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# Kinetics of Human Immunodeficiency Virus Type 1 Reverse Transcription in Blood Mononuclear Phagocytes Are Slowed by Limitations of Nucleotide Precursors

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**Human immunodeficiency virus type 1 infection of mononuclear phagocytes has been implicated in disease manifestations, but postentry viral replication events in these cells have not been well characterized. Productive infection of activated T cells is associated with cell proliferation and accumulation of full-length viral DNA within 6 h. In infected, nondividing quiescent peripheral blood lymphocytes, reverse transcription is aborted prior to full-length viral DNA formation. For nondividing, cultured mononuclear phagocytes, we now report a third pattern of reverse transcription with relatively slow kinetics, in which full-length viral DNA did not accumulate until 36 to 48 h. The reverse transcription rate in mononuclear phagocytes could be accelerated by addition of exogenous nucleotide precursors, but still not to the rate seen in activated T cells. These results indicate that substrate limitations in mononuclear phagocytes slow but do not arrest human immunodeficiency virus type 1 reverse transcription.**

Mononuclear phagocytes, which include blood monocytes and a variety of tissue macrophages, are principal host target cells for infection with human immunodeficiency virus type 1 (HIV-1). Most HIV-1 replication found in extravascular and extralymphoid tissues is associated with mononuclear phagocytes. In addition, these cells are permissive to some extent for infection with most primary HIV-1 strains. Recent reports have shown that HIV-1 strains associated with acute infection are uniformly macrophage tropic (35). Therefore, infection of these cells may be responsible for virus transmission, as well as for some of the clinical manifestations of AIDS, particularly AIDS dementia complex. Other lentiviruses are also thought to cause disease as a result of mononuclear phagocyte infection, including visna virus and caprine arthritis encephalitis virus (15). Because of the correlation with HIV-1 infection of mononuclear phagocytes and disease processes, investigations of events associated with establishment of infection in these cells may provide insights into HIV pathogenesis. However, details of HIV-1 replication in these cells have not been well defined, owing in part to limitations in the number of primary mononuclear phagocytes that can be obtained at one time from a donor, and to insufficient sensitivity in detection assays. Monocytoid cell lines such as U937 have been used to study HIV-1 replication (8, 18), but these cells are fundamentally different from primary mononuclear phagocytes in terms of cell proliferation and permissiveness for infection with primary virus strains. Therefore, we have focused our studies on infection of blood mononuclear phagocytes by using a brain-derived primary strain, HIV-$1_{JR-FL}$, in order to more closely represent HIV-1 replication in vivo. In this report, we show that HIV-1 reverse transcription proceeds slowly in cultured blood mononuclear phagocytes compared with phytohemagglutinin-activated peripheral blood lymphocytes (PBL) and requires 48 h for completion. This is due in part to limitations in the cellular nucleotide pool.

HIV-$1_{JR-FL}$ was isolated following short-term cocultivation of lectin-stimulated normal PBL with frontal lobe brain tissue from a patient who died with AIDS dementia complex (12). This primary isolate replicates efficiently in both cultured blood mononuclear phagocytes and stimulated PBL, but with different kinetics of virus production (Fig. 1). Virus production peaks several days after infection of activated PBL, but virus production is slower and more prolonged in mononuclear phagocytes and peaks at 14 days or later (12, 17).

There are other fundamental differences in HIV-1 replication between PBL and mononuclear phagocytes. Typically, productive retroviral infection requires cell proliferation and integration of reverse-transcribed viral DNA (5, 25, 28). This requirement is also seen with HIV-1 infection of PBL (33). However, mononuclear phagocytes can be productively infected by HIV-1 in the absence of cellular proliferation (31). We assessed cell division by measuring the fraction of cells that proceed through S phase in culture. Cultures of quiescent and activated PBL and mononuclear phagocytes prepared from the same donor, as previously described (17, 33), were pulsed with [$^3$H]thymidine for 24 h and subjected to autoradiography. Approximately 60% of stimulated PBL incorporate labeled nucleotide precursors during a 24-h period, beginning 24 h after HIV-1 infection (Table 1), whether infected with HIV-$1_{JR-FL}$ or heat-inactivated virus supernatants or in mock-infected cultures. In contrast, there is very little cellular DNA synthesis in quiescent PBL; fewer than 1% of quiescent cells proceed through S phase, and the majority of these cells are not productively infected, as previously reported (33).

