Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein

Xiaoyun Wu¹, Hongmei Liu¹,
Hongling Xiao¹, Joan A. Conway¹,
Eric Hunter² and John C. Kappes^{1,2,3,4}

Departments of ¹Medicine and ²Microbiology, University of Alabama at Birmingham, 701 South 19th Street, LHRB 639, Birmingham, AL 35294 and ³Birmingham Veterans Administration Medical Center, Research Service, Birmingham, AL 35233, USA

⁴Corresponding author
e-mail: hema021@uabdp.dpo.uab.edu

The expression and incorporation of retroviral enzymes into virions in the form of Gag/Pol precursor polypeptides is believed to be important for the assembly of infectious viral particles. HIV-1 encodes a 160 kDa Gag/Pol precursor that includes Gag, protease (PR), reverse transcriptase (RT) and integrase (IN). We have developed the use of HIV accessory proteins (Vpr and Vpx) as vehicles to incorporate protein of both viral and non-viral origin into virions by expression *in trans* as heterologous fusion proteins (Wu *et al.*, 1995, 1996a). To analyze the role of Gag/Pol in the formation of infectious virions, we incorporated RT and IN into HIV-1 particles *in trans*, as fusion partners of viral protein R (Vpr). Virions derived from an RT and IN minus proviral clone were infectious and replicated through a complete cycle of infection when RT and IN proteins were provided *in trans*. These results demonstrate that functional RT and IN proteins can be provided *in trans*, and that their expression and incorporation into virions as components of Gag/Pol are not required for the formation of infectious virions. Thus, for the first time, we have demonstrated for a human pathogenic retrovirus that processes of assembly and the function of critical viral enzymes can be unlinked. This finding will provide unique opportunities to explore retroviral RT/IN function and the role of Gag/Pol in the formation of infectious virions in the context of a replicating virus (*in vivo*).

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Introduction

Retroviral enzymes are synthesized and assembled into virions in the form of Gag/Pol precursor polypeptides. All retroviruses encode at least four enzyme activities: protease (PR), polymerase and ribonuclease H (RNase H) activities of the reverse transcriptase (RT) and integrase (IN). The viral PR processes the Gag and Gag/Pol precursor proteins in late stages of the virus life cycle, and is required for the formation of mature infectious virions. During early stages, RT catalyzes the conversion of the viral RNA genome into double-stranded DNA, and IN integrates the

newly synthesized DNA into host cell chromosomes (for review see Goff, 1990; Katz and Skalka, 1994). Human immunodeficiency virus type 1 (HIV-1) encodes and incorporates PR, RT and IN as Pol components of a 160 kDa Gag/Pol precursor polypeptide (Pr160^{Gag/Pol}). Expression of the viral enzymes in the form of a Gag/Pol precursor polypeptide is believed to be important for the formation of infectious retroviral particles. It controls their expression in proper ratios and coordinated assembly into virions (for review, see Dickson *et al.*, 1982). The arrangement of the Gag/Pol precursor is conserved (Gag–PR–RT–IN) and probably facilitates the proper organization of the viral enzymes within the mature virus particle, which is required for their respective enzymatic functions *in vivo*. Studies on HIV-1 suggest the Pr160^{Gag/Pol} precursor protein plays important roles in the morphogenesis of infectious virus. Deletion and site-specific mutations in the RT or IN coding regions may cause defects in virion assembly, production, maturation and composition (Shin *et al.*, 1994; Ansari-Lari *et al.*, 1995; Engelman *et al.*, 1995; Ansari-Lari and Gibbs, 1996; Bukovsky and Göttinger, 1996). However, it has also been reported that similar mutations do not cause apparent abnormalities (Gallay *et al.*, 1995; Masuda *et al.*, 1995; Wiskerchen and Muesing, 1995; Leavitt *et al.*, 1996).

Retroviruses are characterized by the ability to convert their diploid, single-stranded viral RNA genome into a double-stranded DNA copy. This process of reverse transcription is a critical step in the life cycle of all retroviruses. It is catalyzed by an RNA-dependent DNA polymerase (RT), and completed in the context of a nucleoprotein complex after the virus core enters into the host cell. While the initial phases of reverse transcription can be achieved *in vitro* (independently of the virus and host cell) with recombinant RT, template and primer, reverse transcription is more complex in the infected cell (Varmus and Swanstrom, 1982). Complete synthesis of HIV-1 DNA requires viral core components such as nucleocapsid protein (NC), dimeric viral RNA and primer tRNA (for review, see Goff, 1990; Katz and Skalka, 1994). Moreover, the structural milieu provided by the capsid and nucleoprotein complex is also necessary for correct DNA synthesis (Coffin, 1990). Other factors inherent to the formation of the virus particle are also thought to affect reverse transcription. Incorporation of primer tRNA₃^{Lys} is essential, and it is believed that RT and possibly NC as components of the Pr160^{Gag/Pol} precursor mediate selective packaging and positioning of the primer tRNA on the RNA template (Mak *et al.*, 1994; Marquet *et al.*, 1995). The demonstration that the expression of enzymatically active RT in mammalian cells is not sufficient to support the DNA synthesis of RT-defective virus (Ansari-Lari and Gibbs, 1996) suggests that incorporation of RT into virions is necessary for its function, and that Gag/Pol facilitates

the proper association of RT with the ribonucleoprotein initiation complex. A logical conclusion from these results is that Gag/Pol is necessary for both virus enzyme function *in vivo* and the formation of an infectious viral core/ribonucleoprotein structure.

Since RT and IN are part of the Gag/Pol precursor, and therefore the assembling capsid structure, mutations in these domains may have pleiotropic effects and cause defects in virus replication at different steps in the life cycle. As a result, a detailed molecular analysis on the enzymatic functions of RT and IN, independently of Gag/Pol and its role in virion morphogenesis, has not been possible in the context of a replicating virus. Mutations within the RT and IN domains of Gag/Pol that affect assembly may also interfere with RT/IN function. Similarly, mutations that affect enzyme function may disturb normal virion morphogenesis. Therefore, our understanding of how infectious HIV-1 particles are formed remains obscure. Uncoupling virion morphogenesis and enzyme functions would provide a powerful means for exploring RT and IN function as they occur within the infected cell and the viral nucleoprotein-preintegration complex.

