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Human Immunodeficiency Virus Type 1 Replication Is Blocked prior to Reverse Transcription and Integration in Freshly Isolated Peripheral Blood Monocytes

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Peripheral blood monocytes are resistant to productive human immunodeficiency virus type 1 (HIV-1) infection in vitro immediately after isolation. No viral cDNA (either early or late transcripts) was detected by PCR in monocytes exposed to virus on the day of isolation. In contrast, in monocytes cultured for as little as 1 day, initiated and completed reverse transcripts were readily detectable within 24 h of infection with both HIV-1_{Ba-L} and primary isolates. The levels of initiated, partially completed, and completed viral DNA copies found 24 h after infection increased progressively with time in culture before infection. Unlike quiescent T lymphocytes, there appeared to be no block or delay in the integration of viral DNA into the genome of susceptible cultured monocytes. With an *Alu*-PCR method designed to specifically detect proviral DNA being used, integration events were found within 24 h of infection in monocytes cultured for a day or more after isolation. No integration signal was found in freshly isolated monocytes up to 7 days following exposure to the virus. Cloning and sequencing of *Alu*-PCR-amplified DNA confirmed integration in HIV-1-infected cultured monocytes. Our finding that in vitro replication of HIV-1 is clearly blocked prior to the initiation of reverse transcription in freshly isolated peripheral blood monocytes suggests that these cells may not be susceptible to infection in vivo. Further studies to clarify this possibility and the nature of the block to infection should provide useful information for treatment strategies against HIV-1.

Blood monocytes and tissue macrophages from human immunodeficiency virus type 1 (HIV-1)-infected individuals have been shown to harbor the virus (10, 13, 18, 34), and because the cytopathology in these cells appears to be minimal, they have the potential to act as reservoirs of infection and to disseminate the virus to other cells within the infected individual. It is not clear whether, in vivo, the majority of infected monocytes and macrophages is productively infected or whether the virus is maintained in them in some form of latent state and how this balance changes with disease progression (21, 23, 32, 41). In addition, it is also not known whether the low proportion of monocytes in peripheral blood in which viral DNA is detectable (13, 21, 23) is made up of immature cells or represents a more mature subset of monocytes which is more susceptible to infection (31). However, cells of this lineage can be productively infected in vitro with macrophage-tropic isolates (6, 10, 22, 45). Infection of monocytes in vitro appears to be linked, at least in part, to differentiation (29, 33, 36), and by this phenomenon, HIV-1 resembles some of the other members of the lentivirus subfamily of retroviruses which replicate more efficiently in tissue macrophages than in blood monocytes (11, 25). In addition, replication in monocytes is generally enhanced by factors which promote differentiation (16, 19, 32).

The capacity of HIV-1 to infect and persist in nondividing cells presumably contributes to the pathogenesis of this infection. Virus is found in macrophages in various organs, including the brain, where macrophages and microglia are the cells predominantly infected (18, 44). A recent report has clarified the previously contradictory data on the ability of HIV-1 to persist in quiescent, nondividing T lymphocytes (38, 40, 46, 47). In a primary T-cell model of acute infection of quiescent CD4 lymphocytes, HIV-1 was able to complete reverse transcription and persist in a viral DNA form that was stable for over 10 days in culture (37). In the absence of cell activation, transcription was blocked in these cells, but upon stimulation with phytohemagglutinin at any time after infection, productive infection proceeded. This work is consistent with in vivo data showing that in T lymphocytes of the peripheral circulation of HIV-infected individuals, the majority of which are quiescent, viral DNA exists predominantly as an extrachromosomal form (2, 3).

