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Human Immunodeficiency Virus Type 1 Virions Composed of Unprocessed Gag and Gag-Pol Precursors Are Capable of Reverse Transcribing Viral Genomic RNA

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The structural proteins and enzymes of the human immunodeficiency virus type 1 core are translated as part of two polyprotein precursors, Gag and Gag-Pol, which are cleaved by a virally encoded protease. Viruses grown in the presence of inhibitors of the protease contain core particles that are aberrantly assembled, and upon infection of susceptible cells, they do not synthesize viral DNA. Through the use of a proteinase inhibitor (A77003), we determined that the viral reverse transcriptase can efficiently synthesize viral DNA as part of the unprocessed Gag-Pol precursor. We also found that the stabilities of core particles composed of unprocessed precursors were considerably enhanced. These observations suggest that for viruses composed of unprocessed precursors, replication is interrupted before the reverse transcription step.

The core particle of human immunodeficiency virus type 1 (HIV-1) is assembled from polyprotein precursors which are cleaved by a virally encoded protease. The viral genome encodes two of these precursors, Gag and Gag-Pol. When processed, Gag gives rise to the structural proteins of the core. The enzymatic proteins of the core are contained in Gag-Pol; these include the viral protease, the reverse transcriptase (RT), and the integrase (5). The unprocessed precursors assemble at the membrane of the infected cell, and proteolytic processing of Gag and Gag-Pol is initiated during virus assembly.

Mutations which render the protease inactive have been introduced into several retroviruses including HIV-1 (6, 13, 18), Mason-Pfizer monkey virus (19), Moloney murine leukemia virus (M-MuLV) (4, 11), and Rous sarcoma virus (RSV) (20). Although virus particles are produced in the absence of protease activity, complete and accurate processing of these precursors is required to produce fully infectious virus. If protease activity is inhibited or absent, the virions produced are noninfectious and contain aberrantly assembled core particles (4, 6, 10, 11, 13, 18, 20). Electron micrographic evaluation of the protease-deficient retroviruses indicates that instead of the collapsed, central, electron-dense core seen in wild-type virus, these particles typically have an abnormal open, ring-shaped, distended core (6, 10, 11, 18, 20). In addition, the core particles from the protease-deficient virus are relatively resistant to nonionic detergents (16a, 20).

It has also been suggested that the viral RT can function as part of the Gag-Pol precursor. In reactions requiring the unprocessed RT to act as an RNA-directed DNA polymerase, the enzyme has been shown to have activity between 15 and 100% that of the wild-type virus (4, 10, 15, 17, 18, 20). For RSV, Stewart et al. (20) have demonstrated that RT processing is required for viral DNA synthesis. In the only other study in which the endogenous reverse transcription reaction was used to evaluate the unprocessed RT, Crawford and Goff (4) have demonstrated that a protease-deficient mutant of M-MuLV was still able to produce minus-strand strong-stop DNA. This mutant, however, produced no detectable viral DNA upon infection of susceptible cells, a finding that we have extended to HIV-1 using a protease inhibitor and a PCR-based assay to detect viral DNA (10). In the study described in this report, we demonstrated that the unprocessed HIV-1 RT can efficiently reverse transcribe the viral genome in vitro. This indicates that all of the requirements for reverse transcription can be met by the full-length Gag and Gag-Pol precursors. Furthermore, these data suggest that the life cycle of viruses composed of unprocessed precursors is interrupted before the reverse transcription step.

To characterize the virus particles produced in the presence of a protease inhibitor, we used HIV-1 isolate HIV-1_G, which was obtained from an infected patient (a gift of M. Cloyd and R. Buckheit). CEM cells were chronically infected with this isolate as described previously (9). Briefly, 2×10^6 CEM cells in 200 μl of RPMI 1640 containing 10% (vol/vol) fetal calf serum and 200 μl of HIV-1 stock were placed in a well of a 48-well plate. The cells and virus were incubated at 37°C for 2 h. The cells were then placed in 10 ml of fresh medium. The cells were pelleted daily and were resuspended in fresh medium. Maximum virus spread as measured by syncytium formation and cell death occurs at about 5 days postinfection. After 7 days CEM cells which were chronically infected began to appear. These cells were resuspended daily in fresh medium and were used for these experiments at 3 weeks after infection, at which time virus production was maximal. The cells were grown for 5 days in 9.0 μM HIV-1 protease inhibitor, A-77003 (12), and the medium was replaced daily. Virus supernatant was recovered on day 6. The sample was divided into aliquots, and the aliquots were frozen. Virus was concentrated by

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ultracentrifugation through a 15% sucrose cushion at 100,000 × *g* for 1.5 h.

