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# Incorporation of Functional Human Immunodeficiency Virus Type 1 Integrase into Virions Independent of the Gag-Pol Precursor Protein

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**Retroviral integrase (IN) is expressed and incorporated into virions as part of the Gag-Pol polyprotein precursor. IN catalyzes integration of the proviral DNA into host cell chromosomes during the early stages of the virus life cycle, and as a component of Gag-Pol, it is involved in virion morphogenesis during late stages. It is unknown whether the scheme, conserved among retroviruses, for expressing and incorporating IN as a component of the Gag-Pol precursor protein is necessary for its function in the infected cell after viral entry. We have developed human immunodeficiency virus (HIV) virion-associated accessory proteins (Vpr and Vpx) as vehicles to deliver both foreign and viral proteins into the virus particle by their expression in *trans* as heterologous fusion proteins (X. Wu, et al., J. Virol. 69:3389–3398, 1995; X. Wu, et al., J. Virol. 70:3378–3384, 1996; X. Wu, et al., EMBO J. 16:5113–5122, 1977). To analyze IN function independent of its expression as a part of Gag-Pol, we expressed and incorporated IN into HIV type 1 (HIV-1) virions in *trans* as a fusion partner of Vpr (Vpr-IN). Our results demonstrate that the Vpr-IN fusion protein is efficiently incorporated into virions and then processed by the viral protease to liberate the IN protein. Virus derived from IN-minus provirus is noninfectious. However, this defect is overcome by *trans* complementation with the Vpr-IN fusion protein. Moreover, complemented virions are able to replicate through a complete cycle of infection, including formation of the provirus (integration). These results show, for the first time, that full IN function can be provided in *trans*, independent of its expression and incorporation into virions as a component of Gag-Pol. This finding also indicates that the IN domain of Gag-Pol is not required for the formation of infectious virions when IN is provided in *trans*. The ability to incorporate functional IN into retroviral particles in *trans* will provide unique opportunities to explore the function of this critical enzyme in a biologically relevant context, i.e., in infected cells as part of the nucleoprotein/preintegration complex.**

The retroviral enzymes, protease (PR), reverse transcriptase (RT), and integrase (IN), are expressed and incorporated into virions in the form of Gag-Pro and/or Gag-Pol precursor polyproteins (for a review, see reference 6). During and after assembly and budding of the virus particle from the infected cell, the precursor proteins (including the Gag precursor) are processed by PR to form a condensed core structure that contains RT and IN. After viral entry and uncoating, reverse transcription of the viral RNA occurs in the context of a subviral nucleic acid-protein complex (nucleoprotein/preintegration complex) that includes RT, IN, and other viral and cellular proteins (5, 9, 10, 15). IN then catalyzes integration of the nascent viral cDNA into the host cell's chromosomes (for reviews, see references 14 and 18). While this scheme of incorporating RT and IN into virions is conserved among retroviruses, it is unknown whether it is necessary for the formation of a nucleoprotein/preintegration complex capable of supporting viral DNA synthesis and integration.

Human immunodeficiency virus type 1 (HIV-1) encodes and incorporates PR, RT, and IN in the form of a 160-kDa Gag-Pol precursor protein ($Pr160^{Gag\text{-}Pol}$) by a ribosomal frameshift that occurs at a frequency of approximately 5 to 10% relative to that of the Gag precursor ($Pr55^{Gag}$) (16). Although expression of Gag protein alone is sufficient for the assembly of virus-like particles, Gag-Pol is absolutely required for the formation of infectious virions. The HIV-1 $Pr160^{Gag\text{-}Pol}$ precursor protein has important functions during virion morphogenesis. Gag-Pol mediates incorporation of the viral enzymes, and it is part of the assembling capsid structure and, therefore, is directly involved in the formation of infectious virions. Previous studies have shown that mutations within the C-terminal region of Gag-Pol (within the IN domain) may cause defects in virion assembly and maturation (2, 4, 8, 25). These results suggest that the C-terminal region of Gag-Pol is important for the formation of infectious particles. Mutations in *IN* may also affect the function of the mature IN protein during early stages of the virus life cycle. Therefore, such mutations are pleiotropic and may affect different steps in the virus life cycle. Consequently, detailed molecular analyses of the function of the IN protein in the context of a replicating virus have been complicated, and most of our knowledge of IN function comes from in vitro biochemical analysis of the integration reaction (3, 7, 9, 11, 22). However, in vitro conditions do not duplicate those under which IN is active in vivo, and there remains an incomplete understanding of IN function as it occurs within the infected cell, in the context of the viral nucleoprotein/preintegration complex.

