Incompletely Reverse-Transcribed Human Immunodeficiency Virus Type 1 Genomes in Quiescent Cells Can Function as Intermediates in the Retroviral Life Cycle

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Using a quantitative polymerase chain reaction (PCR) method, we have previously shown that a molecularly cloned isolate of human immunodeficiency virus type 1 (HIV-1) can efficiently enter quiescent primary lymphocytes; however, the reverse transcription process is not completed in these cells. In this study, we further characterized the reverse transcription of HIV-1 in quiescent cells, and our results indicate that while initiation of reverse transcription occurs simultaneously in both activated and quiescent lymphocytes, it not only ends prematurely but also proceeds more slowly in quiescent cells. We also performed experiments to address the role of partial reverse transcripts as intermediates in the viral life cycle. We used azidothymidine either before or after infection with HIV-1 to prevent formation of and further DNA synthesis by partial reverse transcripts, respectively. Decreases in virus production from these cells following mitogenic stimulation indicated that partial reverse transcripts can contribute significantly to virus rescue from infected quiescent cells stimulated subsequent to infection. Furthermore, we established that mitogenic stimulation of infected quiescent cells induces reinitiation of DNA synthesis from partial reverse transcripts. However, the virus rescue is inefficient relative to the initial multiplicity of infection, and this is explained by inefficient completion of DNA synthesis from the partial reverse transcript. Thus, the arrest of reverse transcription in quiescent cells may play an important role in HIV-1 pathogenesis by contributing to the inefficient infection of potential target cells in the peripheral blood of HIV-1-infected individuals.

The stage of the cell cycle at the time of infection by retroviruses greatly influences viral replication. Both human and other animal retroviruses are capable of infecting and persisting in quiescent cells without producing progeny virions (7-9, 12, 13, 20, 23, 24, 26). Mitogenic stimulation subsequent to infection can induce progeny virus production. Following infection, incomplete species of viral DNA can be detected in quiescent cells (5, 8, 9, 22, 23). Our recent studies demonstrated that human immunodeficiency virus type 1 (HIV-1) can efficiently enter quiescent primary human lymphocytes but that the reverse transcription process is not completed in these cells. Mitogenic stimulation of quiescent cells harboring this partial reverse transcript induces virus production, suggesting that we characterized a novel latent form of HIV-1. We further demonstrated that the HIV-1 DNA in quiescent lymphocytes is labile and degrades with a half-life of approximately 1 day in vitro (23). This lability of the partial HIV-1 reverse transcript suggests that there may be a mechanism for clearance of the virus in vivo. The vast majority of circulating lymphocytes in vivo are in the quiescent state; thus, this model may explain the low number of infected cells in the peripheral blood of patients with AIDS (10, 11, 18).

In this study, we further characterized the nature of the partial HIV-1 reverse transcript in quiescent cells and performed experiments to determine more precisely the role of this intermediate in the retrovirus life cycle. Comparison of reverse transcription in both activated and quiescent primary T cells reveals that reverse transcription initiates at

about the same time in both cell types, but there is an apparent difference in the rate of this process between the two cell types. Our results also indicate that the partial reverse transcript in quiescent cells can be induced to complete the reverse transcription process following mitogenic stimulation of the infected cells and is capable of leading to progeny virus production; however, when stimulation is applied 15 h postinfection, this rescue is approximately 20-fold less than infection of prestimulated cells during a single virus replication cycle.

MATERIALS AND METHODS

Cells and viruses. Peripheral blood was obtained from normal donors by venipuncture. Peripheral blood lymphocytes (PBL) were isolated by centrifugation over Ficoll-Hypaque and depleted of macrophages by adherence to plastic for 4 h. Quiescent lymphocytes were cultured in RPMI 1640 supplemented with 10% pooled human AB serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2 mM glutamine. To obtain activated cells, PBL from the same blood donor were obtained and stimulated with phytohemagglutinin (PHA) (HA 15, 0.8 µg/ml; Wellcome) for 3 days prior to infection. Following infection, these cells were cultured in the same medium supplemented with 30 U of recombinant interleukin 2 per ml following infection.

