% penicillin and streptomycin. Transient transfection was done using 1mg/ml polyethyleneimine (Polysciences, Warrington, PA) as previously described (4). Cells and viral supernatants were harvested between 45 and 48 h posttransfection and prepared as previously described (17). Briefly, culture supernatants were collected, and cell debris was cleared by low-speed centrifugation and filtration through a 0.45 µm-pore-diameter syringe filter. Viral particles were pelleted through 20% sucrose cushions at 25,000 rpm for 2 h using an L7 ultracentrifuge (Beckman). After removing the supernatants, cells were scraped off plates in 1X SDS sample buffer (12.5% 50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue), and lysates were prepared by using Qiashredder (Qiagen) in accordance with the manufacturer’s protocol. To measure infectivity, a foamy virus-activated \( \beta \)-galactosidase (\( \beta \)-gal) expression assay was performed as previously described (39). Briefly, the FAB indicator cell line has a single integrated copy of a PFV long terminal repeat (LTR)-driven \( \beta \)-gal gene. Infection of these cells leads to the transactivation of the PFV LTR promoter by the viral Tas protein.

**Western blot analysis.** Cell lysates and viral pellets were resuspended in 1X SDS sample buffer before loading onto 9% SDS-polyacrylamide gels. In some experiments
viral pellets were resuspended in 1X phosphate buffered saline (PBS) solution and proteolytically digested with subtilisin (Sigma-Aldrich), according to previously described protocols (32). Western blot analysis was performed as previously described (1), using 1:5000 dilution of rabbit polyclonal anti-Gag antibody (1), 1:800 dilution of mouse monoclonal anti-Pol antibody (31), 1:5000 dilution of rabbit polyclonal anti-IN antibody (27), 1:1000 mouse monoclonal anti-SU antibody (40), 1:1000 mouse monoclonal anti-LP antibody, or 1:2000 dilution of mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse monoclonal antibody (IgG2a subtype) against PFV LP was generated using the GST-LP fusion protein. Proteins were visualized using the Odyssey detection system (Li-Cor, Lincoln, NE), according to the manufacturer’s protocol. Intracellular protein levels were normalized to the level of Gapdh protein as an internal control.

**RT-PCR.** Forty-eight hours after transfection, both cells and virus-containing supernatants were collected and used to extract RNA. The cells were scraped off plates with PBS and centrifuged for 10 min at 500 x g. Cell pellets were lysed and total cellular RNA was extracted using RNeasy mini kit (Qiagen). The amounts of viral supernatants to be pelleted were determined according to each sample’s intracellular Gag level that was quantified by Western blots. The Gag level in the cell was normalized by Gapdh level. Equal numbers of virus particles, as determined by extracellular Gag levels, were treated with RNase-free DNase I and viral RNA was isolated using a QIAamp viral RNA minikit (Qiagen). Extracted cellular or viral RNA (0.7 µg) was reverse transcribed using a poly (A) · poly (dT)₁₂ primer and ThermoScript RT (Invitrogen), according to the
manufacturer’s protocol. The cellular levels of viral RNA were measured by PCR amplification using the same reverse primer for both the unspliced genomic RNA and the spliced pol mRNA (5’-TCCACGTCTCTCTCCTACC-3’) and a forward primer specific for each RNA (5’-AGATAATCAAACAAGAGC-3’ for the unspliced genomic RNA and 5’-ACTACTCGCTGCGTCGAGAG-3’ for the spliced pol mRNA), each giving PCR amplification product of ~300 nt. gapdh mRNA was used as an internal control for cellular RNA. The level of viral genomic RNA packaged into virions was measured using primers around the 5’splice site (5’-

\[\text{GGAGCTCTTCACTACTCGCTGCGTCGAG-3’ and 5’-CAACCAGAGCTTCAACATCAAG-3’}\], yielding PCR products of 501 nt. For PCR amplification, samples were denatured for 5 min at 95° before thermal cycling was done. Temperatures for denaturing, annealing, and extension were 95°, 48°, and 72°, respectively, for 45 sec each cycle and run for 25 cycles. The final extension reaction was done at 72° for 10 min.

**Immunofluorescence microscopy.** Transfected cells were fixed with freshly made 3% paraformaldehyde at room temperature (RT) for 15 min. Cells were quenched and blocked as previously described in (40). Samples were incubated with the primary antibodies at RT for 1 h. After washing, cells were incubated with Alexa Fluor-conjugated secondary antibodies at 1:5000 at RT for 45 min. Samples were then washed in PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Fluorescence was visualized using a fixed-stage Nikon Eclipse E800 microscope.