Although the molecular details of HIV-1 infection of primary CD4 lymphocytes in various states is now quite well understood, comparatively little is known about the infection in cells of the macrophage lineage. It is not known, for example, whether there is an equivalent in monocytes or macrophages to the stable, inducible form of extrachromosomal viral DNA found in quiescent lymphocytes. Until recently, it was not clear whether integration of viral DNA is a requirement for productive infection of nondividing monocytes and macrophages. Previously, the only direct evidence purporting to demonstrate integration in monocyte-derived macrophages (MDM) consisted of Southern hybridization of high- and low-molecular-weight DNA fractions from infected cells (45). Such analysis alone, however, cannot rule out the possibility that unintegrated viral DNA was simply trapped in the chromosomal fraction. In a recent report, Englund et al. (7) used molecular clones of macrophage-tropic isolates with mutations in the

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integrase gene which render them incapable of integration to demonstrate unequivocally that integration is an obligate step in the productive infection of primary human MDM as well as activated peripheral blood CD4 lymphocytes.

However, there is as yet no information on the kinetics of integration in productively infected monocytes or macrophages or whether this step is delayed or blocked in resistant versus susceptible monocytes. Also, it has not been determined if fresh monocytes are less susceptible to infection than MDM or tissue macrophages because of differences in virus entry or in replication steps after entry. We have endeavored to define HIV-1 infection in these important cells further, at the molecular level, by determining the extent of reverse transcription and the kinetics of integration in monocytes and culture-derived macrophages. To detect integration in macrophages, we developed a PCR-based method which takes advantage of the existence of repeated sequences within the human genome (14), the most abundant of which is the *Alu* family of approximately 300-bp elements with almost a million copies, or an estimated 5 to 10% of the human genome (39).

MATERIALS AND METHODS

Cells and infection. Monocytes were isolated on the day of blood collection from HIV-seronegative buffy packs (Red Cross Bank, Melbourne, Australia) by plastic adherence and were either infected immediately after isolation or maintained for various periods before infection in suspension culture as detailed previously (6, 36). Cultures were made up of 90% ± 3% monocytes on the day of isolation, as determined by flow cytometric analysis with anti-CD11c monoclonal antibody (Becton Dickinson, Mountain View, Calif.), and were prepared and maintained under endotoxin-free conditions (36).

Fractions of monocyte cultures from each individual donor were infected immediately postisolation (within 10 h of blood collection) or after 1 to 7 days in culture with HIV-1_{Ba-L} (10) or low-passage primary macrophage-tropic isolates HIV-1_{17}, HIV-1_{19}, or HIV-1_{676} (kindly provided by A. Dunne and D. McPhee, Macfarlane Burnet Centre for Medical Research). Inocula were standardized by p24 antigen content (100 ng of p24 per 10^6 cells, as determined by enzyme immunoassay; Abbott Laboratories, Abbott Park, Ill.) and consisted of cell-free peripheral blood mononuclear cell (PBMC) culture supernatants filtered through a 0.22-μm-pore-size filter and pretreated with 10 U of RNase-free DNase I (Boehringer Mannheim Australia, Castle Hill, New South Wales, Australia) per ml for 20 min at room temperature in the presence of 10 mM $MgCl_2$ to remove contaminating viral DNA (29). Cells were exposed to virus for 2 to 4 h at 37°C and then were washed, resuspended in fresh medium, and returned to polytetrafluorethylene (Teflon) pots (Savillex, Minnetonka, Minn.). The percentage of infected cells in each culture was assessed 14 days later by intracytoplasmic p24 antigen detection by flow cytometry as described previously (6, 36).

Preparation of DNA for PCR. Cell lysates were prepared by a modification of the method described by Rich et al. (29). Cells (5×10^5) were washed with phosphate-buffered saline and then lysed with 50 μl of lysis buffer (50 mM NaCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg of bovine serum albumin [BSA] per ml, 0.45% Nonidet P-40, 0.45% Tween 20, and 50 μg of proteinase K per ml). The lysates were then heated at 56°C for 1 h and then at 95°C for 10 min to inactivate the proteinase K and were stored at −20°C until used in PCR without further purification. Lysates from 8E5 cells, which contain a single copy of proviral DNA per cell (9), were prepared as described above for use in quantitation.