The virus strains HIV-1_{JR-CSF} (15) and HIV-1_{NL4-3} (1) were recovered following electroporation of molecular clones (3). Virus stocks were subsequently obtained from 24-h harvests of supernatant from infected PBL and stored at -70°C. Patient isolates 1 and 2 were obtained from the

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peripheral blood of HIV-seropositive patients seen at the University of California at Los Angeles Medical Center. Patient cells were cocultivated with PHA-stimulated PBL from normal donors. Twenty-four-hour culture supernatants were harvested on day 15 of coculture. Prior to use, virus stocks were filtered and treated with RNase-free DNase I (1 $\mu\text{g}/\text{ml}$; Worthington) for 30 min at room temperature in the presence of 0.01 M MgCl_2 to remove contaminating HIV-1 DNA arising from the lysis of infected cells during preparation. Infection was accomplished by incubating cells with the DNase-treated virus in the presence of 10 μg of Polybrene per ml for 1 to 2 h at 37°C. Infections were standardized by enzyme-linked immunosorbent assay (ELISA) specific for HIV-1 p24^{gag} antigen (Coulter). Limiting-dilution experiments on PBL from various donors have indicated that approximately 1 to 30 pg of p24 is equivalent to one infectious unit of HIV-1_{JR-CSF}. Cells were rinsed twice prior to culturing to remove residual free virus. Heat-inactivated virus controls were prepared by incubation for 30 min at 60°C. Infections with heat-inactivated virus were performed in parallel with infections with live virus.

Nucleic acid preparation. All reagents used for nucleic acid preparation were specifically prepared for polymerase chain reaction (PCR) use and tested to ensure that no HIV-1 DNA contamination was present. Nucleic acids from live and heat-inactivated virus infections were extracted in parallel. Cells were washed in phosphate-buffered saline (PBS), lysed in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris-HCl [pH 8.0]) and subjected to phenol-chloroform extraction and ethanol precipitation. Total nucleic acids resulting from this extraction procedure were used for PCR amplification.

To analyze viral DNA in quiescent lymphocytes infected with HIV-1_{NL4-3}, clinical HIV isolates (see Fig. 2), and pseudotyped HIV-1 (see Fig. 7), an alternative DNA extraction and PCR method was used. Eighteen hours after infection, PBL were washed once with PBS and resuspended at a concentration of 2.5×10^7 cells per ml in gelatin lysis buffer (45 mM NaCl, 9 mM Tris-HCl [pH 8.3], 2.2 mM MgCl_2 , 0.1 mg of gelatin [Sigma] per ml, 0.4% Nonidet P-40, 0.4% Tween 20, 1 mg of proteinase K per ml). The samples were incubated at 60°C for a minimum of 3 h and then placed in a boiling water bath for 10 min. These lysates were used directly in PCR amplification, as described below.

PCR amplification. Quantitative PCR amplification with ³²P-end-labeled primers was performed as previously described (2, 16, 17, 23), except that denaturation was accomplished by heating to 94°C. Twenty-five cycles of amplification were performed for HIV-1 and human β -globin sequence analysis by using radiolabeled oligonucleotide primers. β -Globin-specific primers and the HIV-1-specific primers M667, M661, LA8, LA9, LA45, LA64, and AA55 were previously described (23). The primer AA943 (5'-TGACTTACAAGGCAGCTATAGATC-3') corresponds to nucleotides (nt) 9048 to 9071 in the HIV-1_{JR-CSF} sequence, and AA946 (5'-CTCTGGATCAACTGGTACTAGC-3') corresponds to nt 9265-9244 (antisense). These two primers amplify a 218-bp fragment corresponding to sequences in the HIV-1_{JR-CSF} *nef* gene that proceed and overlap the U3 region of the 3' long terminal repeat (LTR). The location of the HIV-1-specific primers used in these studies is depicted schematically in Fig. 1. Following amplification, radiolabeled products were resolved on a 6% polyacrylamide gel and visualized by autoradiography. HIV-1 DNA standards used to quantitate viral DNA were derived from dilutions of

FIG. 1. Oligonucleotide primers used in PCR analysis. The relative locations and orientations of oligonucleotide primers used to analyze reverse transcription are represented by arrows. The DNA genome of HIV-1_{JR-CSF} is depicted schematically. Open boxes represent the LTRs of the viral DNA, U3 and U5 indicate the 3' and 5' sequences of HIV-1 RNA duplicated during formation of the LTRs, R is the region repeated at both ends of the viral RNA genome, and the solid circle represents the location of the viral primer binding site (nt 637 to 651). The shaded rectangle represents the polypurine tract (nt 9085 to 9099) which directs plus-strand priming. Figure is not drawn to scale.