**Electron microscopy (EM) analysis.** 293T cells were transfected with WT or the mp3 G-P fusion construct in the context of full length proviral genome. 45-48 h after
transfection, the cells were washed with PBS, and fixed with ½ strength Karnovsky’s
fixative (2% paraformaldehyde, 2.5% glutaraldehyde) at RT for 1 h. Cells were scraped
off the plates and collected by centrifugation at 1200 rpm for 5 min at 4°C. The cell
pellets were rinsed in ½ strength Karnovsky’s fixative and dehydrated in graded ethanol
(50 to 100%). The pellet was processed for EM analysis as described in (16). Digital
images were obtained with an electron microscope (JOEL 1230) equipped with a digital
camera (Gatan).

**Particle density analysis.** 293T cells were seeded in 10-cm plates a day prior to
transfection. The supernatants of cells transfected with WT or the mp3 G-P fusion
construct were harvested between 45-48 h posttransfection and pelleted through 20%
sucrose cushions by ultracentrifugation at 25K rpm for 2 h. The viral pellets were
resuspended in PBS and loaded onto a discontinuous density gradient consisting of 20,
30, 40, and 50% (w/v) iodixanol. The samples were subjected to ultracentrifugation at
32K rpm for 19 h. Fourteen fractions were collected from the top, and the density of each
fraction was measured. Proteins from each fraction were precipitated with 10%
trichloroacetic acid (TCA) and resuspended in 1X SDS sample buffer for Western blot
analysis. The Western blot membranes were probed with a polyclonal anti-Gag antibody
for detection of the WT Gag protein and a monoclonal anti-Pol antibody for detection of
the mp3 G-P Gag-Pol fusion protein.
RESULTS

Construction of the PFV Gag-Pol fusion proteins. Processing by FV protease (PR) is limited compared to processing by orthoretroviral PRs which completely cleave Gag to matrix (MA), capsid (CA), and nucleocapsid (NC). Figure 1A shows proteolytic processing of the prototype FV (PFV) Gag and Pol proteins. The partial cleavage near the C-terminus of Gag releases a p3 peptide, converting a 71 kDa Gag protein to a 68 kDa protein. The other cleavage is in the Pol protein between reverse transcriptase (RT) and integrase (IN). Cleavage produces a 40 kDa IN and an 85 kDa PR-RT fusion from a 125 kDa precursor Pol protein.

Normally, the FV pol gene is in the +1 reading frame with respect to the gag gene. We created a deletion of GR box 3 near the C-terminus of PFV Gag. In this mutant, by deletion of one extra nucleotide (nt), the Gag and Pol coding regions were brought into the same reading frame, producing a ΔGR3 Gag-Pol fusion protein (Fig. 1B, ΔGR3 G-P). The 1 nt deletion downstream of the p3 cleavage site led to a change in seven amino acids (aa) downstream of the p3 cleavage site in Gag. Gln-Ser-Ala-Thr-Ser-Ser-Thr was changed to Arg-Val-Pro-Arg-Pro-Pro-Gln in this mutant (Fig. 1B). The in-frame Gag-Pol fusion near the C-terminus of Gag resulted in a truncation of the seventeen aa at the C-terminus of Gag because normally, the segment encoding this portion of Gag overlaps with the coding region for the N-terminus of Pol (Fig. 1B). We also made several other Gag-Pol fusion constructs. The Gag-Pol (G-P) mutant was constructed by the insertion of a segment encoding GR box 3 into the ΔGR3 G-P construct (Fig. 1B). In the mp3 G-P construct, the p3 cleavage site was mutated to prevent Pol from being
released from a Gag-Pol fusion protein (Fig. 1B). Mutations in both the p3 cleavage site and GR box 3 were introduced to create the ΔGR3/mp3 G-P construct (Fig. 1B).