In mononuclear phagocytes, fewer than 1 in 500 cells incorporate [$^3$H]thymidine during a 24-h period, either on day 2 (Table 1) or day 7 after infection (not shown). Despite the nearly complete absence of cell proliferation, virus production is detected in mononuclear phagocytes 3 to 4 days after infection and peaks at 14 days. Because of the marked

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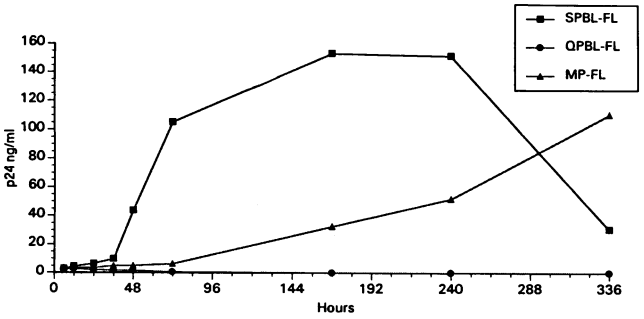


FIG. 1. Kinetics of HIV-$1_{JR-FL}$ replication in primary cells. Quiescent PBL cultures (QPBL-FL) ($10^7$), PHA-activated PBL (SPBL-FL) ($10^7$), or mononuclear phagocytes (MP-FL) ($10^7$) from the same donor were infected with 1.0 μg of HIV-$1_{JR-FL}$ p24 (enzyme-linked immunosorbent assay, Coulter) in the presence of 10 μg of Polybrene per ml for 2 h at 37°C. Contaminating viral DNA in virus supernatants was removed by DNase I treatment (33). Heat-inactivated virus supernatants were treated at 60°C for 1 h. Following infection, cells were washed in serum-free medium and placed in culture. Mononuclear phagocytes were infected after 7 days of adherence and were maintained in Iscove's medium supplemented with 20% fetal calf serum, penicillin-streptomycin, and glutamine. After infection, culture supernatants were assayed for virus at 4, 12, 24, 36, 48, and 72 h and at days 7 and 14.

differences in cellular DNA synthesis and pattern of virus production in stimulated PBL and mononuclear phagocytes, we investigated reverse transcription in these cell types to determine whether there are differences in viral DNA synthesis.

**PCR analysis of HIV-1 DNA.** To compare reverse transcription in PBL and mononuclear phagocytes, we utilized quantitative PCR analysis to compare the kinetics of viral DNA formation, following in vitro infection, with oligonucleotide primers specific for DNA structures present at various stages of the reverse transcription process, as previously described (33, 34). In these earlier studies, we showed that virus entry and initiation of reverse transcription are comparable in stimulated and quiescent PBL, but formation of full-length viral DNA does not occur in the quiescent cells. Furthermore, the kinetics of reverse transcription appear to be slower in quiescent cells (34). We used the same PCR oligonucleotide primer pairs to measure the kinetics of HIV-$1_{JR-FL}$-specific DNA synthesis in mononuclear phagocytes infected with cell-free HIV-$1_{JR-FL}$. Total cellular nucleic acids were isolated from cells at various times after infection and analyzed by PCR (Fig. 2).

TABLE 1. Proliferation of primary blood cells as measured by autoradiography following 24-h incubation with [$^3$H]thymidine[a]

| Infection | % of total cells containing silver grains | | |
|---|---|---|---|
| | Mononuclear phagocyte | Stimulated PBL | Quiescent PBL |
| Mock | <0.1 | 60 | 0.3 |
| HIV-$1_{JR-FL}$ | <0.1 | 62 | 0.2 |
| Heat-inactivated virus | <0.1 | 59 | 0.3 |

[a] Stimulated PBL, quiescent PBL, or macrophages were cultured in the presence of 0.5 μCi of [$^3$H]thymidine per ml for 24 h starting 1 day after infection. Cells were vigorously rinsed with phosphate-buffered saline, fixed with acetone, and dried. The fixed cells were coated with Kodak NTB-2 photographic emulsion, dried, and exposed for 3 h. The emulsion was developed for 2.5 min in Kodak D19, rinsed in distilled water, and fixed for 3 min in Kodak fixer. Cells were stained with Giemsa stain and screened for the presence of silver grains in the overlying emulsion; results are displayed as percentage of total cells containing silver grains. At least 1,000 cells were counted on each slide.