Our laboratory has developed the use of HIV accessory proteins as vehicles to deliver proteins of both viral and non-viral origin into the virus particle by their expression as heterologous fusion protein *in trans* (Wu *et al.*, 1995, 1996a,b). We have shown that Vpr- and Vpx-staphylococcal nuclease (SN) and chloramphenicol acetyl transferase (CAT) fusion proteins can be incorporated into HIV virions and retain their enzymatic activity (Wu *et al.*, 1995). We have also expressed HIV PR in mammalian cells as a fusion partner of both Vpr and Vpx and demonstrated that the fusion proteins possessed PR-specific enzyme activity and incorporated into HIV virions (Wu *et al.*, 1996a,b). In this report we incorporate RT and IN into HIV-1 virions as fusion partners of Vpr and explore whether the expression of RT and IN as components of Pr160^{Gag/Pol} is required for the formation of infectious virions. Our results show that Vpr, RT and IN fusion proteins are incorporated into HIV-1 particles, processed by the viral protease and support virus replication through a single round of infection. Thus, our data indicate that neither the Pr160^{Gag/Pol} precursor protein nor the expression of RT and IN as components of Gag/Pol are required for the formation of infectious virus particles, viral DNA synthesis, integration of the provirus and the production of progeny virions. These results show for the first time that the processes of retroviral assembly and the function of critical viral enzymes can be unlinked. This finding will provide a unique approach for answering complex questions about the HIV replication cycle.

Results

Incorporation of Vpr-RT fusion protein into HIV-1 particles

To express the HIV-1 Vpr and RT proteins as a single translation product, their coding regions were PCR amplified from the pSG3 proviral clone (Ghosh *et al.*, 1993), ligated in-frame and placed under control of the HIV-2 LTR and RRE in the pLR2P expression vector (Wu *et al.*, 1995), generating pLR2P-vprRT (Figure 1A). Thirty three

bp of PR sequence were included at the *vpr-RT* junction to preserve the natural N-terminal protease cleavage site of RT. The expression and incorporation of the Vpr-RT fusion protein into HIV-1 particles was assessed by separately cotransfecting pLR2P-vprRT into 293T cells with two different HIV-1 mutant proviral clones. One of the mutants, pSG3^{ΔRT}, contains an in-frame 539 amino acid residue deletion that encompasses most of RT. The other mutant, pSG3^{S-RT}, contains translational stop codons at the first amino acid positions of both RT and IN (Figure 1A). Progeny virions that were derived from the transfected cells were shown by immunoblot analysis to contain a protein of approximately 81 kDa that was reactive with anti-RT and anti-Vpr antisera (Figure 1B). A molecular mass of 81 kDa is consistent with the combined masses of RT (66 kDa) and Vpr (15 kDa). Proteins of 66 and 51 kDa were also detected with anti-RT antibodies in SG3^{ΔRT}- and SG3^{S-RT}-complemented virions, indicating that the Vpr-RT fusion protein was incorporated and processed by the viral protease. An anti-Vpr reactive 66 kDa protein was also detected and probably represents Vpr-RT^{p51}, a partial cleavage product of the full length fusion protein. As a control, an expression vector containing a translational stop codon at the beginning of RT (pLR2P-vpr^{SRT}, Figure 1A) was cotransfected with SG3^{ΔRT} and SG3^{S-RT}, respectively. Progeny virions contained an anti-Vpr reactive protein (denoted Vpr*) migrating at ~16 kDa (Figure 1B), consistent with the Vpr component of the fusion protein plus the 11 PR amino acid residues which comprise part of the PR cleavage site at the N-terminus of RT. Analysis of the SG3^{S-RT} and SG3^{ΔRT} mutant viruses for Gag protein revealed increased amounts of unprocessed and partially processed p55 and p39. Incomplete processing of Gag appeared to be more profound for the SG3^{ΔRT} virus. Consistent with published reports (Ansari-Lari and Gibbs, 1996), the SG3^{ΔRT} virions also contained significantly reduced amounts of IN protein (data not shown), suggesting a defect in Gag/Pol expression and virion assembly.

Analysis of Vpr-RT-complemented virions

The incorporation of Vpr-RT into SG3^{S-RT} mutant virions was confirmed by buoyant density centrifugation. Peak concentrations of virions were shown to sediment at a density of 1.16–1.17 (fractions 7 and 8) and the corresponding fractions contained peak amounts of the Vpr-RT fusion protein (Figure 2). Analysis of fractions for enzyme activity revealed that the SG3^{S-RT} virions contained enzymatically active RT protein, and activity levels paralleled concentrations of virion-associated Vpr-RT fusion protein. Similar to data shown in Figure 1, both full-length and processed forms of Vpr-RT (p66, p51 and Vpr*) were detected, providing further evidence for protease cleavage subsequent to incorporation into virions. Taken together, the above results indicate that Vpr-RT can be incorporated into HIV-1 particles and processed by the viral protease to form enzymatically active RT (p66/p51).

To test whether Vpr-RT-complemented virions were infectious, they were analyzed by the MAGI assay. The MAGI assay provides a sensitive detection method for HIV-1 infection. It is based on the activation of an integrated LTR-β-galactosidase gene in HeLa CD4-LTR/

Fig. 1. Virion incorporation and proteolytic processing of Vpr-RT fusion protein. (A) The 5' half of the pSG3^{wt} proviral clone, including restriction enzyme sites used for its construction, is illustrated. The pSG3^{ΔRT} clone contains an in-frame 1617 bp deletion (539 amino acid residues) within the RT region. The pSG3^{S-RT} clone contains TAA stop codons at the first amino acid positions of RT and IN, and abrogates translation of the RT-IN domain of Gag/Pol. The Vpr-RT expression plasmid, pLR2P-vprRT, fuses the *vpr* and *RT* coding regions in-frame and preserved the natural protease cleavage site at the N-terminus of RT (PC). The pLR2P-vpr^SRT plasmid is identical to pLR2P-vprRT except that it contains a translational stop codon (TAA) at the first amino acid residue position of *RT*. (B) 4 μg of pSG3^{ΔRT} and pSG3^{S-RT} were separately cotransfected into 293T cells with 2 μg of pLR2P-vprRT, pLR2P-vpr^SRT and pLR2P (vector alone) respectively, by the calcium phosphate DNA precipitation method according to the manufacturer's instructions (Stratagene). 293T cells were also transfected with pSG3^{wt} alone. 48 h later, extracellular virus was concentrated from culture supernatants by ultracentrifugation, lysed and examined by immunoblot analysis using anti-RT (top), anti-Vpr (middle) and anti-Gag (bottom) antibodies as probes.