We have developed the use of HIV virion-associated accessory proteins (Vpr and Vpx) as vehicles to deliver proteins of

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both viral and nonviral origin into the virus particle by their expression in *trans* as heterologous fusion proteins (29–32). We have demonstrated that the HIV protease, as well as enzymatically active staphylococcal nuclease and chloramphenicol acetyltransferase, can be incorporated into HIV virions by expression in *trans* as fusion partners of either Vpr or Vpx (30–32). Most recently, we have demonstrated that RT can support viral DNA synthesis and replication when incorporated into virions in *trans* (29). To examine whether functional IN protein could be incorporated into HIV-1 particles independent of $Pr160^{Gag\text{-}Pol}$, IN was expressed in *trans* as a fusion partner of Vpr (Vpr-IN). Our data show that the Vpr-IN fusion protein is incorporated into wild-type and IN-defective HIV-1 virions, is processed by the viral protease to liberate free IN, and supports virus replication through a complete round of infection, including integration of the provirus and the production of progeny virions from infected cells. These findings demonstrate, for the first time, that expression of the IN domain of Gag-Pol is not required for the formation of infectious particles and that functional IN can be incorporated into virions independently of Gag-Pol.

## MATERIALS AND METHODS

**Cells and antibodies.** 293T and HeLa CD4–LTR–β-gal indicator cells (19) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers, separated by Ficoll-Hypaque density gradient centrifugation, and cultured as described previously (17). The monoclonal antibody ATC1 is reactive with the $p24^{Gag}$ (capsid [CA]) protein of HIV-1 (28, 32). Polyclonal antibodies include anti-RT (contributed by the Division of AIDS, NIAID; reference no. 634), and anti-IN (contributed by Duane Grandgenett; reference no. 757) obtained from the AIDS Research and Reference Reagent Program, and anti-Vpr (R413 [32]) antisera.

**HIV-1 proviral clones and recombinant expression plasmids.** The HIV-1 pSG3 proviral clone (13) was used for production of infectious virus by transfection and for the construction of recombinant expression plasmids. $pSG3^{wt}$ was generated by modifying pSG3 (29) to facilitate mutation of the *pol* region (Fig. 1A). To abrogate translation of the entire *IN* coding region of the $pSG3^{wt}$ clone, a *Bam*HI-*Sal*I DNA fragment (nucleotides 3760 to 5332) containing the IN coding region of pSG3 was amplified by PCR with a mutagenic 5′ primer (containing a TAA translational stop codon at the first amino acid position of the IN coding region) and ligated into *Bam*HI- and *Sal*I-cut $pSG3^{wt}$, generating $pSG3^{S\text{-}IN}$ (Fig. 1A). To abolish the catalytic function of the IN protein, partially overlapping 5′ *Bam*HI-*Csp*451 (from position 3760 to 4135) and 3′ *Csp*451-*Sal*I (from position 4130 to 5332) DNA fragments encompassing the entire IN coding region were amplified from pSG3 by PCR in separate reactions. The internal mutagenic primer pairs introduced a C in place of an A at position 4123 of the pSG3 sequence, which substituted an alanine residue for the aspartic acid at position 116 (D116A). The two DNA fragments were then ligated into *Bam*HI- and *Sal*I-cut $pSG3^{wt}$, generating $pSG3^{D116A}$. The HIV-1 R7-3 clone is a derivative of the HIV-1 HXB2d isolate (24). It encodes a truncated Vpr protein and has no ATG initiation codon for *vpu* (27). $R7\text{-}3^{S\text{-}IN}$ was constructed to abrogate translation of the entire IN coding region. Partially overlapping 5′ *Pst*I-*Bam*HI (from position 1414 to 3763) and 3′ *Bam*HI-*Sal*I (from position 3758 to 5785) DNA fragments encompassing the entire IN coding region were amplified from pR7-3 by PCR in separate reactions and ligated into *Pst*I- and *Sal*I-cut R7-3. The internal mutagenic primers introduced nucleotides AA in place of TT at positions 3776 and 3777 of the R7-3 sequence, which replaced the Phe residue at position 1 of the IN coding region with a translational stop codon. Sequence analysis of $pSG3^{S\text{-}IN}$, $R7\text{-}3^{S\text{-}IN}$, and $pSG3^{D116A}$ confirmed that only the specified mutations were introduced by PCR.

To allow for the efficient coexpression of the Vpr-IN fusion protein with the HIV-1 provirus, the Vpr and IN coding regions were fused and ligated into the pLR2P long terminal repeat (LTR)–Rev-responsive element-based expression vector (31, 32). Briefly, a *Bgl*II-*Xho*I DNA fragment of pSG3 containing the IN coding region was amplified by PCR and ligated into the pLR2P-vprRT vector (29) after removal of the RT coding region by digestion with *Bgl*II and *Xho*I, generating pLR2P-vprIN (Fig. 1A). The PCR-amplified *Bgl*II-*Xho*I DNA fragment with the IN coding region contained 39 bp of RT coding sequence. This additional sequence was included to preserve the natural protease cleavage site between RT and IN. The pLR2P-IN expression vector was constructed by ligating a PCR-amplified *Nco*I-*Xho*I DNA fragment containing the IN coding region into the *Nco*I-*Xho*I polylinker sites of pLR2P. To allow for the initiation of IN protein synthesis, the amplified IN-encoding DNA fragment was designed to include a methionine and a glycine residue on the N terminus of IN.