We used the endogenous reverse transcription reaction to assay the abilities of virus-containing cores composed of unprocessed precursors to reverse transcribe their genomic RNAs (13a). By quantitating viral DNA synthesis at each of the steps of the endogenous reaction, we can assess the ability of the unprocessed RT to carry out RNA-dependent DNA synthesis as well as DNA-dependent DNA synthesis using the enzyme's natural template as a substrate. In addition, the frequency with which template jumping occurs can also be evaluated. In contrast, the exogenous reaction is limited to measuring the ability of the enzyme to act as an RNA-dependent DNA polymerase on a synthetic template.

For the endogenous reaction, filtered virus supernatants were recovered from chronically infected CEM cells. The virus was collected by ultracentrifugation through a cushion containing 20% Renograffin-60 (Squibb) and 80% TEN (10 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl). A portion of the virus pellet was added to the endogenous reaction mixture which contained final concentrations of 50 mM NaCl, 50 mM Tris (pH 8.0), 10 mM dithiothreitol, 5 mM $MgCl_2$, and 0.1 mM each dATP, dCTP, dGTP, and dTTP. The concentration of Triton X-100 was varied from 0 to 0.5%. After incubation at 37°C, the reaction was stopped by the addition of EDTA and proteinase K. An aliquot of the reaction mixture was analyzed by PCR as described previously (1, 20, 21). Different regions of the newly synthesized viral DNA were amplified with primers specific for the sequences of interest. The initial products of reverse transcription (R-U5) were amplified by primers M667-AA55 (25). The region of the genome encompassing the *nef* gene was amplified by using AA943-AA946 (25). LA45-LA24 amplified *tat-rev* sequences (1), and plus-strand DNA which has been extended beyond the primer binding site after the second template switch is identified by the long terminal repeat (LTR)-*gag* primers M667-M661 (25). In order to make certain that the observed similarity in DNA synthesis was not due to strain differences between the untreated and protease-deficient virus stocks (HIV_{JRCSF} and HIV_{HXB2}, respectively), we compared untreated virus derived from these stocks directly in the endogenous reaction. We found that an equal amount of the two strains synthesized equivalent amounts of viral DNA and that, for both viruses, DNA synthesis was maximal at 0.1% Triton X-100 (data not shown).

HIV-1 particles produced from infected cells grown in the presence of greater than 3 μM A-77003 are composed of unprocessed Gag and Gag-Pol precursors (8a, 10). We recovered supernatant from CEM cells chronically infected with HIV-1 that were maintained either in the presence or the absence of 9 μM A-77003 as described above. Virus was concentrated by ultracentrifugation, and the virion proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The extent of processing of the Gag and Gag-Pol precursors was evaluated by Western blot (immunoblot) analysis and is shown in Fig. 1. Virus produced by the untreated cells contained only completely processed proteins. This could be seen when the Western blots were analyzed for Gag processing by staining with a monoclonal antibody directed against the mature capsid protein p24 (lane 1). When these samples were stained with a monoclonal antibody to the RT, only the two forms of the completely processed mature enzyme (p66 and p51) were seen (lane 3). However, when chronically infected cells grown in the presence of 9.0 μM inhibitor were evaluated, Gag was present as either the 55-kDa precursor or one of several 39- to 41-kDa processing intermediates (lane 2). A Western blot of the same sample stained with the monoclonal antibody to the RT did not reveal any processed enzyme (lane 4). As we have demonstrated previously (10), this antibody does not recognize the RT as part of the unprocessed Gag-Pol precursor.