Expression of the PFV Gag-Pol fusion proteins. To determine the ability of the Gag-Pol fusion proteins to participate in virus assembly and support replication, these Gag-Pol fusion constructs were inserted into a full length PFV plasmid in place of the regions encoding Gag and Pol. Expression of the viral sequences is under the control of the cytomegalovirus immediate early promoter (pcPFV) (31). 293T cells were transfected with constructs expressing the Gag-Pol fusion proteins and Western blot analyses were performed on both cell lysates and pelleted supernatants. Unlike orthoretroviruses, FV Env is required for virus budding (1,8). A PFV mutant with a deletion of the env gene (ΔEnv) was used as a negative control to ascertain the level of nonspecific release of viral proteins which could be caused by cell lysis. Cells transfected with the ΔEnv construct expressed Gag in cells at levels equivalent to the WT, but no Gag was released into the supernatant (Fig 2A, lanes 3 and 10). This indicates that any Gag or Pol detected in the supernatants was not the results of non-specific release. The two Gag-Pol fusion mutants containing cleavage sites in both Gag and Pol, showed a cleaved 68 kDa Gag when probed with anti-Gag antibody (Fig. 2A, lanes 4 and 7; ΔGR3 G-P and G-P). The mp3 G-P and ΔGR3/mp3 G-P mutants that disrupted the p3 cleavage site expressed Gag-Pol fusion proteins but no Gag. These Gag-Pol proteins were more readily detected with anti-Pol antibody than anti-Gag antibody for unknown reasons (Fig. 2A and B, lanes 5 and 6). The amounts of the Gag-Pol fusion proteins present in cells were significantly higher than the amounts of Pol present in cells transfected with WT (Fig. 2B, compare lanes 4-7 with lane 2).
The PFV protease is active when expressed as a Gag-Pol fusion protein in the absence of the spliced Pol protein. The sizes of the Gag-Pol proteins expressed in the p3 cleavage site mutants, judged by the position to which they migrate on gels, are consistent with a partial processing by PFV PR at the cleavage site between RT and IN of Gag-Pol proteins (Fig. 1A). This cleavage would result in a 193 kDa full-length Gag-PR-RT-IN fusion protein and a 150 kDa Gag-PR-RT, lacking IN. This interpretation was confirmed by detection of cleaved IN with anti-IN antibody in the pelleted supernatants (Fig. 2C). PR proteolytic processing was also detected in the Gag-Pol fusion mutants that had cleavage sites in both Gag and Pol. The ΔGR3 G-P and G-P mutants produced Pol products that migrated on SDS-PAGE, similar to the corresponding WT products yielding an approximately 80kDa Pol (PR-RT) and a 125 kDa Pol precursor (PR-RT-IN; PrPol) (Fig. 2B, lanes 4 and 7). Similar Pol products were also detected in the p3 cleavage site mutants (Fig. 2B, lanes 5 and 6). These results suggest that in these mutants, Pol was also expressed from the spliced pol mRNA independently of the Gag-Pol fusion protein. Thus, to determine whether protease is active in the context of Gag-Pol, it was necessary to eliminate the spliced Pol product.

To eliminate the expression of WT Pol, we introduced into the mp3 G-P mutant a mutation at the 3’ splice site (ss) used to generate pol mRNA. This new Gag-Pol construct (Δ3’ss mp3 G-P) lacks both 3’ss and the p3 cleavage site near the C-terminus of Gag, resulting in the expression of only a Gag-Pol fusion protein. 293T cells transfected with this new Gag-Pol construct were harvested and the levels of unspliced genomic RNA and spliced pol mRNA in cells were measured by RT-PCR. Both the mp3 G-P and the Δ3’ss mp3 G-P mutants expressed unspliced genomic RNA at levels equivalent to
WT (Fig. 3A, lanes 2-4). However, the 3’ss mutant did not express the spliced pol mRNA while the mp3 G-P synthesized approximately the same amount of the spliced pol mRNA as WT (Fig. 3A, lanes 6-8). Western blot analyses showed that in the cells expressing the Δ3’ss mp3 G-P protein, both 80 kDa Pol and 125 kDa precursor Pol bands were absent (Fig. 3C, compare lane 5 with lane 4). Proteolytic cleavage at the site between RT and IN was seen by detection of cleaved IN with anti-IN antibody in the pelletable supernatants (Fig. 3D, lane 9). The results indicate that the protease expressed in the context of a Gag-Pol fusion protein is active in the absence of the WT Pol.

The PFV Gag-Pol fusion protein can assemble into virus-like particles. To examine whether PFV mutants expressing only a Gag-Pol protein can assemble virus particles, the supernatants of transfected cells were concentrated by sedimentation through a 20% sucrose cushion. Virus pellets were proteolytically digested with a non-membrane permeable endoprotease, subtilisin, to eliminate nonspecific aggregates and cellular debris (32). Expression of the ΔGR3 G-P and the G-P fusion led to the release of a cleaved 68 kDa Gag into the medium (Fig. 2A, lanes 11 and 14). This was expected because the Gag p3 cleavage sites are intact in these forms of Gag-Pol proteins. Despite the fact that there are high levels of the Gag-Pol fusion proteins from the p3 cleavage site mutants in the cells, regardless of presence or absence of the WT Pol, only small amounts of 150 kDa Gag-Pol proteins were detected in the supernatants (Fig. 2B, lanes 12 and 13; Fig. 3C, lanes 12 and 14). These results contrast with the efficient assembly and release of Pol in the WT and the two Gag-Pol fusion mutants that contain cleavage sites in both Gag and Pol (Fig. 2B, compare lanes 12 and 13 with lanes 9, 11, 14; Fig. 3C, lane 8 vs. lanes 12 and 14). However, the amounts of subtilisin resistant Gag-Pol proteins present in
the pellets were significant compared to the amounts seen in the absence of Env (Fig. 2B and 3C, lane 10), suggesting that the Gag-Pol fusion proteins can assemble into virus-like particles (VLPs) protected by membranes.