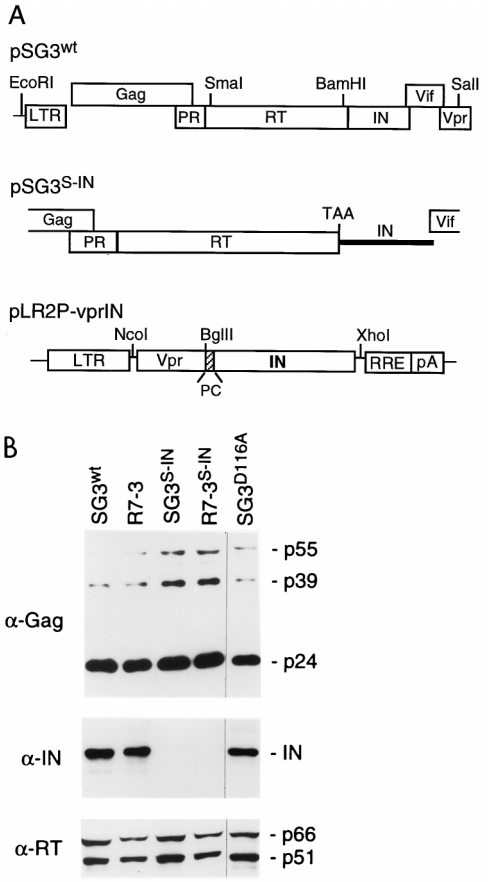


FIG. 1. Immunoblot analysis of IN-minus virions. (A) Construction of the IN-minus proviral clone and the Vpr-IN expression vector. The 5′ half of the $pSG3^{wt}$ proviral clone, including restriction enzyme sites used for its construction, is illustrated. The $pSG3^{S\text{-}IN}$ clone contains a TAA stop codon at the first amino acid position of the IN coding region; this abrogates translation of the entire IN domain of Gag-Pol. The Vpr-IN expression plasmid, pLR2P-vprIN, contains *vpr* ligated in frame with *IN*. Thirty-nine base pairs of upstream RT sequence were included at the Vpr-IN junction to preserve the natural protease cleavage (PC) site at the N terminus of IN. (B) 293T cells were transfected with different wild-type ($pSG3^{wt}$ and pR7-3) and IN mutant ($pSG3^{S\text{-}IN}$, $pR7\text{-}3^{S\text{-}IN}$, and $pSG3^{D116A}$) HIV-1 molecular clones. Forty-eight hours after transfection, 4 ml of supernatant from each culture was collected and subjected to ultracentrifugation over cushions of 20% sucrose. Pellets were lysed and examined by immunoblot analysis using anti-Gag (top), anti-IN (middle), and anti-RT (bottom) antibodies as probes.

**Transfections and virus purification.** DNA transfections were performed on monolayer cultures of 293T cells by 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 respective pLR2P expression plasmid DNAs. Forty-eight hours after transfection, cell culture supernatants were collected and clarified by low-speed centrifugation (1,000 × *g*, 10 min). Virions were pelleted by ultracentrifugation in a Beckman SW-41 rotor (125,000 × *g*, 2 h) through cushions of 20% sucrose and analyzed for HIV-1 p24 antigen concentration by HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA; Coulter Corp.).

**Analysis of virus infectivity (MAGI assay).** Virus infectivity was assessed with the 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 β-galactosidase gene under the control of the HIV-1 LTR. Detailed methods for performing the multinuclear activation of galactosidase indicator (MAGI) assay have been described previously (19). Briefly, supernatants from transfected 293T cell or infected PBMC cultures were clarified by low-speed centrifugation (1,000 × *g*, 10 min), filtered through 0.45-μm-pore-size sterile filters, and analyzed for HIV-1 p24 antigen content by ELISA. Three serial fivefold dilutions of equivalent amounts of virus (25, 5, and 1 ng of 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 mono-

layers were washed with Hanks' balanced salt solution (HBSS) and then cultured in 1 ml of Dulbecco's modified Eagle's medium containing 10% FBS. Forty-eight hours later, cells were fixed with phosphate-buffered saline (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 with the aid of a light microscope. Wells containing between 50 and 250 blue cells were used to calculate the number of HIV-1 infectious units per nanogram of HIV-1 p24 antigen.

**Immunoblot analysis.** Virus particles in transfected cell culture supernatants were concentrated by ultracentrifugation (125,000 × *g*, 2 h) through cushions of 20% sucrose, solubilized in lysis buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol), boiled for 3 min, and separated on 12% polyacrylamide gels containing sodium dodecyl sulfate. Following electrophoresis, proteins were transferred to nitrocellulose (0.2 μm pore size; Schleicher & Schuell) by electroblotting, incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in PBS), and then incubated for 2 h with the appropriate antibodies diluted in blocking buffer. Protein-bound antibodies were detected with horseradish peroxidase-conjugated specific secondary antibodies by enhanced chemiluminescence methods according to the manufacturer's instructions (Amersham).