cloned HIV-1_{JR-CSF} DNA (3) digested with *EcoRI*, which does not cleave viral sequences. This DNA was diluted into carrier tRNA (4 $\mu\text{g}/\text{ml}$) or PBL DNA (10 $\mu\text{g}/\text{ml}$) where indicated in the figure legends. Standard curves for β -globin DNA were derived from dilutions of PBL DNA. HIV-1- and β -globin-specific primers were simultaneously incorporated into each reaction where indicated. For PCR analysis of samples in Fig. 2 and 7, PCR mixtures consisted of 10 μl of the cellular lysate added to 15 μl of low-salt PCR buffer (25 mM Tris [pH 8.0], 2 mM MgCl_2 , 30 mM NaCl, 0.1 mg of bovine serum albumin per ml, 0.25 mM dNTP). Twenty-five cycles of amplification were performed, each consisting of a 1-min denaturation step at 94°C followed by a 2-min anneal-extension phase at 65°C.

Addition of exogenous nucleosides. Quiescent cells (5×10^6) were cultured in the presence or absence of 50 μM each nucleoside (2'-deoxycytidine [Calbiochem], 2'-deoxyadenosine, 2'-deoxyguanosine, and 2'-deoxythymidine [ICN]) or 2 μM the nucleoside analog azidothymidine (AZT) (Sigma) for 3 h prior to infection with HIV-1_{JR-CSF}. Cells were infected for 2 h, rinsed, and cultured for 20 h in appropriate media. DNA was harvested, and 5% of the recovered amount was analyzed by PCR with various primer pairs.

Generation of pseudotyped virus. To generate infectious replication-defective HIV-1 virions, an envelope-negative HIV-1 was pseudotyped with the murine amphotropic envelope. An envelope-negative HIV-1 plasmid (pJRCSF-AFLΔ) was generated by digestion of pYK-JRCSF (4) with *AflIII* (which cleaves at nt positions 6513 and 7485 and once in the vector), followed by religation of the two larger fragments. This resulted in a viral clone deleted in the region encoding amino acids 67 to 368 of gp120. pJRCSF-AFLΔ (12.5 μg) and 12.5 μg of the amphotropic *env* expression vector pJD-1 (6) (obtained from M. Emerman) were coelectroporated (3) into 5×10^6 COS cells. Supernatants were harvested daily for 3 days following cotransfection, and virus stocks were stored at -70°C. These stocks were assayed by ELISA for HIV p24 (Coulter) prior to use. Prior to infection of PBL with this virus, virus stocks were subjected to treatment with DNase (4 $\mu\text{g}/\text{ml}$) (Worthington) for 1 h at room temperature in the presence of 0.01 M MgCl_2 to remove contaminating plasmid. DNase-treated virus stock was used to infect PBL in the presence of Polybrene, as outlined above for wild-type HIV.

RESULTS

Infection of quiescent lymphocytes by HIV-1. We previously showed that infection of primary quiescent PBL by a molecularly cloned isolate of HIV-1 (HIV-1_{JR-CSF}) obtained

FIG. 2. Infection of quiescent PBL with different HIV-1 isolates. Three HIV-1 isolates, HIV-1_{NL4-3} (1) and two primary patient isolates (Pt.1 and Pt.2), were used to infect quiescent PBL from the same normal-blood donor. Twenty-five cycles of PCR amplification in the presence of the R/U5 (initiation) or LTR/gag (full-length) primer pairs were performed on DNA extracted 18 h postinfection. DNA from 2.5×10^5 cells was analyzed in all lanes. HI indicates cells treated with heat-inactivated virus in parallel.

from the cerebrospinal fluid of a patient with AIDS (15) resulted in efficient initiation of reverse transcription but inefficient completion of this process (23). This was shown by differential PCR amplification with primer pairs capable of distinguishing DNA structures formed during different stages of the reverse transcription process by virtue of their relative positions in the HIV-1 genome. The resulting virus genomic structure consisted of a single 3' LTR and additional synthesis of minus-strand viral DNA of heterogeneous length.