In order to examine whether the VLPs released from the Gag-Pol fusions are particulate, release of the Env protein was examined with anti-LP antibody. PFV Env is synthesized as a precursor protein and is subsequently processed by cellular protease to yield the particle-associated leader peptide (LP), surface (SU), and transmembrane (TM) subunits (3). After digestion of virus pellets with subtilisin, the SU bands that were shown in the absence of subtilisin disappeared (Fig. 3E, compare lanes 3, 7, and 9 with lanes 2, 6, and 8), indicating the efficacy of subtilisin digestion. LP proteins were trimmed outside of the membrane after digestion with subtilisin and the smaller p14 LP bands were detected in the supernatants of both the mp3 G-P and ∆GR3 G-P as well as WT (Fig. 3F, lanes 3, 7, and 9), supporting release of VLPs from the Gag-Pol fusion mutants.

The PFV Gag-Pol fusion particles are not infectious. The infectivity of Gag-Pol fusion viruses was measured using the FAB assay (39) and summarized in Table 1. The G-P mutant that retains the PR cleavage sites in both Gag and Pol produced approximately the same amount of infectious viruses as WT. The titer of the ∆GR3 G-P mutant was about ten-fold lower than the WT, implying that GR box 3 might contribute to infectivity. All of the p3 cleavage site mutants including the ∆GR3/mp3 G-P, mp3 G-P, and ∆3’ss mp3 G-P, in which the p3 cleavage site was mutated, did not produce infectious viruses (Table 1). These results indicate that the Gag-Pol fusion viruses were able to replicate as long as PR-mediated proteolytic cleavages took place in both Gag and
Pol of the fusion proteins. However, the Gag-Pol mutants, in which Gag is fused with most of Pol except IN are not replication competent, regardless of presence or absence of the spliced Pol.

**VLPs produced from expression of the PFV Gag-Pol fusion protein have aberrant morphologies in cells.** We examined intracellular localization of the Gag-Pol fusion proteins, using both immunofluorescence microscopy and electron microscopy (EM). Previously, our laboratory reported that PFV Gag assembly occurs at a pericentriolar region (40). Gag proteins accumulate in a perinuclear region and co-localize with γ-Tubulin, a subcellular marker for the microtubule organizing center (MTOC). When HT1080 cells were transfected with a WT proviral vector and were stained with anti-Gag and anti-Pol antibodies, Gag localized in a perinuclear region and accumulated at a site called “the site for Gag assembly” (40) (Fig. 4A). Although less Pol was expressed than Gag, Pol colocalized with Gag at the assembly site (Fig. 4A, Merge). In cells transfected with either the mp3 G-P or the Δ3’ss mp3 G-P constructs, the Gag-Pol fusion proteins localized in a perinuclear region (Fig. 4B and C). EM analysis showed large amounts of electron dense cores in 293T cells transfected with the WT expression construct (Fig. 5A and B). The intracellular virions are referred as capsids lacking Env spikes in the viral membrane. WT virions that budded out through the cell membranes of transfected cells contained Env and were uniform in size and shape (Fig. 5C and D). Cells transfected with the mp3 G-P mutant showed electron dense material near the nucleus, but there were no regularly shaped capsids visible in the EM (Fig. 5E-H). In cells transfected with the mp3 G-P mutant, it was difficult to detect VLPs budding through the cell membrane.
The PFV Gag-Pol VLPs have a density slightly less than WT and are deficient in genomic RNA. To determine whether the Gag-Pol proteins found in supernatants (detected by Western blot analysis) were in VLPs, we performed isopycnic gradient centrifugation analysis. The supernatants of 293T cells transfected with WT or the mp3 G-P construct were pelleted 2 days after transfection and prepared for analysis on iodixanol density gradients. The densities of each number fraction prepared for the two samples were comparable (Fig. 6A). The WT Gag proteins were recovered in fractions 5 to 9 with densities of 1.12 to 1.2 g/ml, peaking in fractions 5 and 6 with densities of 1.12 and 1.14 g/ml (Fig. 6B), as predicted for PFV particles (17,38). About a four times larger volume of viral supernatants was collected from cells transfected with the mp3 G-P construct and used in the density analyses. The VLPs of the mp3 G-P were slightly lighter than WT, with densities from 1.1 to 1.14 g/ml (Fig. 6C). The results imply that VLPs that contain the Gag-Pol fusions have a density slightly less than WT.