**Analysis of HIV-1 proviral DNA integration in a single cycle of infection.** *Sal*I-*Hpa*I DNA fragments of $pSG3^{wt}$ and $pSG3^{S\text{-}IN}$ (nucleotides 5578 to 8195) were excised and replaced with *Sal*I-*Hpa*I DNA fragments PCR amplified from the HIV-1-gpt clone (20), generating $pSG3^{gpt}$ and $pSG3^{S\text{-}IN\text{-}gpt}$, respectively. This fragment contains the simian virus 40 origin of replication and promoter and codes for the xanthine-guanine phosphoribosyltransferase (gpt) resistance marker. Pseudotyped virus stocks were prepared by transfecting 293T cells with 2 μg of pCMV-VSV-G and 4 μg of the wild-type and IN mutant gpt-containing viral clones. For complementation studies, the pLR2P-vprIN expression vector was also included in the transfection mixture (triple transfection). After 48 h, the culture supernatants were collected, filtered through 0.45-μm-pore-size sterile filters, normalized to 100 ng of p24 capsid protein per ml, serially diluted, and used to infect 30% confluent cultures of HeLa CD4–LTR–β-gal cells. The infected cells were then maintained in gpt selection medium and stained to identify resistant (integration-positive) colonies exactly as described previously (27).

## RESULTS

**Production of IN-minus HIV-1 virions.** A number of studies have examined the role of IN in the HIV-1 life cycle, most of which were based on the analysis of cloned proviruses that contained mutations in the IN domain of Gag-Pol. In this study, we analyzed the function of IN when it was incorporated into virions in *trans*, as a fusion partner of Vpr. To test whether it was possible to package functional IN as a Vpr-IN fusion protein, an HIV-1 mutant clone was constructed in which synthesis of the entire IN domain was abrogated. By completely eliminating the virus-encoded IN, it was possible to directly assess the incorporation and proteolytic processing of the Vpr-IN fusion protein. Moreover, this also enabled the function of the *trans* IN to be analyzed without a virus-encoded IN exerting either dominant-negative or complementation effects. Since mutation of the IN coding region can cause defects in virion assembly and production, we first characterized the nature of IN-minus virions. Two different mutant proviral clones with identical mutations were constructed for the initial analysis. The $pSG3^{S\text{-}IN}$ and $pR7\text{-}3^{S\text{-}IN}$ mutants were transfected into 293T cells. Wild-type $pSG3^{wt}$, pR7-3, and $pSG3^{D116A}$ were transfected as controls. Immunoblot analysis of progeny virions confirmed the absence of IN protein in purified $SG3^{S\text{-}IN}$ and $R7\text{-}3^{S\text{-}IN}$ virions (Fig. 1B). Compared with wild-type $SG3^{wt}$ virions, IN-minus virions contained slightly larger amounts of unprocessed (p55) and incompletely processed (p39) Gag protein and similar amounts of processed RT protein (p66 and p51). The amount of Gag protein incorporated and processed for the $SG3^{D116A}$ virus appeared to be similar to that of the wild-type virus. Cultures transfected with the $pSG3^{S\text{-}IN}$ and $pR7\text{-}3^{S\text{-}IN}$ mutants generated easily detectable amounts of progeny virions. The concentrations of mutant $SG3^{S\text{-}IN}$ and $R7\text{-}3^{S\text{-}IN}$ virions were reproducibly two- to fourfold lower than those of the respective wild-type virions (data not shown). This level of reduction in virion production from transfected 293T cells is consistent with data reported by others (1, 4, 8). These results demonstrate the production of IN-minus virions and the subsequent proteolytic processing of the Gag and truncated Gag-Pol (Gag–PR-RT) precursor proteins.

TABLE 1. Uptake of IN-minus HIV-1

| Virus | Concn of capsid protein[a] | | | |
|---|---|---|---|---|
| | Expt 1 | | Expt 2 | |
| | HeLa-CD4 | HeLa | HeLa-CD4 | HeLa |
| SG3 | 9.9 | Neg.[b] | 10.4 | Neg. |
| $SG3^{S\text{-}IN}$ | 8.2 | Neg. | 9.4 | Neg. |

[a] Intracellular capsid protein (nanograms per $5 \times 10^5$ cells) was quantified by analyzing infected cell lysates by HIV-1 p24 antigen ELISA.
[b] Neg., the intracellular capsid protein concentration was below the limit of detection by ELISA.

**Entry of IN-minus HIV-1 into CD4-positive cells.** To examine whether IN-minus virions were impaired in their ability to gain entry into CD4-positive cells, the intracellular p24 antigen concentration was measured 4 h after virus infection by methods similar to those described previously (26). HeLa CD4-LTR–β-gal indicator cells were infected in 60-mm-diameter dishes with IN-minus and wild-type virus stocks normalized to 500 ng of p24 antigen. As a control, HeLa cells (CD4 negative) were also incubated with IN-minus and wild-type viruses. After 2 h of incubation at 37°C, the cell were washed three times in HBSS and then treated with trypsin to facilitate removal of extracellular virus. The cells were resuspended in 2 ml of FBS, pelleted, and washed four times with 10 ml of HBSS containing 1% FBS. The cells were lysed and then analyzed by HIV-1 p24 ELISA (Coulter). Table 1 shows the results of two independent experiments. No significant differences among the IN-minus, IN D116A mutant, and wild-type viruses were detected. CD4-negative HeLa cells were negative for cell-associated p24 antigen by ELISA. These results indicate that IN-minus virions are not defective at the level of virus entry. This result was essential for our determination of whether functional IN protein could be incorporated into IN-minus virions in *trans.*