A recent study suggested that full-length HIV-1 DNA was synthesized in quiescent cells after infection (19). We tested the possibility that strain differences may account for the discrepant results by infecting quiescent cells with three other HIV-1 strains. Infection of quiescent PBL from a normal donor with the molecularly cloned HIV-1 strain HIV-1_{NL4-3} (1), as well as with two virus isolates obtained by culture of PBL from two different HIV-seropositive patients, was analyzed by using quantitative PCR (2, 16, 17, 23) to determine the ability of these isolates to complete the reverse transcription process. The oligonucleotide primer pair M667/AA55 (Fig. 1) is specific for the viral LTR sequences in the R/U5 region, the first region synthesized during reverse transcription. This primer pair will detect the first 140 bases of viral minus-strand DNA synthesized following initiation of reverse transcription. Therefore, any and all reverse transcripts of HIV-1 would be detected with this primer pair. The primer pair M667/M661 amplifies sequences in the LTR/gag region of the HIV-1 genome and flanks the viral primer binding site. To generate an amplified product with these primers, contiguous LTR and gag sequences are necessary. Thus, complete or nearly complete reverse transcription is required to generate the 200-bp PCR product by using this primer pair. Analysis with the R/U5 (initiation-specific) primer pair resulted in PCR signals well above those of the heat-inactivated controls for all three virus isolates (Fig. 2, top). PCR analysis of the same DNA samples with the LTR/gag (full-length) primer pair resulted in minimal signals above those of the controls (Fig. 2, bottom). Thus,

while all three strains could enter quiescent cells and initiate reverse transcription, completion of this process was inefficient. We therefore conclude that premature termination of reverse transcription appears to be a general property of HIV-1 infection of quiescent lymphocytes.

Kinetics of reverse transcription. We have taken advantage of the ordered sequence of events in the reverse transcription process (for a review, see reference 21) to analyze the stages of reverse transcription by using oligonucleotide primers specific for DNA structures present at various stages of reverse transcription. To analyze the kinetics of reverse transcription in activated and quiescent cells, two primer pairs in addition to the LTR/gag (M667/M661) and R/U5 (M667/AA55) primer pairs were utilized. These primer pairs detect different regions of the minus strand of viral DNA found in partial reverse transcripts. The primer pair AA943/AA946 detects sequences at the 3' end of the HIV-1 genome, corresponding to the region of the *nef* gene immediately 5' to and overlapping the U3 region of the 3' LTR. Viral reverse transcription must proceed through the first template-switching event to form DNA detectable by these primers. The primer pair LA45/LA64 amplifies a region of the HIV-1 genome corresponding to *tat/rev* coding sequences just 5' of envelope coding sequences. This primer pair will detect further extension of the minus strand of viral DNA. We utilized these additional primer pairs to further investigate the kinetics of reverse transcription in quiescent versus activated human T lymphocytes.

Cellular DNA from both quiescent and activated cells infected with HIV-1_{JR-CSF} was harvested at various time points postinfection and analyzed by quantitative PCR. All samples were obtained during the first 20 h postinfection, prior to progeny virus production (23). The M667/AA55 primer pair, the indicator of initiation of reverse transcription, revealed that reverse transcription begins early after infection, within the first 2.5 h in both activated and quiescent cells (Fig. 3). The primer pair that detects complete or nearly complete reverse transcription (M667/M661) (Fig. 3, bottom) indicates that full-length HIV-1 DNA is evident within 6 h postinfection in activated cells but is not found in quiescent cells. We also analyzed these same samples by using the primers specific for the *tat/rev* region (LA45/LA64) (Fig. 1) which detect the approximate midpoint of elongation of the minus strand of HIV-1_{JR-CSF} (nt 5964 to 6079). These primers, while positive by 6 h in activated cells, resulted in no signal following infection of the quiescent cells even at 24 h (25). The same DNA samples reveal that reverse transcription of the *nef* region of HIV-1 (detected by the AA943/AA946 primer pair) is completed within 6 h in the activated cells but is not completed until 9 h postinfection in quiescent cells (Fig. 3, middle). Similar analyses with cells from a different normal-blood donor exhibited this same 3-h delay. Thus, while there was no detectable difference in initiation of reverse transcription, there was a 3-h delay in kinetics of the early stages of elongation between activated and quiescent cells. Thus, reverse transcription proceeds more slowly and terminates prematurely in quiescent cells.