Early products of viral reverse transcription were detected with a PCR oligonucleotide primer pair specific for the R/U5 region of the long terminal repeat (LTR) (M667/AA55). This primer pair will amplify the first region of viral DNA synthesized during reverse transcription and requires only 140 nucleotides (nt) of minus-strand DNA synthesis. This DNA region is synthesized during the first 4 h following HIV-$1_{JR-FL}$ infection in both stimulated PBL and mononuclear phagocytes (Fig. 2). Increased levels in PBL at later times reflect newly initiated reverse transcripts due to HIV-1 spread through the culture. In mononuclear phagocytes, however, there was no further increase in newly initiated reverse transcripts formed between 4 and 72 h. In this case, there was no increase in total viral DNA transcripts and thus no reinfection, consistent with the delayed kinetics of virus production compared with PBL. Therefore, entry and initiation of reverse transcription in both PBL and mononuclear phagocytes occur within 4 h of virus inoculation.

The primer pair M667/M661 flanks the primer binding site and thus detects only full-length or nearly full-length viral DNA (33). Full-length HIV-1 DNA begins to accumulate by 4 h postinfection in stimulated PBL, as seen previously (34); however, full-length viral DNA was not detected in mononuclear phagocytes until 24 h after infection. Levels of full-length HIV-1 DNA increased markedly by 48 h. Thus, the majority of viral DNA was completely synthesized between 36 and 48 h after infection.

PCR primers LA45/LA64 and LA8/LA9 detect viral DNA structures at intermediate stages of reverse transcription. The primer pair LA45/LA64 is specific for *tat/rev* viral DNA, which is formed early after the first reverse transcription template switch, and LA8/LA9 detects sequences in the *gag* gene, which are formed just prior to completion of minus-strand viral DNA synthesis. Viral DNA detected by *tat/rev* primers (LA45/LA64) was formed by 24 h in mononuclear phagocytes, with no further increase for up to 72 h. Using *gag* primers (LA8/LA9), we detected very little viral DNA at 24 h, with increases at 36 and 48 h. Thus, reverse transcription in mononuclear phagocytes proceeds with slower kinetics than that in PBL, with accumulation of full-length viral DNA occurring primarily between 36 and 48 h, versus 4 to 6 h in T cells. The slow kinetics of full-length viral DNA formation in mononuclear phagocytes correlate with the delayed appearance of new virus, which is not detected in culture supernatant until 72 h or more postinfection.

Zidovudine (azidothymidine or AZT) has been shown to block HIV-1 replication in mononuclear phagocytes (19), as well as in PBL by inhibiting reverse transcription. Completion of viral DNA synthesis in mononuclear phagocytes would be predicted to be sensitive to AZT for a more prolonged period of time, providing further support for the delayed kinetics of reverse transcription. We specifically examined the effect of AZT treatment on virus production and accumulation of reverse transcription intermediates in mononuclear phagocytes. Viral DNA was assayed by quantitative PCR following infection of mononuclear phagocytes cultured in the presence of 10 μM AZT. The cells were pretreated with AZT for 6 h prior to infection with HIV-$1_{JR-FL}$ or were treated with AZT beginning 24 h after infection. Accumulation of early products of reverse transcription in mononuclear phagocytes, as determined with the oligonucleotide primer pair M667/AA55, was not affected by AZT treatment (Fig. 3, top). However, this treatment prevented accumulation of full-length viral DNA (Fig. 3, bottom, M667/M661), even if cells were not treated