**Incorporation of Vpr-IN fusion protein into IN-minus virions, and restoration of infectivity.** To test for the incorporation of the Vpr-IN fusion protein into HIV-1 particles, 293T cells were cotransfected with pLR2P-vprIN and $pSG3^{S\text{-}IN}$. Immunoblot analysis of progeny virions detected two anti-IN-reactive protein species. The larger protein had an apparent molecular mass of approximately 47 kDa, consistent with the combined masses of IN and Vpr. The smaller protein was approximately 32 kDa, indicating cleavage of the virion-associated Vpr-IN fusion protein by the viral protease and liberation of IN (Fig. 2A). When expressed without Vpr as a fusion partner, IN failed to incorporate into $SG3^{S\text{-}IN}$ particles at detectable levels. Analysis of pLR2P-IN-transfected cells confirmed IN expression (data not shown). As a control, $SG3^{wt}$ virions derived from cotransfection of $pSG3^{wt}$ with the Vpr-IN and IN expression vectors, respectively, were also analyzed. Similar to IN-minus virions, complemented $SG3^{wt}$ virions incorporated and processed the Vpr-IN fusion protein. Analysis of mutant and wild-type virions for Gag revealed that IN-minus virions contained slightly increased amounts of incompletely processed Gag protein (p39). Similar processing of the Gag protein was detected for complemented and noncomplemented wild-type and mutant virions, suggesting that the Vpr-IN fusion protein did not affect virion assembly or maturation.

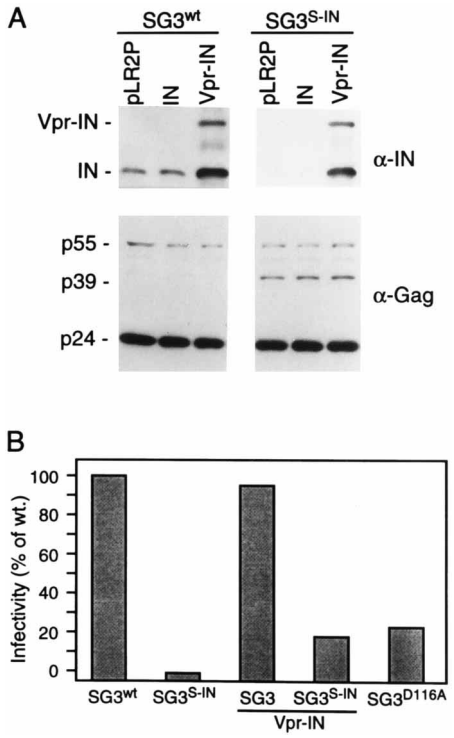


FIG. 2. *trans* complementation of IN-minus HIV-1 virions with the Vpr-IN fusion protein. (A) $pSG3^{S-IN}$ and $pSG3^{wt}$ were separately cotransfected into 293T cells with pLR2P-vprIN, pLR2P-IN, and pLR2P (vector alone), respectively. Forty-eight hours later, extracellular virus in the supernatants of each culture was concentrated by ultracentrifugation, lysed, and analyzed by immunoblot analysis with anti-IN (top) and anti-Gag (bottom) antibodies as probes. (B) To analyze whether the Vpr-IN fusion protein could complement $SG3^{S-IN}$ virions, $pSG3^{S-IN}$ and $pSG3^{wt}$ were separately transfected into 293T cells and cotransfected with pLR2P-vprIN. As an additional control, $pSG3^{D116A}$ was also transfected. Forty-eight hours later, progeny virions were collected from culture supernatants and analyzed by the MAGI assay. The results are expressed as percent infectivity relative to that of the $SG3^{wt}$ virus, which was arbitrarily set to 100. The absolute number of infection-positive cells for $SG3^{wt}$ was 2,184 per 5 ng of p24 antigen equivalents of input virus.

IN-minus HIV-1 virions do not support productive infection (1, 21). Since IN-minus virions rarely produce infection-positive cells when analyzed by the MAGI assay (1, 8), we utilized this method as a rapid means of evaluating whether Vpr-IN could complement the defect in particle infectivity. The MAGI assay provides sensitive and quantitative detection of virus infection within a single round of HIV-1 infection. It is based on activation of an integrated LTR–β-galactosidase gene in HeLa CD4–LTR–β-gal cells by Tat protein expression (19). For the purpose of our analysis, complementation was defined to mean that the defect(s) in replication of virions derived from IN-minus provirus was overcome by the *trans* IN. $SG3^{S-IN}$ virions produce very few β-galactosidase-positive cells, only 0.1 to 0.5% of the number produced by equivalent amounts of wild-type $SG3^{wt}$ virions. However, Vpr-IN complemented viral infectivity to 18% of the level of the wild-type virus (Fig. 2B). Virions derived by cotransfection of 293T cells with $pSG3^{S-IN}$ and pLR2P-IN did not exhibit increased infectivity above that of $SG3^{S-IN}$ virions. The incorporation of Vpr-IN into wild-type $SG3^{wt}$ virions did not markedly change infectivity, indicating that the Vpr-IN fusion protein did not have strong dominant-negative effects. Consistent with other reports, $SG3^{D116A}$ virions were positive by the MAGI assay, indicating that viral DNA synthesis and Tat protein expression are sufficient to induce β-galactosidase expression (1, 8, 27).