Role of partial reverse transcripts in the virus life cycle. The formation of incomplete reverse transcripts following infection of quiescent cells appears to be a common phenomenon exhibited by multiple HIV-1 isolates (see Fig. 2). Quiescent T lymphocytes harboring such structures are capable of producing viable progeny virus if stimulated to proliferate by a mitogen added in vitro (23, 24). However, we had not formally proven that this partially reverse-transcribed species is responsible for the rescue of progeny virus following

FIG. 3. Kinetics of reverse transcription in primary cells. Quiescent and stimulated cells from the same normal-blood donor were infected with HIV-1_{JR-CSF} (2 μ g of p24^{gag} antigen per 10^7 cells). DNA was harvested at various time points within the first 20 h, as indicated. DNA from 5×10^4 cells was subjected to 25 cycles of amplification with the R/U5-, *nef*-, or LTR/*gag*-specific oligonucleotide primer. Standard curves of HIV-1 DNA from 10 to 25,000 copies were run in parallel. HIV-1 standards contained tRNA (4 μ g/ml) as carrier. β -Globin-specific primers were included in each reaction to standardize input cell DNA. Dilutions of uninfected PBL DNA were analyzed in parallel as controls. DNA samples are identical in all three panels. HI indicates DNA from cells treated with heat-inactivated virus.

activation of the infected cell. There are three viral genomic structures that could be found in infected quiescent cells: the incompletely reverse-transcribed viral species, viral genomic RNA that has not initiated the reverse transcription process, and a small amount of what appears to be full-length viral DNA which is likely formed in the few activated cells that exist in fresh PBL populations. We undertook a series of experiments to determine which of these structures was contributing to the production of progeny virus following stimulation of an infected quiescent cell population.

Full-length viral DNA is not responsible for progeny virus rescue. The three possible viral DNA structures in quiescent cells mentioned above are illustrated schematically in Fig. 4A. After formation of partial reverse transcripts in quiescent cells, reverse transcription that is newly initiated following mitogenic stimulation should be inhibited by the presence of the nucleoside analog AZT. Therefore, AZT treatment following infection would block DNA synthesis and replication of virus either from the incompletely reverse-transcribed species or from de novo synthesis from viral genomic RNA but not from completely synthesized viral DNA. Thus, any progeny virus produced would be derived from expression of previously completely reverse-transcribed viral DNA. Quiescent cells were infected with the HIV-1_{NL4-3} isolate, and the cells were incubated for 15 h to allow formation of partially or completely reverse-transcribed viral DNA. After this incubation period, the cells were divided into two pools, and AZT was added to one of the pools. Both cultures were subsequently stimulated with

PHA and cultured in the presence of soluble CD4 (obtained from Eric Daar, Cedars Sinai Hospital) to inhibit virus spread. Supernatants derived from these cultures were analyzed daily for virus production. Infected cells treated in this manner exhibited complete inhibition of virus production in the presence of AZT, while cells cultured in the absence of AZT produced large quantities of progeny virus (Fig. 4B). The sensitivity of the p24 ELISA is such that virus production can be detected even in the absence of virus spread and reinfection (see below). A similar experiment with cells from a different normal donor resulted in nanogram quantities of virus per milliliter by day 4 in untreated cells, with no virus production from cells treated with AZT following infection. Thus, assuming that AZT is not affecting integration, the viral intermediate in this system does not appear to be completely reverse-transcribed viral DNA but may be either non-reverse-transcribed viral genomic RNA or incomplete reverse transcripts.

The partial reverse transcript in infected quiescent cells can function as an intermediate in the virus life cycle. We next determined whether viral genomic RNA that had not yet initiated the reverse transcription process or whether the incompletely reverse-transcribed genomic species was the intermediate responsible for virus rescue. A second AZT inhibition experiment was performed (schematically illustrated in Fig. 5A). During infection of quiescent cells by HIV-1, formation of both partially and completely reverse-transcribed species would be blocked by AZT pretreatment, but viral genomic RNA that did not initiate reverse transcrip-