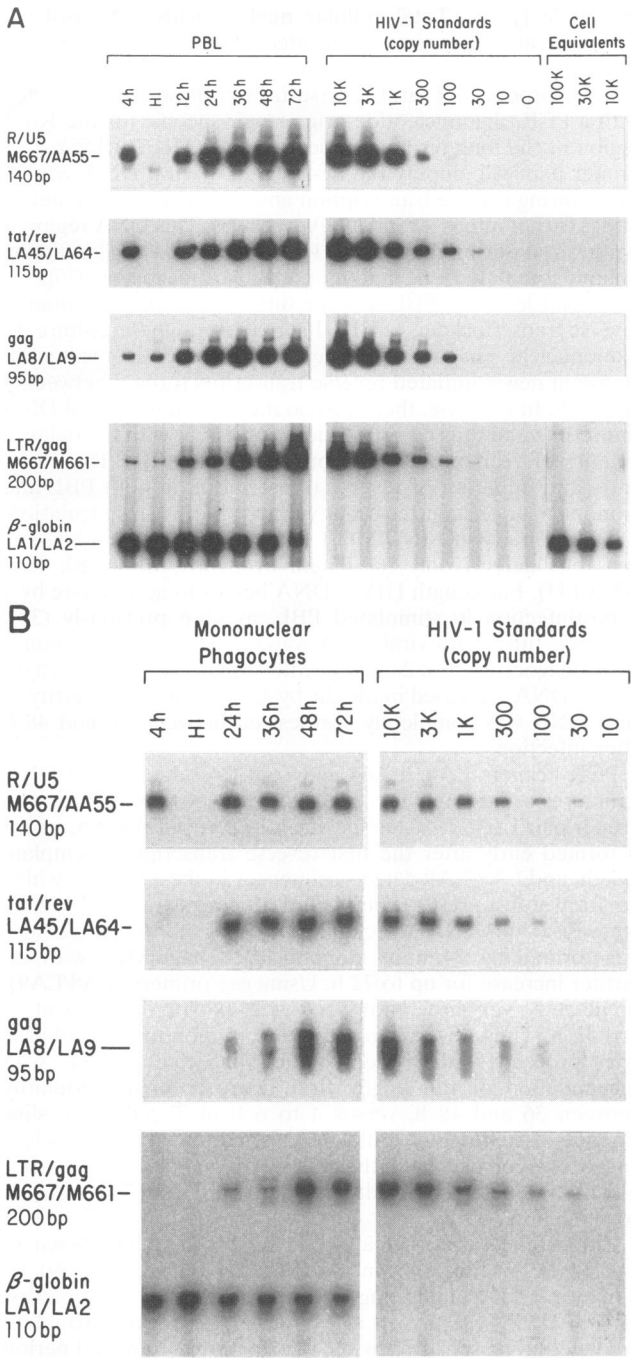


FIG. 2. Kinetics of reverse transcription in primary cells. Quantitative PCR amplification with $^{32}$P-end-labeled primers was performed as previously described (16, 20, 34). DNA was harvested from stimulated PBL (A) and mononuclear phagocytes (B) (20, 34) (infected as described in the legend to Fig. 1) at various time points in the first 72 h after infection, as indicated. DNA from $10^5$ cells was subjected to 25 cycles of amplification (94°C for 1 min and 65°C for 2 min) with either M667/M661 (LTR/*gag*), LA8/LA9 (*gag*), LA45/LA64 (*tat/rev*), or M667/AA55 (R/U5) oligonucleotide primer pairs. Cloned HIV-1 DNA standards (linearized pYKJRCSF) from 10 to 10,000 copies containing tRNA (20 μg/ml) as carrier were amplified in parallel. Human β-globin primers (24) were included in reactions with LTR/*gag*-specific primers. DNA samples were identical in all four panels. "HI" indicates DNA from cells infected with heat-inactivated virus extracted 4 h after infection. Results are a representative experiment of 12 performed.