β-gal cells by Tat protein that is expressed subsequent to HIV-1 infection (Kimpton and Emerman, 1992). It should be noted that β-gal activation requires only synthesis and transcription of nascent viral DNA and not integration of the provirus (Ansari-Lari *et al.*, 1995; Engelman *et al.*, 1995; Wiskerchen and Muesing, 1995). However, virus containing mutations in the catalytic center of IN does produce 5- to 10-fold fewer infection-positive cells compared with wild-type virus. In five independent experiments, SG3^{S-RT}-complemented virions were found to be infection-positive by the MAGI assay to levels between 2 and 4% of that of wild-type virus, while SG3^{ΔRT}-complemented virions were not positive above background (Figure 3). Both the SG3^{S-RT}- and SG3^{ΔRT}-complemented virions exhibited levels of RT activity that were ~7-fold greater than wild-type virus. As a control, the infectivity of SG3^{wt} virus complemented with Vpr-RT was analyzed and found to be similar to that of non-complemented SG3^{wt} virus. Thus, Vpr-RT does not inhibit virus infectivity in a dominant-negative manner. These results suggested that the RT-IN-deficient virions (SG3^{S-RT}) complemented with Vpr-RT are infectious and synthesize viral DNA in infected cells, albeit inefficiently. Several possibilities were considered to explain why complementation was so inefficient. First, it may not be possible to provide functional RT effectively by its incorporation as a fusion

partner of Vpr. Second, IN (either as the C-terminal domain of Gag/Pol or as a mature protein) may be necessary for reverse transcription in infected cells, and therefore mutant viruses complemented with RT may not be able to effectively support DNA synthesis (SG3^{ΔRT} incorporates significantly reduced amounts of IN). Third, the mutant viruses may be defective in assembly and/or maturation in ways that do not support viral DNA synthesis.

Incorporation of Vpr-RT and Vpr-IN restores virus infectivity

To examine whether incorporating IN *in trans* together with RT would augment the infectivity of SG3^{S-RT} mutant virions, pSG3^{S-RT} proviral DNA was transfected into 293T cells with both the Vpr-RT and Vpr-IN expression vectors (triple transfection, Vpr-RT/IN). As controls, pSG3^{wt} was transfected with the Vpr-RT and Vpr-IN expression vectors, and with the pLR2P vector alone. Wild-type and SG3^{S-RT} mutant progeny virions efficiently incorporated and processed both Vpr-RT and Vpr-IN fusion proteins (Figure 4A). Greater amounts of liberated RT and IN were detected in complemented virions as compared with non-complemented wild-type SG3^{wt} virions. The complemented mutant virions were found to be infectious, as determined by the MAGI assay. In five independent

Fig. 2. Analysis of *trans*-complemented HIV-1 particles for Vpr-RT fusion protein. **(A)** 293T cells were cotransfected with pSG3^{S-RT} and pLR2P-vprRT. 48 h after transfection, extracellular virus was pelleted by ultracentrifugation (125 000 g, 2 h) through cushions of 20% sucrose, resuspended in TNE, layered over 20–60% linear gradients of sucrose and centrifuged for 18 h at 100 000 g. Sucrose fractions (0.8 ml) were collected and divided into two aliquots. One aliquot set was diluted in PBS and subjected to ultracentrifugation (125 000 g, 2 h). Virus pellets were lysed and examined by immunoblot analysis using anti-RT (top), anti-Vpr (middle) and anti-Gag (bottom) antibodies as probes. **(B)** The other aliquot set of sucrose fractions was analyzed for density with a refractometer and RT enzyme activity using previously described methods (Dubay *et al.*, 1992).

experiments, the infectivity of the complemented mutant virions was 13–19% of that of SG3^{wt} (data not shown). *Trans*-complemented wild-type virions retained a level of infectivity near (>95%) that of non-complemented wild-type virus (data not shown). SG3^{ΔRT} virions derived from transfection with both the Vpr-RT and Vpr-IN expression vectors remained non-infectious (data not shown).

The above results suggested that the incorporation of the IN protein together with RT was required to support the infectivity of RT-IN mutant virions. Therefore, to optimize the condition for the incorporation of similar relative amounts of both fusion proteins (Vpr-RT and Vpr-IN), different concentrations of the Vpr-RT and Vpr-IN expression vectors were transfected into 293T cells with constant amounts of pSG3^{S-RT} (4 μg). Figure 4B shows that as increasing concentrations of the Vpr-RT/IN expression vectors were transfected, progeny virions were produced that contained greater relative amounts (standardized by p24 antigen concentration) of the *trans*-RT and IN proteins. Virion-associated RT protein was assessed by immunoblot analysis (data not shown) and

Fig. 3. Infectivity of Vpr-RT-complemented viruses. 293T cells were transfected with pSG3^{wt} and cotransfected with pLR2P-vprRT plus pSG3^{S-RT}, pSG3^{ΔRT} and pSG3^{wt}, respectively. 48 h after transfection, supernatants were collected, filtered through 0.45 μm filters, equilibrated for p24 antigen [viral capsid (CA) protein] concentration and analyzed for RT enzyme activity (hashed bars) and infectivity (shaded bars) by the MAGI assay. The results represent the means of three independent experiments.

for enzyme activity (Figure 4B). At the higher concentrations, RT activity exceeded that of wild-type by ~7-fold. Under conditions which optimized the incorporation of both RT and IN the maximum level of infectivity reached 21% of that of wild-type virus. These results, for the first time, demonstrate that virions derived from RT-IN minus proviral DNA are infectious when both RT and IN are provided *in trans*. This result may also suggest that either a significant fraction of the SG3^{S-RT} virions were defective, or that RT and IN can be provided with only limited effectiveness *in trans*.