The efficiency of the *trans* IN to complement the IN-minus provirus was further assessed by cotransfecting 293T cells with different concentrations of pLR2P-vprIN and constant amounts of $pSG3^{S-IN}$. The analysis of progeny virions demonstrated that the conditions which maximized the incorporation of Vpr-IN also generated the highest proportion of infectious virions (Fig. 3). Under optimal conditions, $SG3^{S-IN}$ virions were complemented to 21% of the level of the wild-type virus. These results demonstrate that when packaged into virions as a fusion partner of Vpr, IN can support the infectivity (as measured by the MAGI assay) of virions derived from IN-minus provirus. However, this result does not demonstrate whether the complemented virus supports integration of the viral DNA.

**Replication of complemented virus through a complete cycle of infection.** Integration of the HIV-1 provirus is essential for virus production from infected cultures of peripheral blood lymphocytes and macrophages (27). Therefore, primary cultures of human PBMC were used to further assess the infectivity and replication competence of Vpr-IN-complemented $SG3^{S-IN}$ virus. As controls, equivalent amounts of transfection-derived SG3, $SG3^{S-IN}$, and $SG3^{D116A}$ viruses were also analyzed. Over a 5-day period, pelletable HIV-1 CA protein was detected in PBMC cultures infected with $SG3^{wt}$ virus and complemented $SG3^{S-IN}$ virus (Fig. 4A). In sharp contrast, only background levels of virus were detected in cultures infected with the IN mutant viruses ($SG3^{S-IN}$ and $SG3^{D116A}$). To assess whether infectious virus was generated through recombination, virions in the culture supernatants on days 3 and 5 (200-pg equivalents of p24 antigen) were analyzed for infectivity. Only the $SG3^{wt}$ virus was found to be infectious (Fig. 4B). Taken together, these results suggest that IN function, including integration of the provirus, can be provided in *trans*, independent of Gag-Pol.

***trans* IN supports formation of the provirus.** The replication of complemented IN mutant virus through a complete cycle of infection in PBMC strongly suggested that the enzymatic function of IN can be provided in *trans*. To directly test this notion, the IN mutant proviral clone $SG3^{S-IN-gpt}$ was constructed and used to assess integration in a single-round integration assay.

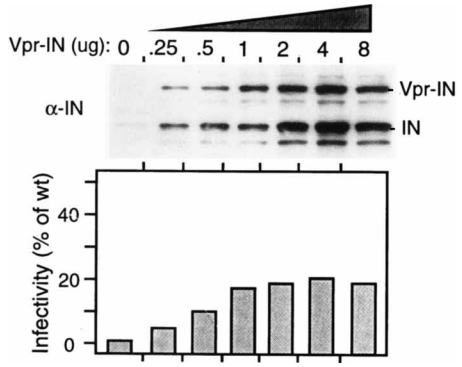


FIG. 3. Restoration of $SG3^{S-IN}$ infectivity by *trans* complementation with Vpr-IN. Different amounts of pLR2P-vprIN DNA (ranging from 0.25 to 8 μg) were cotransfected into 293T cells with constant amounts of $pSG3^{S-IN}$ DNA (4 μg). Forty-eight hours later, culture supernatants were collected, clarified by low-speed centrifugation (1,000 × *g*, 10 min), analyzed by HIV-1 p24 antigen ELISA, normalized for p24 antigen concentration, and divided into two aliquot sets. One set was processed to pellet virions and examined by immunoblot analysis with anti-IN antibodies. The other set was analyzed by the MAGI assay. Infectious units are expressed as a percentage relative to that of the $SG3^{wt}$ virus.