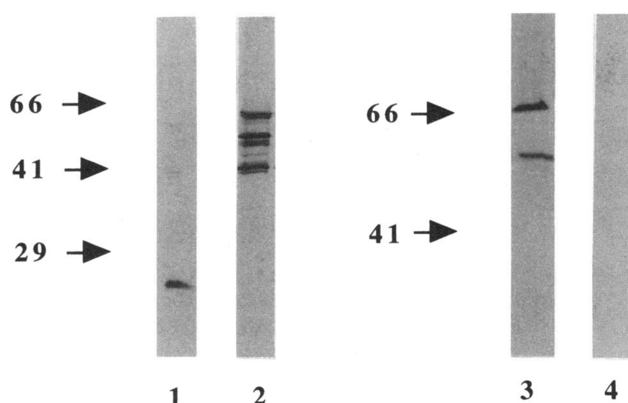

FIG. 1. Processing of Gag and Gag-Pol precursors in the presence of A-77003. Virus produced either in the presence (lanes 2 and 4) or the absence (lanes 1 and 3) of A-77003 was collected by ultracentrifugation and was subjected to Western blot analysis. The total amounts of viral proteins added to each lane were normalized by using serial, threefold dilutions of each sample. The samples were then stained with monoclonal antibodies directed against either the p24 capsid protein (lanes 1 and 2) or the RT (lanes 3 and 4). Molecular weight markers (in thousands) are shown.

We next characterized the process of reverse transcription in virus grown in the presence of the inhibitor. Varying results have been obtained by an exogenous template-based assay to measure the activity of the RT in viruses composed of unprocessed precursors (4, 10, 15, 17, 18, 20). We therefore decided to use the endogenous reverse transcription reaction in order to assay the abilities of these viruses to synthesize DNA. Pelleted samples of virus grown in treated and untreated cells were normalized for p24 antigen content and were prepared for the reaction as described above. Three microliters of pelleted virus containing 750 pg of p24 was used in each reaction mixture. The ability of the p24 antigen enzyme-linked immunosorbent assay to measure the capsid protein as part of the Gag precursor was verified by comparing Western blots of serial dilutions of both samples (data not shown). Using PCR primers specific for different parts of the HIV-1 genome, we were able to distinguish between viral DNA which represents the initiation of reverse transcription (Fig. 2, R-U5 primers), the first template switch (*nef* primers), extension of DNA synthesis after the first template switch (*tat-rev* primers), and nearly complete transcripts (LTR-*gag* primers). Equivalent amounts of viral DNA were synthesized by the virus grown in treated and untreated cells at each of the steps of reverse transcription. Furthermore, with the exception of the initial products of reverse transcription, viral DNA synthesis was maximal at 0.01% Triton for both samples and at each of the steps of reverse transcription. Of note, some DNA synthesis was apparent in the absence of detergent. This has been reported previously and may reflect disruption of the virus particles during specimen collection (2, 3, 21). The initiation of reverse transcription is less sensitive to detergent in the virus grown in treated cells. For this product, the amount of viral DNA synthesized is not affected by concentrations as high as 0.5% (data not shown).