Efficient rescue of RT-IN minus provirus by complementation with Vpr-RT-IN fusion protein

Among all retroviruses RT-IN is synthesized as a single translation product. In the case of avian viruses, the proteolytic processing of the RT-IN precursor protein is quite different from other retroviruses. RT-IN is not processed by the viral protease to completion, approximately half remains intact as the β-subunit of RT while half is cleaved to form the smaller α-subunit, and a separate IN protein (Katz and Skalka, 1994). The mature RT is thus an α-β heterodimer with the larger subunit retaining an extra C-terminal copy of the IN domain. Thus, we considered that the relationship of RT-IN expression could be important and play a role in the formation of infectious virions. To explore this possibility, we tested whether the expression of RT and IN as a contiguous fusion protein would lead to an increase in the infectivity of complemented virions. The pLR2P-vprRT-IN expression vector was cotransfected into 293T cells with pSG3^{S-RT} and pSG3^{wt} respectively. The Vpr-RT-IN fusion protein was expressed, incorporated into virions, and processed by the viral protease nearly to completion (Figure 5A). Vpr-RT-IN-complemented SG3^{S-RT} virus generated 1521 positive cells (per five p24 ng equivalents) in the MAGI assay, compared with 2142 generated by infection with the same amount of SG3^{wt} virus (Figure 5B). In five independent experiments, the infectivity of complemented virions was significantly higher than before (~3- to 4-fold), and ranged

Fig. 4. *Trans*-complementation of RT-IN-defective HIV-1 with Vpr-IN and Vpr-RT fusion proteins. (A) 4 μ g of pSG3^{wt} and pSG3^{S-RT} were separately cotransfected into 293T cells with pLR2P (vector control), and transfected in combination with 2 μ g each of pLR2P-vprRT and pLR2P-vprIN, respectively (Vpr-RT/IN, triple transfection). 48 h later, extracellular virus was concentrated from culture supernatants, lysed and analyzed by immunoblot analysis using equal amounts of p24 antigen-equilibrated virus. Replica blots were probed with anti-RT (top), anti-IN (middle) and anti-Gag (bottom) antibodies. (B) Increasing DNA concentrations of both pLR2P-vprRT and pLR2P-vprIN (ranging from 0.1 to 3.2 μ g) were transfected into 293T cells together with constant amounts of pSG3^{S-RT} (4 μ g). 48 h later, culture supernatants were collected, filtered through 0.45 μ m filters and analyzed for HIV-1 p24 antigen concentration. Samples were equilibrated for p24 antigen and aliquoted. One aliquot set was ultracentrifuged to pellet virions and examined by immunoblot analysis using anti-IN antibodies. The other set of aliquots was analyzed for RT activity (hashed bars) and by the MAGI assay (darkened bars). Results are expressed as a percentage relative to an equal amount of wild-type SG3^{wt} virus.

Fig. 5. Efficient rescue of RT-IN-defective HIV-1 by *trans*-complementation with Vpr-RT-IN fusion proteins. (A) 4 μ g of pSG3^{wt} and pSG3^{S-RT} were separately cotransfected into 293T cells with 2 μ g of pLR2P-vprRT-IN, pLR2P-vpr^SRT-IN and pLR2P, respectively. 48 h later, culture supernatants were collected, clarified by filtration through 0.45 μ m filters, equilibrated for HIV-1 CA protein (by p24 antigen ELISA) and aliquoted. One aliquot set was subjected to ultracentrifugation (125 000 g, 2 h) over cushions of 20% sucrose, lysed and examined by immunoblot analysis. Replica blots were probed with anti-RT (top), anti-IN (middle) and anti-Gag (bottom) antibodies. (B) Five p24 ng equivalents of wild-type SG3^{wt} virus (top), mutant SG3^{S-RT} virus complemented with Vpr-RT-IN (middle) and Vpr^SRT-IN (bottom) were analyzed by the MAGI assay. After staining with X-gal, monolayer cultures were photographed using a phase contrast microscope. (C) Increasing amounts of both pLR2P-vprRT-IN DNA (ranging from 0.025 to 3.2 μ g) were cotransfected into 293T cells with constant amounts pSG3^{S-RT} (4 μ g). 48 h later, culture supernatants were collected, clarified by filtration through 0.45 μ m filters and analyzed by HIV-1 p24 ELISA. Samples were equilibrated for p24 antigen (CA protein) and aliquoted. One set of aliquots was ultracentrifuged to pellet virions and examined by immunoblot analysis using anti-RT and anti-IN antibodies. The other aliquot set was analyzed for RT activity (hashed bars) and for infectivity by the MAGI assay (darkened bars). MAGI assay results are expressed as infectivity relative to wild-type SG3^{wt} virus that was included for control. RT activity results are expressed as a percentage relative to an equal amount of SG3^{wt} virus.