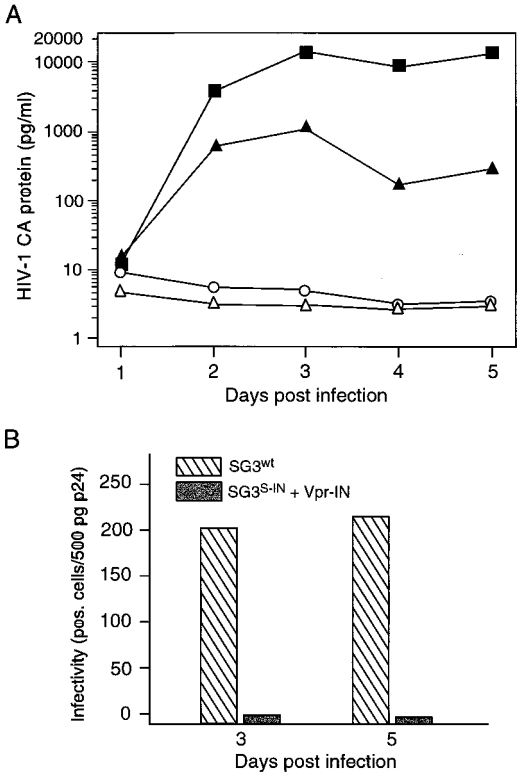


FIG. 4. Replication of IN-complemented virus through a complete cycle of infection in PBMC. (A) Phytohemagglutinin-stimulated PBMC ($10^6$) were infected overnight at 37°C with equal amounts (500 ng of p24 antigen equivalents) of transfection-derived wild-type $SG3^{wt}$ virus (closed squares), $SG3^{D116A}$ mutant virus (open circles), $SG3^{S\text{-}IN}$ IN-minus virus (open triangles), and $SG3^{S\text{-}IN}$ virus complemented with Vpr-IN (closed triangles). Cells were then washed five times by centrifugation, and on days 1 to 5 postinfection, 250-μl samples were collected from the culture supernatants, 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 for p24 antigen concentration. CA protein concentrations were plotted for days 1 to 5. On day 3, the culture medium was exchanged for fresh medium. (B) On days 3 and 5, culture supernatants were collected, filtered through 1.2-μm-pore-size sterile filters, and analyzed by HIV-1 p24 antigen ELISA. Two hundred-picogram equivalents (p24 antigen) of the wild-type SG3 and Vpr-IN complemented viruses were then analyzed by the MAGI assay.

This and similar single-round integration assays have been used extensively by others to quantify HIV-1 proviral DNA integration events (21, 23, 27). The gpt resistance gene within the HIV-1 genome allows for the outgrowth of infected cells in the presence of gpt selection medium if viral DNA is synthesized, integrated, and expressed. The single-cycle integration assay therefore enables a direct assessment of proviral integration events per unit of input virus. For analysis, IN-minus $pSG3^{S\text{-}IN\text{-}gpt}$ DNA was cotransfected into 293T cells with either the pCMV-VSV-G *env* vector or both the pLR2P-vprIN and pCMV-VSV-G expression vectors. As a positive control, $pSG3^{gpt}$ was also cotransfected with pCMV-VSV-G. Two days after transfection, culture supernatants were collected, filtered through 0.45-μm-pore-size filters, and divided into two aliquots. One aliquot set was subjected to ultracentrifugation over 20% cushions of sucrose and examined by immunoblot analysis. Virions derived from $pSG3^{S\text{-}IN\text{-}gpt}$ did not contain detectable IN, while virions derived from cotransfection of pLR2P-vprIN and $pSG3^{S\text{-}IN\text{-}gpt}$ did (data not shown). The second aliquot set was normalized for p24 antigen concentration and used to infect HeLa CD4–LTR–β-gal indicator cells in gpt selection medium. Table 2 summarizes the results, which were highly reproducible in three independent experiments. $SG3^{S\text{-}IN\text{-}gpt}$ virions produced gpt-resistant colonies when complemented with VSV-G-Env and Vpr-IN, confirming integration of the viral DNA into the infected cells' chromosomes. Complemented virions produced 10.5% of the number of resistant colonies compared with wild-type $SG3^{wt}$ virions. Pseudotyped $SG3^{S\text{-}IN\text{-}gpt}$ virions produced only rare gpt-resistant colonies, 0.02% of the wild-type level. Thus, complementation resulted in greater than a 500-fold increase in integration frequency. These results are consistent with the PBMC results and confirm that the *trans* IN supports integration of proviral DNA into host cell chromosomes.

## DISCUSSION

Previous studies in our laboratory have exploited HIV virion-associated accessory proteins (Vpr and Vpx) as vehicles to deliver proteins of both viral and nonviral origin into virions by their expression in *trans* as heterologous fusion proteins (29, 30, 32). This report extends our earlier results and shows that HIV-1 IN can be incorporated into virions as a fusion partner of Vpr. The virion-associated fusion protein (Vpr-IN) is processed by the viral PR to liberate IN, similar to the Gag-Pol precursor protein. Complemented virions are able to replicate through a complete cycle of infection, including formation of the provirus. Therefore, our findings demonstrate, for the first time, that fully functional IN can be incorporated into HIV-1 particles independent of the Gag-Pol precursor protein and that the IN domain of Gag-Pol is not required for the formation of infectious virions when IN is provided in *trans*. These findings will provide unique opportunities to gain an understanding of the role of both the IN domain of Gag-Pol in the formation of infectious virions and the mature IN protein during early stages of the virus life cycle.