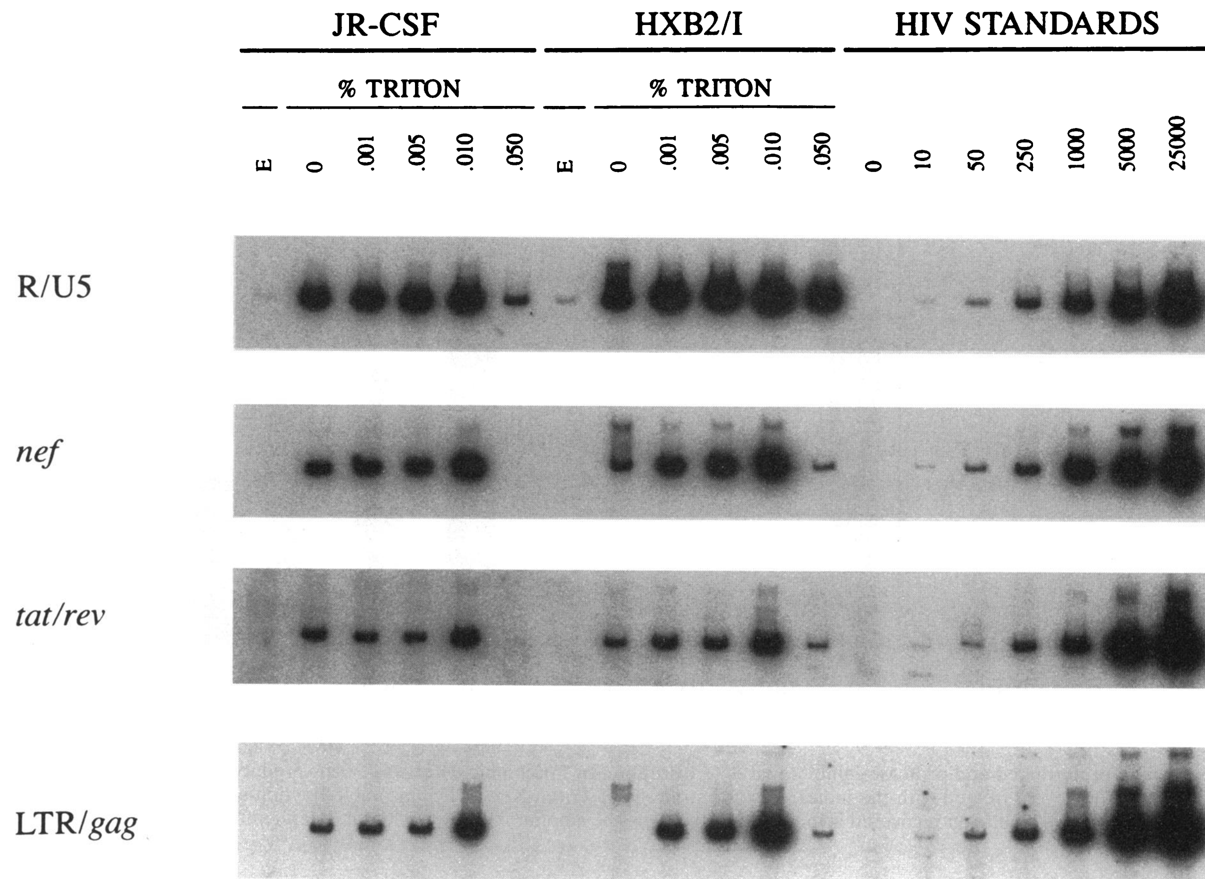

FIG. 2. PCR analysis of endogenous reverse transcription reactions. The endogenous reactions were performed as described above. The reactions were analyzed by using each of the four primer pairs (R-U5, *nef*, *tat-rev*, and LTR-*gag*) in the presence of EDTA (25 mM) or at various concentrations of Triton X-100. Endogenous reaction products derived from virus produced in the absence (JRCSF) and the presence (HXB2/I) of A-77003 were analyzed. EDTA at 250 mM was used as a negative control (lane E). This was diluted to 0.8 mM in the final PCR.

It has been demonstrated that although the retrovirus core particle is extremely susceptible to low concentrations of nonionic detergents, a mutation at the protease active site results in the production of virus particles whose cores are relatively resistant to detergent (16a, 20). We extended this observation to HIV-1 treated with protease inhibitors by determining the stabilities of viral cores that are composed of unprocessed precursors. p24 antigen determinations were performed on virus supernatants produced by cells infected with the HIV_{HXB2} strain and grown either in the presence or the absence of A-77003. The samples were divided into four equal aliquots. These supernatant samples were placed at 37°C for 1 h at concentrations of either 0.005, 0.01, or 0.1% Triton X-100 or without detergent. The intact core particles were collected by ultracentrifugation through a sucrose cushion and were resuspended in RIPA buffer. The amount of pelletable p24 antigen present in the sample incubated without detergent was set equal to 100% (Fig. 3). For the virus grown in untreated CEM cells, greater than 95% of the p24 protein was solubilized at detergent concentrations of as low as 0.005%. In contrast, the sample containing unprocessed core precursors was much less susceptible to the effects of the detergent; one-third of the viral core proteins were recovered by centrifugation at a detergent concentration of 0.1%.

Inhibitors of the HIV-1 protease have been developed and are entering clinical trials (7, 12, 15a, 22, 23). Although it has been well-established that protease-deficient retroviruses are noninfectious and are aberrantly assembled, the mechanism behind their loss of infectivity remains obscure. Our data indicate that virions composed of unprocessed Gag and Gag-Pol precursors are capable of efficiently synthesizing viral DNA. On the basis of an estimate of 2×10^6 virions per ng of p24, we find that both protease-deficient and untreated viruses produce about one copy of DNA per 10 to 100 virions (14). This compares favorably with the very low ratio of infectious to noninfectious HIV-1 particles (e.g., one infectious unit for every 10^4 to 10^7 particles) (14). In addition to demonstrating the ability of the RT to function as part of the precursor, our data and those of other investigators (4, 15a) also suggest that the precursors are able to package and anneal the tRNA primer required for reverse transcription. It is possible that processing of the RT may have occurred at a level below that which can be visualized by Western blot analysis. Since comparable amounts of viral DNA were synthesized in both the treated and the untreated virus samples, it is unlikely that the few virions that may contain a small amount of processed RT in the protease-deficient virus sample could account for our results. In order to make certain that the conditions of the reaction did not result in the release of protease inhibition, we repeated the endogenous reaction and evaluated the degree of