RT-PCR after DNase treatment was performed to measure the ability of the Gag-Pol mutants to encapsidate genomic RNA into virions. No viral RNA was detected in supernatants of the ΔEnv control (Fig. 6D). The levels of unspliced genomic RNA in the virions released from the ΔGR3 G-P and G-P fusions (which retained the p3 cleavage sites) were comparable to that of the WT (Fig. 6D, lanes 3, 5 and 8). However, neither of the p3 cleavage site mutants, mp3 G-P and ΔGR3/mp3 G-P, were able to encapsidate genomic RNA into virions (Fig. 6D, lanes 6 and 7). Thus, the VLPs that are slightly lighter than WT are deficient in genomic RNA.

PFV Gag-Pol can coassemble with Gag into virions to produce infectious particles.
Since orthoretroviral Gag can assemble into VLPs and Gag-Pol coassembles with Gag [reviewed in (9)], we asked whether, in the presence of PFV Gag, the PFV Gag-Pol protein could coassemble with Gag into virus particles. Equimolar amounts of a plasmid expressing PFV Gag (pGag) and Gag-Pol proviral plasmids (mp3 G-P or Δ3’ss mp3 G-P) were cotransfected into 293T cells and we measured the levels of the viral proteins in cells and viral assembly and release by Western blot analyses. Both the PFV Gag and Gag-Pol proteins were detected in the cotransfected cells and in the supernatants (Fig. 7A and B, lanes 5, 7, 12 and 14). Thus, VLPs were released from cells that were cotransfected with the pGag plasmid and the Gag-Pol mutant constructs. In Δ3’ss mp3 G-P cells that coexpressed Gag, although WT Pol protein was absent, the protease expressed in the Gag-Pol cleaved Gag (Fig. 7A, lane 7). Virus titers from cotransfected cells with the Gag and Gag-Pol were measured. We have previously shown that a PFV Gag mutant that has an R50A mutation in the cytoplasmic targeting/retention signal (CTRS) prevents virus assembly at the MTOC, resulting in no virus production (5). When the R50A full-length proviral construct was cotransfected with pGag, capsid assembly and virus budding was restored and titer was about 11% of WT (Table 2). In order to compare virus titers, we used this R50A PFV that was released from cells cotransfected with pGag as a control since the Gag-Pol fusions are deficient in assembling normal VLPs (Table 2). Virus titers from cells cotransfected with equimolar ratio of plasmids expressing pGag and either the mp3 G-P or Δ3’ss mp3 G-P were 9.7 x 10^3 and 4.6 x 10^3 units/ml; these are 36 and 17% of the R50A PFV cotransfected with pGag, respectively (Table 2). When molar ratios of the expression plasmids for pGag and the Δ3’ss mp3 G-P fusion were changed to increase the amounts of Gag in the transfected
cells, the titers were higher (Table 2). Cells cotransfected at a molar ratio of 20:1 (pGag:
$\Delta 3^{ss}$ mp3 G-P fusion), which should give a ratio of Gag and the Gag-Pol proteins that is
similar to the ratio found in orthoretroviruses, restored infectivity to the levels seen when
the Gag expression plasmid was cotransfected with R50A PFV. These results show that
eexpression of a free Pol was not needed for PFV infectivity and the Gag-Pol protein can
provide the enzymatic activities required for PFV replication.
DISCUSSION

FV Pol is synthesized from a spliced pol mRNA and does not contain Gag sequences. This is unlike orthoretroviruses, in which Pol is synthesized as part of a Gag-Pol protein. In orthoretroviruses, if the Gag-Pol protein is expressed by itself, it cannot assemble into virus particles. In the present study, we examined the behavior of PFV Gag and Pol in the context of a Gag-Pol protein. We found that regardless of coexpression of the spliced Pol protein, a PFV Gag-Pol protein can assemble into VLPs, although these particles have aberrant morphology and are not infectious. In the absence of normal spliced pol, the viral protease encoded in PFV Gag-Pol is active and PFV Gag-Pol coassembled with Gag leads to the production of infectious viruses. We do not know whether PFV Gag-Pol is incorporated into virions by binding to Gag as in orthoretroviruses, or by binding to RNA as does WT Pol.