FIG. 4. Full-length reverse transcripts are not responsible for virus rescue following mitogenic stimulation of infected quiescent cells. (A) Schematic representation of experimental strategy of AZT treatment postinfection. (Top) Infected quiescent cells contain three potential forms of the HIV-1 genome: viral RNA that does not initiate reverse transcription, the partial reverse transcript, and a minute amount of full-length viral DNA. If a quiescent cell population harboring these structures is stimulated with PHA, virus production ensues. (Bottom) If the infected quiescent cell is treated with AZT postinfection and subsequently stimulated with PHA (in the presence of soluble CD4 to block virus spread), newly initiated reverse transcription will be blocked. Consequently, progeny virus should be released only from cells harboring full-length viral DNA prior to stimulation. (B) Virus production from cells treated with AZT postinfection. Quiescent PBL (10^7) were infected with $1.5 \mu\text{g}$ of HIV-1_{NL4-3} on day 0. Sixteen hours postinfection, the cells were treated for 2 h in the presence or absence of AZT ($5 \mu\text{M}$). PHA stimulation was then applied (day 1), and soluble CD4 ($10 \mu\text{g/ml}$) was added daily to prevent virus spread. Culture supernatants were harvested daily and analyzed by ELISA specific for HIV-1 p24^{gag} protein.

FIG. 5. Pretreatment of quiescent PBL with AZT. (A) (Top) Schematic representation of AZT pretreatment strategy. AZT pretreatment will prematurely terminate reverse transcription in quiescent cells, resulting in aborted reverse transcripts. Viral RNA that did not initiate reverse transcription will remain unaltered. At a time subsequent to infection, cells are rinsed and treated with nucleosides to compete with any remaining intracellular AZT. PHA treatment is then applied. Virus production should result only from viral genomes that did not initiate reverse transcription prior to mitogenic stimulation, as those that did initiate would be terminated by the AZT. (Bottom) Virus production from AZT-pretreated quiescent cells. Quiescent PBL (2×10^7) were cultured in the presence or absence of $5 \mu\text{M}$ AZT for 3 h on day 0. Cells were then infected with HIV-1_{NL4.3} ($2 \mu\text{g}$ of p24) for 2 h, rinsed, and cultured for 16 h in RPMI 1640 supplemented with 10% pooled human AB serum in the presence or absence of AZT. Following culture, cells were rinsed three times and cultured for 2 h in the presence of $50 \mu\text{M}$ nucleosides. The cells were then split into two pools. Cells (10^7) from each pool were stimulated with PHA, and $1 \mu\text{g}$ of soluble CD4 per ml was added to limit virus spread (day 1). Culture supernatants were harvested daily and analyzed by ELISA for viral p24 antigen. (B) AZT pretreatment inhibits reverse transcription in quiescent PBL. Quiescent PBL (5×10^6) were cultured in the presence (+N) or absence of $50 \mu\text{M}$ all four nucleosides or $2 \mu\text{M}$ AZT (+AZT) or both treatments (+N/AZT) for 3 h prior to infection with $1 \mu\text{g}$ of HIV-1_{JR-CSF}. Heat-inactivated (HI) controls were infected in parallel. Twenty hours postinfection, DNA was harvested and quantitative PCR was performed with 10^5 cell equivalents by using the primer pairs indicated. HIV-1 copy number standards were analyzed in parallel.

tion would remain uninhibited. Assuming that AZT treatment does not directly affect the stability of the viral genomic RNA, following the removal of the AZT subsequent mitogenic stimulation would be expected to induce production of progeny virus only from viral genomic RNA newly initiating the reverse transcription process, since the other genomic species would be aborted. Quiescent lymphocytes were preincubated in the presence or absence of AZT for 2 h prior to infection with HIV-1_{NL4-3}. The infected cells were incubated overnight under the same conditions in the presence or absence of AZT, rinsed extensively, and then exposed to high concentrations of nucleosides to compete with any residual AZT that might remain inside the cells. Figure 5B illustrates that AZT pretreatment inhibits reverse transcription in quiescent cells and that addition of excess exogenous nucleosides efficiently prevents this inhibition. The infected cells were stimulated with PHA and assayed for virus production by ELISA, as described above. Infected quiescent cells treated in this manner exhibited a three- to fivefold decrease in virus production following AZT pretreatment (Fig. 5A, graph), indicating that the majority of the progeny virus produced in the absence of AZT pretreatment was not derived from new reverse transcription from 'naive' viral RNA. A duplicate experiment utilizing cells from a different HIV-1-seronegative normal-blood donor yielded similar results: a two-and-a-half-fold decrease in virus production from AZT-treated cells.

On the bases of these results and the data indicating that complete viral DNA is not responsible for most of the virus rescue (Fig. 4), we conclude that the partially reverse-transcribed viral species is an intermediate that can be induced to form progeny virus following mitogenic stimulation of infected quiescent lymphocytes.