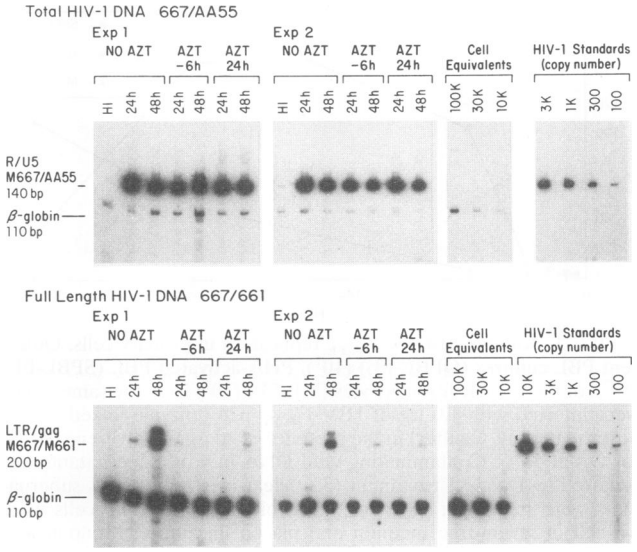


FIG. 3. Treatment of mononuclear phagocytes with AZT. Mononuclear phagocytes from two different donors (Exp 1 and Exp 2) were infected with HIV-$1_{JR-FL}$ as described for Fig. 1. Cultures were maintained in Iscove's medium and 20% fetal calf serum throughout (NO AZT) or pretreated with 10 μM AZT 6 h prior to infection and continuously after (AZT-6h) or starting 24 h after infection (AZT 24h). DNA was isolated from infected cultures at 24 or 48 h, and the same samples were analyzed by PCR with M667 and AA55 (140 bp, upper panel) or with M667 and M661 (200 bp, lower panel). Human β-globin primers were included in each reaction. Cultures inoculated with heat-inactivated virus (HI) were analyzed at 24 h. HIV-1 copy number and cell DNA standards were analyzed in parallel. Results are a representative experiment of three performed.

until 24 h following infection. Virus production was also blocked in these AZT-treated cells (data not shown). Therefore, AZT treatment, even if initiated 24 h after infection, was able to block full-length viral DNA synthesis in mononuclear phagocytes. AZT treatment did not affect formation of the earliest viral reverse transcripts, presumably because incorporation of AZT does not occur within this short (140-nt) stretch of cDNA. Inhibition of reverse transcription and virus production by AZT 24 h after infection provides further support for a slower rate of reverse transcription in mononuclear phagocytes by demonstrating that full-length viral DNA accumulates largely after 24 h.

We tested another HIV-1 strain to determine whether slower kinetics of reverse transcription in mononuclear phagocytes were strain dependent. Reverse transcription kinetics were similarly slow in mononuclear phagocytes infected with the recombinant virus HIV-$1_{NFN-SX}$ (data not shown). This strain can enter mononuclear phagocytes efficiently because it contains most of the *env* gene of HIV-$1_{JR-FL}$ (17); however, the *pol* gene is derived entirely from the infectious clone pNL4-3 (1). Therefore, the slow kinetics of reverse transcription are not related to specific characteristics of HIV-$1_{JR-FL}$ *pol* gene functions.

We next investigated the potential role of nucleotide pools in regulation of reverse transcription. Since mononuclear phagocytes generally do not divide, a substantial nucleoside pool for cellular DNA synthesis is not required. One possible explanation for slow reverse transcription in blood mononuclear phagocytes could be a limitation in intracellular nucleotide precursors. To test this possibility, mononuclear phago-