Fig. 6. Replication of Vpr-RT-IN-complemented virus through a complete cycle of infection. (A) HeLa CD4-LTR/ β -gal indicator cells were infected with transfection-derived wild-type SG3^{wt} virus (squares), SG3^{S-RT} virus complemented with Vpr-RT-IN (triangles) and Vpr^SRT-IN (circles) respectively, washed and cultured in DMEM. At 2 h (0 days), and 1, 2 and 4 days post-infection, 250 μ l samples of the supernatants were collected from each culture. Samples were diluted to 1 ml in PBS and subjected to ultracentrifugation at 125 000 g for 50 min in a Beckman TL-45 rotor. Pellets were resuspended in 0.4 ml of PBS containing 0.5% Triton X-100 and analyzed by HIV-1 p24 antigen ELISA. (B) 4 days after infection of the HeLa CD4-LTR/ β -gal indicator cells, 200 μ l of the culture supernatants (500 pg equivalents of p24 antigen) were analyzed for infectivity by the MAGI assay. Monolayer cultures were stained with X-gal and photographed. (C) PHA-stimulated PBMC (1×10^6) were infected overnight at 37°C with 100 ng p24 antigen equivalents (derived by transfection) of wild-type SG3^{wt} virus (filled squares), SG3^{S-RT} virus complemented with Vpr-RT-IN (filled triangles), SG3^{S-RT} virus (open squares) and SG3^{D116A} IN mutant virus (open circles). Cells were then washed 5 times by centrifugation, and on days 1, 3, 5, 7 and 9 post-infection, 250 μ l samples of the culture supernatants were collected, diluted to 1 ml in PBS and subjected to ultracentrifugation at 125 000 g for 50 min in a Beckman TL-45 rotor. Pellets were resuspended in 0.4 ml of PBS containing 0.5% Triton X-100 and analyzed by HIV-1 p24 antigen ELISA. (D) On days 5 and 9 post-infection of the PBMC cultures, supernatants were collected, filtered through 0.45 μ m sterile filters and analyzed by HIV-1 p24 antigen ELISA. 500 pg equivalents of the wild-type SG3^{wt} (hashed bars) and Vpr-RT-IN complemented SG3^{S-RT} viruses (filled bars) were analyzed for infectivity by the MAGI assay.

from 65 to 79% compared with SG3^{wt} virus. The infectivity of Vpr-RT-IN-complemented SG3^{wt} virus was similar to that of non-complemented wild-type virus (data not shown). To control for the generation of infectious virus through mutations that could have been generated during reverse transcription, mutant SG3^{S-RT} virions derived by cotransfection with the pLR2P-vpr^SRT-IN vector were also analyzed. No infectious virus was detected (Figure 5B).

This experiment was repeated using different concentrations of the Vpr-RT-IN expression vector (ranging from 0.025 to 3.2 μ g). Analysis of equal amounts of transfection-derived progeny virions for infectivity indicated a dose-response effect (Figure 5C). The amount of virion-associated Vpr-RT-IN (and processed RT and IN) paralleled virus infectivity. Under optimal conditions, infectivity was restored to a level of 85% of that of wild-type virus. It is noteworthy that at lower concentrations of Vpr-RT-IN (0.1–0.4), the relationship of RT activity to virus infectivity more closely resembled that of wild-type virus than when Vpr-RT and Vpr-IN were provided separately (compare Figures 4 and 5). At the higher concentrations, increased

RT activity did not correlate with further increases in virus infectivity.

Since formation of the provirus is not required for a positive MAGI result, the restoration of virus infectivity by *trans* complementation indicated at least the synthesis of full-length double-stranded viral DNA and the expression of viral genes products such as Tat. To further assess the replication competence of *trans*-complemented virions, supernatants of the MAGI assay cultures were monitored for progeny virions. Increasing concentrations of pelletable p24 antigen were detected from cultures infected with Vpr-RT-IN-complemented and wild-type viruses over a 4 day period (Figure 6A). Virions in the day four culture supernatants (500 pg equivalents of p24 antigen) were analyzed for infectivity by the MAGI assay. Importantly, only the wild-type virus was found to be infectious, confirming that the restoration of virus infectivity by complementation was not the result of recombination (Figure 6B).

Primary cultures of peripheral blood mononuclear cells (PBMCs) were used to assess further the infectivity and replication competence of complemented virions. Virions

Table I. Analysis of Vpr-RT-IN-complemented HIV-1 particles

Virus	Vpr-RT-IN	MAGI (%wt)	MAGI (+AZT)	p24 antigen	RT activity	RT/p24
SG3 ^{wt}	–	2163 ^a (100)	0	2030 ^b	2793 ^c	1.4
SG3 ^{wt}	+	2055 (95±5)	0	1022	6194	6.1
SG3 ^{S-RT}	+	1752 (81±6)	0	1554	9013	5.8
SG3 ^{ΔRT}	+	130 (6±3)	0	1286	9388	7.3
SG3 ^{S-IN}	+	1536 (71±7)	0	ND	ND	ND
PM3	+	0	ND	ND	ND	ND

^aNumber of infection-positive MAGI indicator cells produced by infection with 5 ng equivalents (p24 antigen) of virus derived from transfected 293T cell cultures. The values represent the mean of at least three independent MAGI assays for any given virus. Percent infectivity relative to wild-type virus is indicated in parentheses.

^bHIV-1 p24 antigen concentration (ng/ml) of virions derived from transfected 293T cell cultures and sedimented in linear gradients of sucrose at 1.16–1.17 g/ml.

^cRT activity (c.p.m. per 25 μl×10^{–3}) of virions sedimented in linear gradients of sucrose at 1.16–1.17 g/ml.

derived from RT-IN minus provirus (pSG3^{S-RT}) complemented with Vpr-RT-IN were used to infect primary cultures of PHA-stimulated normal human PBMCs. As controls, viruses derived from transfection of pSG3^{S-RT} (non-complemented), wild-type pSG3^{wt} and integration-defective pSG3^{D116A} proviral DNAs were also analyzed. The pSG3^{D116A} IN mutant contains an alanine substitution at position 116 of IN. This mutation (D116A) is within the catalytic center of IN, and abolishes integration activity (Engelman and Craigie, 1992; Cannon *et al.*, 1994). Over a 5 day period of time, substantial amounts of virions were detected in PBMC culture infected with wild-type and Vpr-RT-IN-complemented SG3^{S-RT} virus (Figure 6C). On day 3, the amount of virions produced by infection with complemented virus approached that of the wild-type. In sharp contrast, only background levels were detected in cultures infected with the non-complemented SG3^{S-RT} and the D116A IN mutant virus. Day 5 and 9 culture supernatants (500 pg equivalents of p24 antigen) were analyzed by the MAGI assay. Only the wild-type virus was found to be infectious (Figure 6D). Taken together, these results clearly show that virus particles derived from RT-IN minus proviral DNA are not severely defective and efficiently support the formation of infectious particles when RT and IN are provided *in trans*. Also, these results indicate that the expression of RT-IN as a single translation product can enhance the function of these two enzymes in the virus life cycle.