When we cotransfected cells with equimolar ratio of PFV Gag and a Gag-Pol plasmid (in the absence of WT Pol), resultant viruses had 17% infectivity compared to a control virus that coexpressed R50A PFV and Gag protein (Table 2). Another laboratory also reported that coexpression of equal amounts of PFV Gag-Pol and Gag, using an PFV 4-plasmid expression system, yielded a virus with a titer 10% of WT (33). Because all of the viral enzymatic activities are required for the production of infectious particles, all three individual enzymes of Pol (PR, RT, and IN) must be active when expressed as a Gag-Pol in the absence of spliced pol. In the WT virus, PR cleaves Gag once near the C-terminus of Gag at the p3 cleavage site and Pol once at the site between RT and IN (Fig. 1A). Previously, our laboratory showed that IN needs to be cleaved from precursor Pol protein for viral replication (27). Both cleavages can occur when PR is expressed as part of a Gag-Pol protein, consistent with results published by Löchelt et al. (19).
Like orthoretroviral PRs, PFV PR is active only as a homodimer (11,18,37). This implies that PFV Pol-Pol interactions can occur within the context of the Gag-Pol protein and that these interactions give rise to protease activity.

When the Gag-Pol proteins were expressed in the absence of Gag from HIV-1 and spleen necrosis virus, the Gag-Pol proteins were efficiently processed by the viral protease (14,22,25,35). On the contrary, the Gag-Pol protein of MLV was not processed by PR if it was expressed in the absence of Gag (7). In all cases, there was no virion assembly or VLP release when orthoretroviral Gag-Pol proteins were expressed in the absence of Gag. In contrast, PFV Gag-Pol protein can assemble into VLPs with aberrant morphologies (Fig. 5). Recently Swiersy et al. (33) also reported that expression of similar PFV Gag-Pol proteins (with an inactivated protease) gave rise to aberrant intracellular structures, but the Gag-Pol proteins were unable to assemble into normal capsid structures. In reference (33), Gag-Pol proteins pelleted from the supernatants were mostly degraded by subtilisin digestion, suggesting Gag-Pol proteins did not lead to the production of fully-formed membrane-enclosed VLPs in the absence of Gag. In contrast, the Gag-Pol VLPs that we detected are subtilisin resistant. It is not clear why the results obtained in the experiments done in the two laboratories using similar PFV Gag-Pol were different. One possible explanation might be the differential expression of Gag-Pol proteins detected in the two studies. In our experiments, when Pol was expressed as a Gag-Pol, the level of Gag-Pol in the cell was significantly higher than the level of Pol seen with a WT virus. This was expected because expression of Pol from the spliced message is regulated at a transcriptional level. However, the level of Gag-Pol using an PFV 4-plasmid expression system reported by Swiersy et al. (33) was similar to the level of WT Pol, and this level may have been too low for efficient assembly.
Although PFV Gag-Pol can assemble aberrant VLPs that are released into the supernatants, the Gag-Pol mutants do not efficiently encapsidate genomic RNA into VLPs. 

Domains in the C-terminus of Gag are involved in genomic RNA packaging (17,31). The encapsidation of Pol is not prerequisite for RNA packaging (2,17). There are three GR boxes near the C-terminus of FV Gag. These motifs are characteristic of FV and thought to be functionally equivalent to the Cys-His motifs found in the NC protein of orthoretroviruses that are involved in several essential steps in the viral life cycle, including RNA packaging [reviewed in (13)]. The FV GR boxes have been shown to be involved in multiple steps of viral replication, including the encapsidation of genomic RNA, Pol packaging, reverse transcription, and viral assembly (17,20,31). Because the fusion of Gag and Pol could prevent the GR boxes from functioning in RNA encapsidation, we were surprised to find that VLPs produced from a Gag-Pol protein had peak fractions of similar densities as WT virus, although perhaps a bit lighter than WT, despite the deficiency of genomic RNA. Although RNA is a structural element in orthoretroviral particles (21), viral genomic RNA is not required. Viral particles that do not package viral RNA, package cellular mRNAs (28). We have not yet examined the presence of cellular mRNAs in PFV Gag-Pol viruses.

We found that the expression of Gag-Pol proteins that contain cleavage sites in both Gag and Pol led to the production of virions with a titer similar to WT (Table 1, G-P). This implies that PFV Gag-Pol expression alone is compatible with viral assembly and replication as long as there is appropriate processing of the fusion protein in cells. This is consistent with recent results obtained by others using similar PFV Gag-Pol constructs (33). We coexpressed Gag-Pol with Gag in the absence of Pol, varying the ratios of Gag to Gag-Pol from 20:1 (similar to that found in orthoretroviruses) to 1:1 (Table 2). In HIV-1, when the normal ratio of Gag to Gag-Pol
(around 20:1) was altered by expressing increased amounts of Gag-Pol, viral assembly was disrupted and infectivity was greatly reduced (10,14,29). In contrast to what is seen with orthoretroviruses, increasing the amounts of PFV Gag-Pol relative to Gag did not abrogate infectivity. Taken together, the data show that FV can use an orthoretroviral-like Gag-Pol expression mechanism to make infectious viruses, even though, under normal circumstances, spumaretroviruses have evolved to use a different mechanism for Pol synthesis and assembly. Wild type FV reverse transcribes RNA during or after viral assembly and budding, leading to infectious DNA virions. It will be of interest to determine whether FV RT expressed in a Gag-Pol context is also active at this stage.