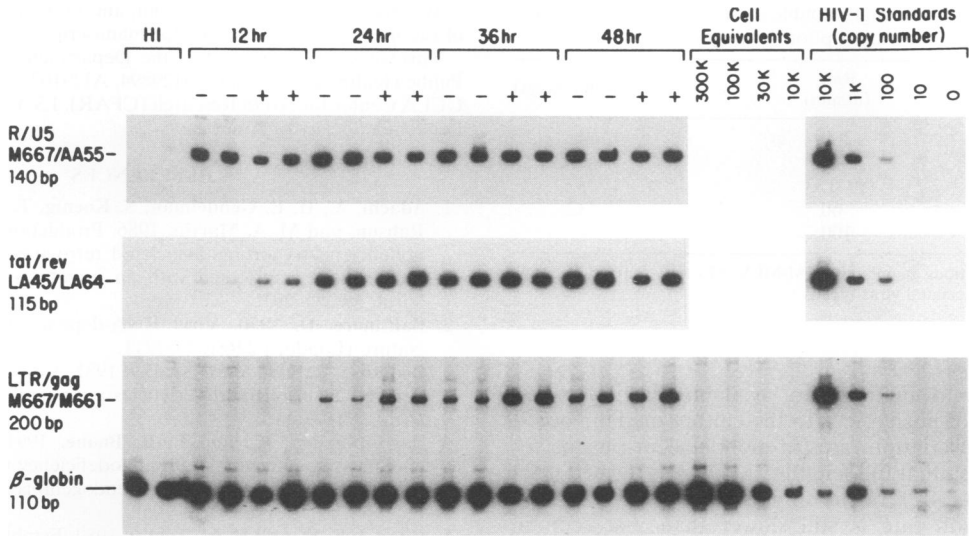


FIG. 4. Kinetics of reverse transcription following nucleoside loading. Mononuclear phagocytes infected as described for Fig. 1 were cultured in duplicate in the presence or absence of 50 μM all four nucleosides for 3 h prior to infection and continuously postinfection. DNA was isolated at the indicated times and analyzed by PCR with the primers indicated. Amplification of cell equivalent and HIV-1 copy number standards were performed in parallel. Results are a representative experiment of three performed.

cytes were cultured in the presence or absence of 50 μM (each) all four nucleosides (2′-deoxycytosine, 2′-deoxyadenosine, 2′-deoxyguanosine, and 2′-deoxythymidine) for 3 h prior to infection with HIV-$1_{JR-FL}$, and the effect of the kinetics of reverse transcription was examined by PCR at 12-h intervals (Fig. 4). Amplification with the R/U5 primer pair (M667/AA55), which detects the first viral DNA region formed in reverse transcription, demonstrated that there was no new initiation of reverse transcription between 12 and 48 h, either with or without addition of exogenous nucleosides. Thus, nucleoside addition did not affect the number of viral templates initiating reverse transcription.

Using the *tat/rev* primer pair, LA45/LA64, which detects structures formed at an intermediate step of reverse transcription, or the LTR/*gag* primer pair, M667/M661, which detects complete or nearly complete reverse transcription, we found that reverse transcription proceeds more rapidly in the presence of increased concentrations of nucleosides, with accumulation of full-length viral DNA at 24 to 36 h rather than at 36 to 48 h as seen in untreated cells. However, this increased rate is still slower than that seen in stimulated PBL in which reverse transcription is completed in 4 to 6 h (11, 33) (Fig. 2A). Therefore, limited levels of nucleotide precursors contribute to but are apparently not entirely responsible for the relatively slow kinetics of reverse transcription seen in mononuclear phagocytes.

Rapid completion of reverse transcription in PBL is associated with cell proliferation, and we considered whether increased mononuclear phagocyte proliferation occurred with addition of exogenous nucleosides to the culture medium. However, this treatment did not result in an increase in cell number, on the basis of cell number counted in duplicate wells in 24-well trays. Thus, nucleoside-induced effects on the kinetics of reverse transcription in mononuclear phagocytes do not appear to be associated with increases in cell proliferation.

The present study has demonstrated differences in kinetics and extent of HIV-1 reverse transcription in blood-derived mononuclear phagocytes and in activated and quiescent T cells. In activated T lymphocytes which divide, productive HIV-1 infection is associated with accumulation of full-length viral DNA within 12 h (Fig. 2). Our previous studies with nondividing quiescent PBL showed that the block to productive infection was the result of incomplete reverse transcription, with formation of a labile viral DNA intermediate (33). In quiescent PBL, reverse transcription was initiated as efficiently as in stimulated PBL, but elongation proceeded more slowly and was not completed (34). This is similar to what was seen following infection of serum-starved cells with either Rous sarcoma virus or spleen necrosis virus, which did not support efficient synthesis of virus-specific DNA, but rather resulted in short DNA fragments and less total viral DNA (5, 25, 30). Infection did occur in these quiescent cells, and progeny virus could be recovered if the cells were stimulated at early times after infection, similar to our results seen with HIV-1. Therefore, functions important for completion of reverse transcription and productive infection in these cases correlate with cell proliferation. In HIV-1-infected mononuclear phagocytes, reverse transcription proceeds more slowly than in activated T cells, but unlike quiescent PBL, reverse transcription in mononuclear phagocytes is completed in the absence of cell proliferation and results in productive infection.