Efficiency of virus rescue following mitogenic stimulation. Our previous studies have shown that quiescent cells stimulated at later time points postinfection produce less progeny virus (23, 24) than those stimulated early after infection. In those experiments, reinfection and numerous rounds of virus production occurred following stimulation. To determine the efficiency of rescue of progeny virus from cells stimulated following infection in a single-cycle infection, stimulated and quiescent PBL were infected with a replication-defective HIV-1_{JR-CSF} pseudotyped with a murine amphotropic envelope glycoprotein. The entry of virus into the two cell types was equivalent as assayed by PCR (25). Quiescent cells were stimulated with PHA 15 h postinfection. Maximal virus production was approximately 7 ng/ml in prestimulated PBL and 0.4 ng/ml in cells stimulated following infection (Fig. 6). These results indicate that rescue of virus production by stimulation 15 h following infection is approximately 20-fold less efficient than virus production in preactivated cells. Thus, the intermediate described above, although biologically active, is much less efficient at completing the virus life cycle than a virus infecting an activated cell.

Partially reverse-transcribed viral genomes can be induced to reinitiate DNA synthesis. The partially reverse-transcribed viral genome is biologically active yet apparently inefficient in giving rise to a round of virus production. To investigate this phenomenon, we performed a molecular analysis. Quiescent cells were infected with the replication-defective HIV-1_{JR-CSF} and the infected cells were split into two pools, one of which was left quiescent. The other was stimulated with PHA 15 h postinfection, after formation of the intermediate. DNA was harvested prior to and 2 and 4 days after the PHA was added, and DNA was subjected to quantitative PCR analysis by utilizing primer pairs that distinguish dif-

FIG. 6. Quiescent or stimulated PBL (10^7) were infected with 2.5 ml of JRCSF-AFLA stock (100 ng/ml) on day 0 and analyzed for p24 by ELISA on the days indicated. Prestimulated cultures are indicated by solid squares. The infected quiescent cells were stimulated with PHA 15 h postinfection (on day 1) and are indicated by open triangles.

ferent regions of the viral genome. The level of R/U5 DNA in stimulated cells is comparable to that in cells left quiescent (Fig. 7), again indicating that *de novo* initiation of DNA synthesis from viral RNA is not responsible for induction of virus production. Primer pairs specific for the *tat/rev* region show only a slight increase in viral DNA following stimulation, as most transcripts have already proceeded through this region; however, the primer pair specific for the *gag* region of the viral genome (LA8/LA9) shows approximately a fivefold increase in copy number in cells stimulated following infection compared with cells left quiescent. Primer pairs that amplify full-length viral DNA (M667/M661) did not detect a difference above background between stimulated and quiescent cells, indicating that although further extension is induced, the completion of reverse transcription is very inefficient. Cells from a second normal-blood donor, infected with pseudotyped virus generated in a separate transfection, behaved similarly. This result accounts for the low efficiency of virus rescue seen when stimulation is applied 15 h postinfection (Fig. 6). Thus, the partially reverse-transcribed viral genome can be induced to further DNA synthesis by a mitogenic signal applied well after infection, but completion of DNA synthesis is very inefficient.

DISCUSSION

Mechanism responsible for arrest of reverse transcription.

The cardinal feature of retrovirus infection is conversion of the single-stranded RNA genome into double-stranded DNA by the action of viral reverse transcriptase. Studies initiated by Temin (20) and subsequently pursued by Temin and others (5, 7-9, 12, 13, 20, 22, 24, 26) indicated that the state of activation of a cell at the time of infection by retroviruses influences reverse transcription and virus expression. Our previous study identified an inability of HIV-1 to complete the reverse transcription process in quiescent T lymphocytes and more completely defined the structure of the viral

FIG. 7. Extension of HIV-1 DNA synthesis following mitogenic stimulation. Quiescent lymphocytes were infected with Env-minus HIV-1 pseudotyped with the amphotropic murine leukemia virus envelope (as described in Materials and Methods) on day 0. On day 1 postinfection, cells were split into two pools and PHA stimulation was applied to one pool (S). The other pool of cells remained quiescent (Q). DNA was harvested from each pool (10^5 cell equivalents) on the days postinfection indicated and analyzed by PCR using various primer pairs. HI indicates cells treated with heat-inactivated virus in parallel and harvested on day 1 postinfection. STDS, standards.