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Table 1. Infectivity of extracellular viruses in the supernatants from cells transfected with Gag-Pol proviruses

<table>
<thead>
<tr>
<th>Construct</th>
<th>Changes from WT</th>
<th>Presence of spliced Pol</th>
<th>Infectivity (unit/ml)*</th>
<th>Relative infectivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>Yes</td>
<td>(2.5 ± 0.5) · 10⁵</td>
<td>100</td>
</tr>
<tr>
<td>ΔEnv</td>
<td>no Env</td>
<td>Yes</td>
<td>&lt; 10</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td>G-P</td>
<td>Gag-Pol fusion</td>
<td>Yes</td>
<td>(2.2 ± 0.9) · 10⁵</td>
<td>91</td>
</tr>
<tr>
<td>ΔGR3 G-P</td>
<td>Gag-Pol fusion lacking GR box 3</td>
<td>Yes</td>
<td>(2.3 ± 0.5) · 10⁴</td>
<td>9</td>
</tr>
<tr>
<td>mp3 G-P</td>
<td>Gag-Pol fusion lacking p3 cleavage site</td>
<td>Yes</td>
<td>&lt; 10</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td>ΔGR3/mp3 G-P</td>
<td>Gag-Pol fusion lacking both GR box 3 and p3 cleavage site</td>
<td>Yes</td>
<td>&lt; 10</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td>Δ3’ss mp3 G-P</td>
<td>Gag-Pol fusion lacking p3 cleavage site and 3’s splice site for pol mRNA</td>
<td>No</td>
<td>&lt; 10</td>
<td>&lt; 0.004</td>
</tr>
</tbody>
</table>

* Infectivity was measured by the FAB assay as described in (39). The numbers are the average ± standard deviation of four independent assays.
Table 2. Infectivity of viruses produced from cells cotransfected with Gag-Pol proviruses and a Gag expression vector (pGag)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Infectivity (unit/ml)*</th>
<th>Relative infectivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>(2.4 ± 0.5) · 10^5</td>
<td>885</td>
</tr>
<tr>
<td>pGag</td>
<td>&lt; 10</td>
<td>&lt; 0.04</td>
</tr>
<tr>
<td>R50A PFV</td>
<td>&lt; 10</td>
<td>&lt; 0.04</td>
</tr>
<tr>
<td>mp3 G-P</td>
<td>&lt; 10</td>
<td>&lt; 0.04</td>
</tr>
<tr>
<td>∆3’ss mp3 G-P</td>
<td>&lt; 10</td>
<td>&lt; 0.04</td>
</tr>
<tr>
<td>pGag : R50A PFV = 1:1</td>
<td>(2.7 ± 0.7) · 10^4</td>
<td>100</td>
</tr>
<tr>
<td>pGag : mp3 G-P = 1:1</td>
<td>(9.7 ± 2.2) · 10^3</td>
<td>36</td>
</tr>
<tr>
<td>pGag : ∆3’ss mp3 G-P = 1:1</td>
<td>(4.6 ± 0.5) · 10^3</td>
<td>17</td>
</tr>
<tr>
<td>pGag : ∆3’ss mp3 G-P = 5:1</td>
<td>(1.8 ± 1.1) · 10^4</td>
<td>65</td>
</tr>
<tr>
<td>pGag : ∆3’ss mp3 G-P = 10:1</td>
<td>(2.6 ± 0.9) · 10^4</td>
<td>97</td>
</tr>
<tr>
<td>pGag : ∆3’ss mp3 G-P = 20:1</td>
<td>(3.4 ± 1.2) · 10^4</td>
<td>126</td>
</tr>
</tbody>
</table>

* Infectivity was measured by the FAB assay as described in (39).

The numbers are the average ± standard deviation of three independent assays.
Figure 1. Construction of PFV Gag-Pol fusion proteins. (A) Diagrams of WT Gag and Pol proteins and the Gag-Pol fusion protein. Both the 5' and 3' splice sites (ss) for pol mRNA are indicated. The three glycine/arginine-rich (GR) boxes are indicated by small black boxes near the C-terminus of Gag. The WT Pol protein is translated in the +1 reading frame from relative to Gag. ATG indicates the start codon for each open reading frame. A deletion of 1 nt brings Gag and Pol into the same reading frame to generate a Gag-Pol fusion protein. The proteolytic cleavage sites for viral protease are indicated by arrows and dotted lines. PR, protease; RT, reverse transcriptase; IN, integrase. (B) The amino acid sequences at the fusion junctions of the Gag-Pol fusion proteins. Amino acids are shown by single-letter codes. The recognition sequences for the p3 cleavage site at the C-terminus of Gag are shown in boxes; WT sequences are in gray-filled box and the mutated sequences are in unfilled box. The coding sequences for Pol proteins are underlined.