Reverse transcription has been studied in vitro following detergent lysis (termed the endogenous reaction) of retroviruses. The efficiency of in vitro reverse transcription was affected by a number of factors (2, 3, 9, 10, 13, 21–23, 26). Under optimal conditions, the rate of polymerization was approximately 240 nt/min. However, the rate of full-length double-stranded DNA synthesis was slower (75 to 100 nt/min) (Table 2) and less efficient, possibly because of the requirement for template switching. The rate of HIV-1 in activated T cells has been shown to be 55 to 77 nt/min (11, 34), similar to that seen for other retroviruses (Table 3).

The rate of HIV-1 reverse transcription in mononuclear phagocytes that we measure here is 7 nt/min, which is considerably slower than in T cells. Our results indicate that one reason for delayed kinetics of reverse transcription in mono-

TABLE 2. Rates of full-length double-stranded retroviral DNA formation by reverse transcription (endogenous reaction)

| Virus[a] | Rate (nt/min) | Reference(s) |
|---|---|---|
| EIAV | 200 | 4 |
| M-MLV | 75–100 | 9, 23 |
| RAV-2 | 89–133 | 3 |
| HIV-$1_{SF2}$ | 60 | 32 |
| HIV-$1_{IIIB}$ | 100 | 4 |

[a] EIAV, equine infectious anemia virus; M-MLV, Moloney murine leukemia virus; RAV-2, Rous-associated virus type 2.

nuclear phagocytes is limitations of available nucleotide precursors. Addition of nucleosides to the culture medium accelerates reverse transcription rates in mononuclear phagocytes to 10 to 13 nt/min so that full-length viral DNA is formed in 24 to 36 h rather than 48 h or more. However, this accelerated reverse transcription rate is still slower than that seen in stimulated PBL and transformed cell lines. Cellular nucleotide pools are relatively high in proliferating T cells (estimated concentration of 5 nmol/$10^6$ cells) (14) and may contribute to the observed rapid reverse transcription rate. In the endogenous reaction of both murine leukemia virus and avian sarcoma virus, accumulation of full-length reverse transcripts required a deoxynucleotide concentration greater than 1 to 2 mM (6, 7, 21, 23). However, nucleotide pools are quite low in mononuclear phagocytes (19) (estimated concentration of 1 pmol/$10^6$ cells) (27) and appear to be limiting for reverse transcription. It is also possible that inefficient nucleoside uptake and/or phosphorylation or increased degradation may contribute to the slower rate of reverse transcription, although this rate is increased when mononuclear phagocytes are cultured in the presence of 50 μM deoxynucleosides. In contrast, addition of exogenous nucleotide precursors does not result in completion of reverse transcription in quiescent PBL (34), suggesting that there may be quantitative differences in the size or utilization of intracellular nucleotide pools or that factors regulating reverse transcription may be different in PBL and mononuclear phagocytes. It is also possible that there may be other factors in addition to nucleotide availability, such as a cellular reverse transcriptase inhibitor or a lack of a cellular reverse transcription potentiator in mononuclear phagocytes and quiescent PBL.

Infected mononuclear phagocytes may persist in a variety of tissues in vivo and serve as a reservoir for HIV-1. Further characterization of slow HIV-1 reverse transcription in mononuclear phagocytes may suggest novel strategies to prevent establishment of infection.

TABLE 3. Rates of full-length double-stranded retroviral DNA formation by reverse transcription (intracellular reaction)

| Virus | Cell type | Rate (nt/min) | Reference |
|---|---|---|---|
| HIV-$1_{W13}$ | H9 | 77 | 11 |
| HIV-$1_{JR-CSF}$ | PBL | 51 | 34 |
| ASV[a] | Quail tumor cells | 30 | 29 |
| HIV-$1_{JR-FL}$ | Mononuclear phagocytes | 7 | |
| HIV-$1_{JR-FL}$ | Mononuclear phagocytes and dNTP[b] | 10–13 | |

[a] ASV, avian sarcoma virus.
[b] dNTP, deoxynucleoside triphosphate.

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