In this study, we chose to assess whether functional IN could be provided in *trans* by performing complementation studies using a mutant proviral clone that was deficient in the synthesis of the entire IN domain. This avoided dominant-negative and/or complementation effects that a viral-encoded mutant IN might exert on the *trans* IN protein. However, it had been suggested that synthesis of the IN domain of Gag-Pol is required for the efficient production of virus particles from transfected cells (4). In HeLa cells, the truncation of IN was reported to cause a 15-fold reduction in particle release, while in 293T cells and Cos-1 cells, the reduction was only about 5-fold (4). In our hands, using two different mutant clones, nanogram quantities of IN-minus virus were produced by transfection into 293T cells. Our results consistently revealed only a two- to fourfold reduction in production of IN-minus virions compared with the wild-type level. This modest reduction did not impair our ability to purify and analyze progeny virions. Con-

TABLE 2. Relative integration frequency of HIV-1 complemented with IN in *trans*

| Virus | Relative integration frequency when complemented with[a]: | |
|---|---|---|
| | VSV-G | VSV-G + Vpr-IN |
| $SG3^{gpt}$ | $58 \times 10^4$ (100) | NT[b] |
| $SG3^{S\text{-}IN\text{-}gpt}$ | $0.012 \times 10^4$ (0.02) | $6.2 \times 10^4$ (10.7) |

[a] Values are the numbers of resistant colonies per 100 ng equivalents of input virus. The numbers in parentheses indicate the numbers of resistant colonies relative to that of the pseudotyped $SG3^{gpt}$ virus, which was arbitrarily set to 100.
[b] NT, not tested.

sistent with other reports, our immunoblot analysis indicated that IN-minus virions are similar (but not identical) to wild-type virions in the proteolytic processing of the Gag and Gag-Pol (Gag–PR-RT) precursor proteins (8, 12, 21). The only notable difference detected was that mutant virions contain slightly increased amounts of unprocessed and incompletely processed Gag protein (p55 and p39). It was also notable that the modest reduction in virion production and the slight defect in Gag processing were not corrected by expressing and incorporating IN in *trans*. This result would suggest that such a defect is due to the assembly of a truncated Gag-Pol precursor protein (Gag–PR-RT) and not the absence of the IN protein from virions.

The retroviral enzymes are expressed and incorporated into virions in a polyprotein precursor form. After proteolytic processing and maturation of the virus core structure, RT and IN constitute integral components of the nucleoprotein complex in which they carry out their respective functions. It had not previously been tested whether the incorporation of RT and IN as components of Gag-Pol is required to support the formation of a functional nucleoprotein/preintegration complex. By providing IN in *trans*, we were able to analyze this directly for the first time. IN-minus $SG3^{S\text{-}IN}$ virions are virtually noninfectious. However, this defect was overcome by complementation with *trans* IN, albeit not to wild-type levels. Interestingly, even under optimized conditions of complementation, virus infectivity was restored to only 21% of the wild-type level. Two possible explanations for this were considered. First, Vpr may not fully mimic Gag-Pol as a vehicle for incorporating and positioning IN in virions. Second, virions produced from proviral DNA that abrogates the synthesis of the IN domain may be defective in ways that cannot support infectivity with high efficiency.

The phenotype of $SG3^{S\text{-}IN}$ virions is distinct from that of $SG3^{D116A}$ mutant virions. $SG3^{S\text{-}IN}$ virions enter cells normally but are unable to induce β-galactosidase expression. On the other hand, $SG3^{D116A}$ mutant virions induce β-galactosidase expression at approximately 20% of the wild-type virus level. Since the induction of β-galactosidase expression in the MAGI assay does not require integration of the HIV-1 provirus, this result suggests that IN-minus virions are defective in other ways besides integration. Recently, others have shown that certain mutations within the IN domain of Gag-Pol, including those in the N-terminal zinc finger motif as well as deletion mutations, severely impair viral DNA synthesis in infected cells (8, 21, 23). Without the synthesis of the $SG3^{S\text{-}IN}$ viral DNA, it would not be possible to express the Tat protein and, in turn, induce β-galactosidase expression in the MAGI assay. In unpublished studies, we have confirmed that the $SG3^{S\text{-}IN}$ virus is severely defective in cDNA synthesis after entry into host cells. This result is consistent with our MAGI assay results. Additional studies will be required to analyze how the IN protein affects viral DNA synthesis in vivo.

By infecting PBMC with complemented $SG3^{S\text{-}IN}$ virions, the *trans* IN was found to restore virus replication through a complete cycle of infection. Since integration of the HIV-1 provirus is required for virion production in cultures of peripheral blood lymphocytes and macrophages of primary origin (27), these results strongly suggest that the *trans* IN can catalyze the integration of nascent viral DNA into host cell chromosomes. This result was confirmed through the analysis of complemented $SG3^{S\text{-}IN}$ virions by a single-cycle integration assay. Taken together, these results clearly demonstrate that complemented virus which is genetically defective in IN can infect natural target cells and replicate through the complete virus life cycle.

We have developed this novel *trans* complementation approach to incorporate enzymatically active protein into HIV particles by their expression in *trans* (29–32). This report extends our earlier findings and shows that fully functional HIV-1 IN can be incorporated into virions independent of the Gag-Pol precursor protein. Thus, we have uncoupled the role of the IN domain of Gag-Pol in virion morphogenesis from that which the mature IN protein plays during the early events of the virus life cycle. Although in vitro studies are important for dissecting the mechanism of integration, they have obvious limitations for recapitulating in vivo IN activity. In vivo, IN exists as a nucleoprotein/preintegration complex, and other components of this complex of host and viral origin influence IN function (5, 9, 12, 15). These findings will provide unique opportunities to analyze IN function in the infected cell with an intact nucleoprotein/preintegration complex and to unveil important aspects of IN function that have not been recognized previously.

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