Figure 2. Analyses of intracellular and extracellular viral proteins by Western blot analyses. 45-48 h after the transfection of 293T cells with expression constructs for either WT or mutant viruses, cell lysates and viral supernatants were collected. Viral pellets were digested with subtilisin before fractionated on 9% SDS-polyacrylamide gels. The proteins were transferred to a membrane and the membrane was probed with anti-Gag antibody (A), anti-Pol antibody (B), or anti-IN antibody (C). The cellular protein Gapdh was used as a loading control (data not shown). PrPol,
precursor Pol (PR-RT-IN); Pol, cleaved Pol (PR-RT). Molecular weight markers (MWM) are shown in kilodaltons.

Figure 3. Analyses of intracellular RNA and expression and incorporation of viral proteins into virions of the Δ3’ss mp3 G-P mutant. 45-48 h after the transfection of 293T cells with expression constructs for either WT or mutant viruses, (A) Total cellular RNA was extracted, reverse transcribed, and PCR amplified using a pair of specific primers for an unspliced genomic RNA and a spliced pol mRNA. gapdh mRNA was used as an internal standard. Cells and viral supernatants were collected from transfected cells and prepared for Western blot analyses. Viral pellets were resuspended in PBS and divided into two aliquots. One aliquot was digested with subtilisin before loading on 9% SDS-polyacrylamide gels and the other aliquot without subtilisin digestion ran on the gel. The blot membranes were probed with anti-Gag antibody (B), anti-Pol antibody (C), anti-IN antibody (D), anti-SU antibody (E), or anti-LP antibody (F). The cellular protein Gapdh was used as a loading control (data not shown). For Western blot analyses (B-D), a half amount of WT viral supernatants compared to other mutants were used to prepare samples with or without subtilisin digestion. PrPol, precursor Pol (PR-RT-IN); Pol, cleaved Pol (PR-RT). DNA ladders (in base pairs) and molecular weight markers (MWM) (in kilodaltons) are shown.

Figure 4. Immunofluorescent microscopy of the cells transfected with the PFV Gag-Pol constructs. 293T cells were transfected with WT (A), the mp3 G-P (B), or the
Δ3’ss mp3 G-P constructs (C) and co-stained with rabbit polyclonal anti-Gag antibody (green) and mouse monoclonal anti-Pol antibody (red). Overlapped images for each set of panels are shown in ‘Merge’ box. The scale bars are shown in the right corner of each merged image. All images were captured using a Nikon E800 microscope.

Figure 5. Electron microscopic analyses of cells transfected with the PFV Gag-Pol construct. 293T cells were transfected with constructs expressing WT or the mp3 G-P Gag-Pol protein. The images are presented in pairs. Each image in the right column shows a close up of the electron dense materials seen in the images shown in the left column. The WT images are in the top 4 images (A-D), and the mp3 G-P images are in the lower 4 images (E-H) are indicated. The nuclei are indicated as N. The scale bars in µm are shown in the left corner of each image. Digital images were obtained with a JOEL 1230 electron microscope.

Figure 6. Particle density analyses of virions released from transfected cells expressing the PFV Gag-Pol. 293T cells were transfected with constructs expressing WT or the mp3 G-P. Viral samples were applied to an isopycnic gradient and subjected to ultracentrifugation. A total of 14 fractions were collected from the top of each sample gradient. The numbers in the table below the X-axis of the graph (A) show densities of each fraction in both gradients in g/ml. The viral proteins from each fraction were precipitated with 10% TCA, and fractionated on a 9% SDS-polyacrylamide gel. The proteins were transferred to a membrane and the Western
blot was done with anti-Gag antibody to detect the WT protein (B) or anti-Pol antibody to detect the mp3 G-P protein (C). (D) RT-PCR was performed to measure the levels of genomic RNA packaged into virions. Molecular weight markers (MWM) are shown in kilodaltons. DNA ladders in base pairs are indicated.

Figure 7. Expression and packaging of Gag and Pol proteins from cells cotransfected with the Gag-Pol construct and the pGag plasmid. Virus pellets were digested with subtilisin before loading onto 9% SDS-polyacrylamide gels. Viral proteins were visualized by Western blot analyses using anti-Gag antibody (A) or anti-Pol antibody (B). PrPol, precursor Pol (PR-RT-IN); Pol, cleaved Pol (PR-RT); Gag-Pol, Gag-PR-RT-IN and Gag-PR-RT. Molecular weight markers (MWM) are shown in kilodaltons.