Complementation of different pol mutant viruses with Vpr-RT-IN

To explore how other mutations in Gag/Pol affect viral infectivity, different mutant viruses were complemented with Vpr-RT-IN and analyzed by the MAGI assay. RT-IN-defective pSG3^{S-RT}, RT-deleted pSG3^{ΔRT}, IN minus pSG3^{S-IN}, protease mutant PM3 (Kohl *et al.*, 1988) and wild-type pSG3^{wt} viruses were analyzed. The pSG3^{S-IN} clone contains a TAA translational stop codon at the beginning of the IN coding region, which abrogates expression of the entire IN protein. It was analyzed to test further the relationship between the synthesis and incorporation of RT-IN as a single translation product and virion infectivity. Data presented in Figures 4 and 5 indicated that RT and IN complemented the infectivity of RT-IN minus virus most effectively when provided as a contiguous fusion protein. Using SG3^{S-IN}, we tested whether this was true for virus that contained an intact

RT coding region and lacked only IN. The PM3 mutant contains only a point mutation in the PR domain of Gag/Pol, which inactivates PR enzyme activity and produces immature virus particles (Kohl *et al.*, 1988). It was selected for comparison with SG3^{ΔRT}, which is also defective in Gag processing. As reported for other RT deletion mutants of HIV-1, SG3^{ΔRT} virions assemble less of the Gag/Pol precursor protein and are defective at the level of virion assembly (Ansari-Lari and Gibbs, 1996). Each of the mutant proviral clones were separately cotransfected with pLR2P-vprRT-IN using the conditions that optimized infectivity of complemented SG3^{S-RT} virus (above). Culture supernatants were collected two days later, clarified by low-speed centrifugation and aliquoted. One set of aliquots was analyzed to confirm incorporation of the Vpr-RT-IN fusion protein (data not shown) and for p24 antigen concentration (except for the PM3 virus). Equivalent amounts of the different complemented viruses were analyzed by the MAGI assay as described above, and in the presence of 5 μM AZT. The other set of aliquots was centrifuged over 20–60% linear gradients of sucrose. Fractions were collected and analyzed for HIV-1 p24 antigen concentration and RT activity. The results of these analyses demonstrated that the ability of Vpr-RT-IN to support infectivity and DNA synthesis differs markedly among the mutant viruses (Table I). As indicated in the above experiments, incorporation of Vpr-RT-IN into wild-type SG3 virions did not substantially reduce particle infectivity, even though virions contained increased amounts of RT and IN protein. Similar to our earlier results, the infectivity of SG3^{S-RT} virus was restored to a level of 81% of that of wild-type virus. Even though the complemented SG3^{ΔRT} virions contained significant amounts of processed Vpr-RT-IN fusion protein (RT and IN), however their infectivity was rescued to a level of only 6% of that of wild-type virus. Vpr-RT-IN failed to rescue the PM3 protease mutant virus above background levels of the MAGI assay. The infectivity of complemented SG3^{S-IN} virions was restored to a level of 71% of that of wild-type. Interestingly, this was 3- to 4-fold higher than when SG3^{S-IN} virions were complemented with Vpr-IN (data not shown). This result provides additional evidence to suggest an important role for the expression and incorporation of RT-IN as a contiguous fusion protein. Complete inhibition of DNA synthesis of all Vpr-RT-IN-complemented viruses by AZT demonstrated that full-

length viral DNA synthesis was completed within infected host cells, similarly to wild-type virus (Trono, 1992).

Discussion

Our laboratory has developed the use of the HIV accessory proteins (Vpr and Vpx) as vehicles to deliver protein of both viral and non-viral origin into virions by expression in a *trans* configuration. In this report we demonstrate the incorporation of functional RT and IN proteins into HIV-1 particles by expression as fusion partners of Vpr, and the analysis of Pr160^{Gag/Pol} in the formation of infectious virions. Our results reveal, for the first time, that the expression and incorporation of RT and IN as components of a Gag/Pol precursor polyprotein is not required for the formation of infectious virions. When expressed *in trans* as fusion partners of Vpr, RT and IN are efficiently incorporated into virions, processed by the viral protease and form enzymatically active proteins that support virus replication through a complete round of infection in both CD4 positive cell lines and PBMCs. The restoration of infectivity to virions derived from RT-IN minus provirus also indicates that a truncated Gag/Pol precursor protein (Gag-Pro) is sufficient to generate mature infectious virions upon complementation. These results have significant implications for our understanding of Gag/Pol function and the assembly and maturation of retroviruses. Moreover, they reveal that it is possible to uncouple the role that the RT and IN domains of Gag/Pol play in virion morphogenesis from the functions of the mature RT and IN proteins during early stages of the virus life cycle.

It is generally believed that the scheme conserved among retroviruses for expressing and incorporating the viral enzymes supports a highly ordered subviral architecture that is essential for the morphogenesis of infectious virions. As components of the HIV-1 Gag/Pol precursor, the RT and IN domains appear to serve important roles in virion assembly and particle maturation. Both deletion and point mutations in RT and IN have been reported to alter virion production, maturation and composition (Shin *et al.*, 1994; Ansari-Lari *et al.*, 1995; Engelman *et al.*, 1995; Ansari-Lari and Gibbs, 1996; Bukovsky and Göttinger, 1996). While it is possible that such mutations may alter the structure of the Gag/Pol precursor and subsequently its function, our results also reveal that the expression of the RT-IN domain of Gag/Pol is not required for the formation of infectious HIV-1 particles when RT and IN are provided *in trans*. The ability of HIV-1 to assemble a truncated Gag/Pol precursor protein (Gag-Pro) may not seem too surprising since other retroviruses, including ALV, MMTV, MPMV and HTLV, also express and assemble a Gag-Pro precursor protein.