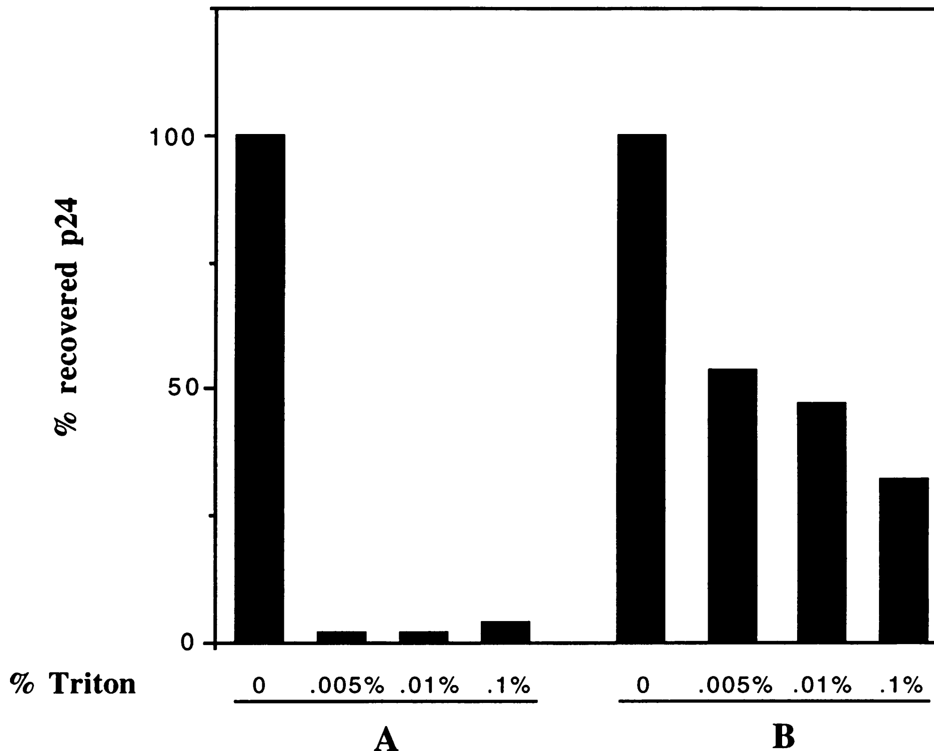

FIG. 3. Stability of untreated and protease-inhibited virus to disruption by a nonionic detergent. Virus produced in the absence (A) and the presence (B) of A-77003 were treated with the indicated concentrations of Triton X-100. The insoluble fraction was recovered by ultracentrifugation. The percentage of p24 antigen recovered is indicated by the bars; the amount recovered without detergent treatment is set equal to 100%.

processing in both the treated and the untreated virus stocks by Western blot analysis. No additional processing of the viral core proteins was observed after the reaction (data not shown).

Previous studies have failed to demonstrate the synthesis of viral DNA upon infection of susceptible cells with protease-deficient retroviruses (4, 10). Viral replication, therefore, is blocked at an early step in the life cycle. Since the RT is functional as part of the unprocessed Gag-Pol precursor, the lack of viral DNA synthesis after infection may be due to some difference between the conditions of the endogenous reaction and the intracellular environment in which reverse transcription occurs. As we have noted, viruses grown in the presence of the inhibitor are considerably more stable than viruses grown in untreated cells. In the endogenous reaction, the RT must have access to nucleotides, and therefore, some disruption of the virus particle by detergent is required. During infection, the viral core must also allow nucleotides access to the nucleoprotein replication complex. The enhanced stability of the core particles composed of unprocessed precursors may impede uncoating of the core once it has entered the target cell. Alternatively, the block to infection may be due to the inability of the core to arrive at a site within the target cell at which reverse transcription can proceed. For example, if virions composed of unprocessed precursors are unable to fuse efficiently with the membrane of the target cell, viral DNA synthesis would not occur. We are evaluating whether the presence of unprocessed Gag and Gag-Pol precursors interferes with this early step in the virus life cycle. Furthermore, we are using microinjection techniques to determine whether, once inside a susceptible cell, viral DNA synthesis can proceed.

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