genome in these cells (23). The current study was undertaken to further characterize this phenomenon. Our data demonstrate that reverse transcription initiates with similar kinetics in both activated and quiescent T cells. Reverse transcription in mitogen-stimulated T cells is completed within 6 h following infection with HIV-1 (Fig. 3), in agreement with observations reported recently by Kim et al., who used Southern blot hybridization (14). Our data further indicate that in quiescent cells, synthesis of U3 DNA and some additional *nef*-encoding sequences 5' to the polypurine tract (a region synthesized immediately following the first template-switching event during reverse transcription) occurs with delayed kinetics. In addition, there is decreased extension in quiescent cells and premature termination of the reverse transcription process. It is still not known what factor(s) causes the premature termination of reverse transcription in quiescent cells. Possible mechanisms to consider include low concentration or sequestering of deoxyribonucleotides in quiescent T cells, certain structures in the viral RNA, binding of virion core proteins that halt reverse transcription, the presence of reverse transcriptase potentiators in activated cells, or inhibitors in quiescent cells. Our previous study showed that a graded decrease in the extent of reverse transcription seems to occur in these cells (23), suggesting that there is not a discrete stopping point such as would be seen if RNA secondary structure or binding of virion protein to specific regions of the RNA was blocking the progression of reverse transcriptase. We have analyzed

reverse transcription in quiescent cells by using additional primer pairs, and these also indicated a graded decrease occurring in this process (25). The current studies do not address the possible interaction of a cellular factor(s) with the viral capsid or directly with reverse transcriptase to influence reverse transcription.

Biologic role of partial reverse transcripts. A question of critical importance is whether the incompletely reverse-transcribed structure is a viable intermediate in the virus life cycle. Our previous studies indicated that quiescent cell populations harboring this structure also harbor a labile virus intermediate which can be rescued to produce virus following stimulation. Here, we demonstrate directly that the incompletely reverse-transcribed species is likely to be an intermediate for further reverse transcription and subsequent virus production. Postinfection treatment of quiescent cells with AZT followed by mitogenic stimulation of the cell abrogated rescue of progeny virus. This indicates that HIV which completed the reverse transcription process prior to stimulation is not responsible for the majority of virus production, as expression from completely reverse-transcribed DNA should be unaffected by treatment with nucleoside analogs. In addition, AZT treatment of quiescent cells prior to infection resulted in minimal virus production, suggesting that the majority of progeny virus was not produced by RNA that newly initiates reverse transcription after cell activation. Thus, the partial reverse transcript contributes significantly to production of progeny virus, and, therefore, we conclude that it is an intermediate in the virus life cycle.

Lability of partial reverse transcripts. The further DNA extension of partial reverse transcripts, as well as the rescue of progeny virus from infected quiescent cells, is inefficient. This is consistent with previous results which indicate that rescue of progeny virus is less efficient with stimulation at later times following infection (24). This inefficient rescue may be due to lability of the partially reverse-transcribed genome. Our previous work indicated that the viral DNA has a half-life of approximately 1 day in quiescent cells (23). Our current results suggest that DNA loss is not the only cause of inefficient rescue from cells stimulated within 1 day of infection. We find that although the minus strand of viral DNA is still present, further extension of the minus strand is inefficient. This could be explained by degradation of the viral RNA template, or alternatively, by lability of reverse transcriptase or other viral proteins that remain in association with the viral genome and are required for further extension to occur. On the basis of the virus rescue and PCR analysis of reverse transcription in a single-cycle infection, we estimate that only 5% of the initial infectious dose of virus can be rescued 15 h following infection of quiescent cells.

Extrapolation of these *in vitro* results to the *in vivo* situation is informative. We previously hypothesized that the lability of the partially synthesized viral DNA could explain the low proportion of infected cells in the peripheral blood of infected individuals. The even more extreme functional lability of the partially reverse-transcribed viral genome in quiescent cells indicates that these structures would be only marginally involved in virus latency but would play a critical role in clearance of the virus from infected quiescent cells. Only previously activated cells or cells that are activated shortly after infection by the virus would sustain a productive infection. In the remainder of the infected quiescent cells, viral replication would be aborted. This scenario could explain the low proportion of infected cells observed in infected individuals, and the resultant lowering of the

virus load would provide a means for self-limiting infection. Such a low-level persistent infection would thereby contribute to prolonging clinical latency in infected individuals.

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