A critical step in the formation of infectious retroviral particles is the activation and autoprocessing of the PR from the Gag/Pol precursor. Regions upstream and downstream of PR have been shown to affect its function *in vitro* and *in vivo*. Linker insertion mutagenesis of Gag has identified regions that affect activation and autoprocessing of PR from a Gag-Pro precursor protein *in vitro* (Luban *et al.*, 1993) and viral assembly and release *in vivo* (Reicin *et al.*, 1995). Similarly, domains upstream of PR in HIV-1 Gag/Pol which influence PR autoprocessing (Zybarth and Carter, 1995) have been identified. We, and

others, have shown that deletion or site-specific mutations of regions downstream of PR can affect virus maturation by interfering with the proteolytic processing of the Pr55^{Gag} precursor protein (Ansari-Lari and Gibbs, 1996). Our results indicate that virions derived from complemented (Vpr-RT) and non-complemented RT-IN minus provirus contain increased amounts of unprocessed Gag protein. Nevertheless, the complemented virions are infectious, synthesize viral DNA, support formation of the provirus and produce progeny virions. The restoration of infectivity to a level near that of wild-type virus indicates either that most virions mature sufficiently even though they express a truncated form of the Gag/Pol precursor (Gag-Pro), or that the Vpr-RT-IN fusion protein can overcome minor defects related to incomplete proteolytic processing. These findings reveal that the RT and IN regions of Gag/Pol are not required for the maturation of the virus particle and PR activation and function. It was not unexpected that the infectivity of SG3^{ART} virions could be rescued to only 6% of the level of wild-type virus by incorporating Vpr-RT-IN *in trans*, since large deletions within the RT domain of Gag/Pol cause defects in Gag/Pol expression and virion assembly. Such complementation strategies may help to understand better how apparent defects in virion assembly and maturation affect viral infectivity.

The ability of the Vpr-RT-IN fusion protein to restore virus infectivity more efficiently than when RT and IN are provided separately may suggest an important role for RT-IN in the formation of infectious virions. This is supported by our finding that the infectivity of virions derived from IN minus provirus (SG3^{S-IN}) can be rescued to a level of 82% of that of wild-type virus by complementation with Vpr-RT-IN (Table I), compared with only 20% when complemented with Vpr-IN fusion protein (data not shown). Immunoblot analysis of virion-associated Vpr-RT-IN fusion protein indicated that it is almost completely processed by the viral protease, while as much as 30–50% of the Vpr-RT and Vpr-IN fusion proteins remained uncleaved when provided separately. Since both Vpr-RT and Vpr-RT-IN contain identical cleavage sites at the N-terminus of RT (the natural PR-RT cleavage site), these results indicate that RT-IN augments processing by the viral PR, and release of RT-IN. It is plausible that the folding of RT-IN induces a conformation in Vpr-RT-IN that facilitates its release by augmenting proteolysis at the PR-RT cleavage site. If this is correct, it may also augment the early release of RT-IN from the Gag/Pol precursor during virus maturation, and suggest the existence of a transient RT-IN intermediate that may affect the formation of infectious particles.

Recent studies have shown that viruses which contain deletions or certain point mutations within the IN domain of Gag/Pol synthesize significantly reduced amounts of viral DNA in infected cells (Masuda *et al.*, 1995; Leavitt *et al.*, 1996). This phenotype has not been recognized by *in vitro* analyses. Our results show that complementation with IN *in trans* can restore infectivity in the MAGI assay to IN mutant virions, including those which contain mutations within the N-terminal zinc-finger motif (data not shown). This result suggests that the *trans* IN can function to complement a defect in viral DNA synthesis. Since integration of the provirus is required for productive

HIV-1 infection of cultured peripheral blood lymphocytes of primary origin (Wiskerchen and Muesing, 1995), the ability of complemented virus to infect PBMCs and produce progeny virus strongly suggests that the *trans* IN protein can also catalyze the integration of newly synthesized viral DNA into host cell chromosomes. In independent studies, using a single cycle integration assay, we now have direct data to support this ascertain (Liu *et al.*, 1997).

Many questions regarding the morphogenesis of infectious HIV-1 particles and factors inherent to the virus core structure that affect reverse transcription in infected cells remain unanswered. Difficulties in manipulating components of Gag/Pol independently of the precursor form of the protein, in the context of a replicating virus, have complicated these investigations. Our results indicate that when incorporated into virions via fusion with Vpr, RT and IN are organized and function within the virion and nucleoprotein–preintegration complex similarly to when incorporated as components of a Gag/Pol precursor protein. These findings will provide new opportunities for understanding HIV assembly, enzyme function and events in the maturation pathway that lead to the formation of infectious virions.

Materials and methods

Cells, viruses and antibodies

The 293T and HeLa CD4-LTR/β-gal indicator cell lines (Kimpton and Emerman, 1992) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin and 0.1 mg/ml of streptomycin. Human PBMCs were obtained from healthy adult volunteers by ficoll-hypaque density-gradient centrifugation, stimulated with lectin and maintained in culture as described earlier (Jackson *et al.*, 1990). Anti-p24 monoclonal antibody (R1C7) is reactive with the p24^{Gag} protein of HIV-1 (Wu *et al.*, 1994, 1995). Polyclonal antibodies included anti-Vpr antiserum (ref. R413) (Wu *et al.*, 1995), anti-RT (contributed by the Division of AIDS, ref. #634) and anti-IN antisera (contributor Duane Grandgenett, #757) that were obtained from the AIDS Research and Reference Reagent Program.

HIV-1 proviral mutant clones and expression plasmids

The HIV-1 pSG3 proviral clone contains all genes typical of HIV-1 with the exception of *vpu* (Ghosh *et al.*, 1993). The pSG3 clone was modified to facilitate manipulation of the RT and IN coding regions. Using PCR, unique *SmaI* and *BamHI* restriction sites were introduced (without changing amino acid sequence) within the RT coding region at amino acid positions 15 and 554, respectively, generating pSG3^{wt} (Figure 1A). Virus derived from transfection of pSG3^{wt} exhibited replication kinetics identical to the parental SG3 virus in CD4 positive cell lines and PBMCs. The RT-deleted provirus, pSG3^{ΔRT}, was generated by ligating an *EcoRI*–*BamHI* PCR DNA product containing the 5' end of pSG3 (LTR through the first 45 bp of RT) into *EcoRI*–*BamHI*-cut pSG3^{wt}. The pSG3^{ΔRT} mutant deletes 539 amino acid residues of RT (between residues 15 and 554), and retains an open reading frame through IN. To abrogate translation of the entire IN coding region, a *BamHI*–*SalI* IN-containing DNA fragment of pSG3 was amplified by PCR using a mutagenic 5' primer (containing a TAA translational stop codon at amino acid position 1), and ligated into *BamHI*–*SalI*-cut pSG3^{wt}, generating pSG3^{S-IN}. A translational strong stop codon (TAA) was then introduced into pSG3^{S-IN} at the first amino acid position of RT by ligating an *EcoRI*–*SmaI* DNA fragment PCR amplified from pSG3 with a mutagenic 3' primer into the *EcoRI*–*SmaI* sites of pSG3^{S-IN}. This mutant clone, designated pSG3^{S-RT}, contains stop codons at the first amino acid positions of both RT and IN (Figure 1A). The HIV-1 PM3 clone contains a mutation in the active site of PR (D25N, Kohl *et al.*, 1988). All mutant clones were sequenced to confirm that only the mutation of interest was present in the final construct.

For efficient coexpression of Vpr–RT and IN fusion proteins with HIV-1 provirus, we utilized the pLR2P expression vector (Wu *et al.*,

1995, 1996a). This vector contains the HIV-2 LTR and RRE and an SV40 poly(A) signal. The HIV-2 LTR and RRE elements were chosen because they are responsive to both HIV-1 and HIV-2 Tat and Rev proteins, respectively (Wu *et al.*, 1995). For expression of RT as a fusion partner with Vpr, a *BglII*–*XhoI* RT-containing DNA fragment and a *NcoI*–*BglII* *vpr*-containing DNA fragments were amplified from pSG3^{wt} by PCR and ligated together into *NcoI*–*XhoI*-cut pLR2P vector (Wu *et al.*, 1995), generating pLR2P–vprRT. This construction placed *vpr* and RT in-frame, preserved the N-terminal protease cleavage (PC) site of RT by including 33 bp of PR sequence 5' of RT and introduced a translational stop codon at the C-terminal position of RT (Figure 1A). The control plasmid pLR2P–vpr^{SRT} was constructed by placing a translational stop codon at the first amino acid position of RT within pLR2P–vprRT. For this purpose an RT-containing *BglII*–*XhoI* DNA fragment was PCR amplified from pSG3^{S-RT} and ligated into *BglII*–*XhoI*-cut pLR2P–vprRT, generating pLR2P–vpr^{SRT}. For expression of the RT–IN domain as a fusion partner with Vpr, an IN-containing *BamHI*–*XhoI* DNA fragment of pSG3^{wt} was amplified by PCR and ligated into *BamHI*–*XhoI*-cut pLR2P–vprRT, generating pLR2P–vprRT–IN. For expression of IN as a fusion partner with Vpr, an IN-containing *BglII*–*XhoI* DNA fragment of pSG3 was amplified by PCR and ligated into *BglII*–*XhoI*-cut pLR2P–VprRT. This construction preserved the N-terminal protease cleavage (PC) site of IN by including 18 bp of RT sequence 5' of IN.

Transfections and virus purification

DNA transfections were performed on monolayer cultures of 293T cells using the calcium phosphate DNA precipitation method according to the manufacturer's recommendations (Stratagene). Unless otherwise noted, all transfections were performed with 4 μg of proviral DNA and 2 μg of the relevant pLR2P recombinant DNA. Unless otherwise noted, culture supernatants were collected 48 h after transfection, clarified by low speed centrifugation (1000 g, 10 min) and analyzed for HIV-1 p24 antigen by ELISA (Coulter Inc.) to quantify the major capsid (CA) protein. Virions were then pelleted by ultracentrifugation in a Beckman SW-41 rotor (125 000 g, 2 h) through cushions of 20% sucrose. Viral pellets were either solubilized in lysis buffer for immunoblot analysis or resuspended in TNE buffer (10 mM Tris–HCl, pH 7.2, 100 mM NaCl, 1 mM EDTA) for further purification by buoyant density sedimentation in linear (20–60%) sucrose gradients, using previously described methods (Liu *et al.*, 1995).

Analysis of virus infectivity

Virus infectivity was assessed using a HeLa CD4-LTR/β-gal indicator cell line. This cell line enables quantitative measurement of HIV-1 infection in a single cycle of infection based on activation of an integrated LTR–β-galactosidase gene in the HeLa cell line [multinuclear activation of galactosidase indicator (MAGI) assay]. Detailed methods for performing the MAGI assay have been described (Kimpton and Emerman, 1992). Briefly, supernatants from transfected 293T cell or infected MAGI assay cultures were clarified by low-speed centrifugation (1000 g, 10 min), filtered through 0.45 μm sterile filters and analyzed for HIV-1 p24 antigen content by ELISA (Coulter Inc.). Three equivalent serial 5-fold dilutions of virus (25, 5 and 1 ng p24 antigen) were incubated at 37°C on 20% confluent monolayer cultures of HeLa CD4-LTR/β-gal indicator cells seeded in 12-well plates. After 4 h, cell monolayers were washed with Hanks' Balanced Salt Solution (HBSS), and then cultured in 1 ml of DMEM containing 10% FBS. 48 h later, cells were fixed with PBS containing 1% formaldehyde and 0.2% glutaraldehyde, washed with PBS and incubated for 50 min at 37°C with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Blue-stained cells (nuclei) were counted using a light microscope. Wells containing between 50 and 250 blue cells were used to calculate the number of HIV-1 infectious units per ng of HIV-1 p24 antigen.

Western immunoblot analysis

Unless otherwise noted, virus particles were concentrated from transfected cell culture supernatants by ultracentrifugation (125 000 g for 2 h) through cushions of 20% sucrose using an SW-41 Beckman rotor. Viral pellets were solubilized in lysis buffer and analyzed by immunoblotting as described previously (Wu *et al.*, 1995).

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