Detection of cDNA synthesis. Semiquantitative PCR was used to detect reverse transcripts in lysates of infected monocytes at various times postinfection. The cell lysates (10 μl) described above were assayed in 50-μl reaction mixtures comprising 50 mM NaCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM (each) deoxynucleoside triphosphates (dNTPs), 0.25 μM sense and antisense primers, and 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Following initial denaturation at 95°C for 2 min, the samples were subjected to 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min in a Perkin-Elmer Cetus thermocycler. A final extension step of 7 min at 72°C was also included. For quantitation, 10-fold dilutions of 8E5 lysates, corresponding to 10^1 to 10^4 copies of HIV-1 DNA, were amplified with each set of monocyte lysates under the same conditions.

After amplification, 10-μl samples were run on 2% agarose gels, transferred to nylon membrane (Hybond N^+; Amersham Australia, Castle Hill, New South Wales, Australia), and hybridized with a full-length HIV-1 probe (derived from plasmid pHXB2) labelled with ^{32}P by random priming (Megaprime; Amersham). Hybridization was performed at 65°C for 16 h in 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA)–5× Denhardt's solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone)–0.5% sodium dodecyl sulfate (SDS). Membranes were then washed twice for 10 min at room temperature in 2× SSPE–0.1% SDS, once for 15 min at 65°C in 1× SSPE–0.1% SDS, and once for 15 min at 65°C in 0.1× SSPE–0.1% SDS before being exposed to X-ray film (Hyperfilm MP; Amersham).

The primer pairs used to determine the extent of reverse transcription in infected monocytes were the U5 sense (5′ ACTCTGGTAACTAGAGATCCCT CAGA 3′; nucleotides [nt] 578 to 603 of the consensus B sequence, 1993 Human Retroviruses and AIDS Sequence Database [24]) and PBS antisense (5′ GTC CCTGTTCGGGCGCCAC 3′; nt 653 to 635) primers, which amplify the first region of the HIV-1 genome reverse transcribed, SK68 and -69, which amplify in the *env* region and thus detect intermediate-length transcripts, and SK38 and -39, which amplify in the *gag* region and will detect only transcripts which have gone almost to completion (27). The amount of DNA in the lysates was standardized with HLA-DQα primers GH26 and -27 and oligonucleotide probe GH64 (30).

Detection of integrated DNA (provirus) by *Alu*-PCR. To specifically detect integrated DNA but not unintegrated forms, a method making used of the ubiquitous repeat elements found in the human genome was developed. Primers were designed to amplify between *Alu* repeat sequences, the most abundant of these repeat elements (14), and adjacent long terminal repeat (LTR) sequences. This method is similar to one described previously (5), although it was developed independently. Rather than using nested PCR, we used two outward-facing *Alu* primers to increase the chances of amplification with an LTR sequence, since the repeat elements can occur in either orientation relative to the integrated provirus. A 5- to 10-μl amount of the same lysates described above was amplified in 50-μl reaction volumes comprising 50 mM NaCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM (each) *Alu* primers, 0.8 μM U3 primer, and 2 U of *Taq*. Samples were first denatured at 95°C for 2 min and then were amplified for 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min, with a final 7-min extension step at 72°C. The primers used for *Alu*-PCR were *Alu* 278 antisense (5′ GCCTCCCAAAGTGCTGGGATTACAG 3′; nt 47 to 23 [26]), *Alu* 3′ sense (5′ TCCCAGCTACTGGGGAGGCTGAGG 3′; nt 164 to 187 of the *Alu* consensus sequence [15]), and U3 antisense (5′ CCTGGTGTGTAGTTCT GCCAATCAG 3′; nt 100 to 76, 1993 HIV consensus B sequence [24]). For analysis of lysates of cells infected with primary isolates, nested *Alu*-PCR, as described below, was used to enhance sensitivity for the detection of integration events.

Following *Alu*-PCR, 10-μl samples were electrophoresed in 1% agarose gels, transferred to nylon membranes, and hybridized with a ^{32}P-labelled, LTR-specific oligonucleotide probe (5′ TGGAAGGGCTAATTTACTCCCAAAAAA GACAAGATATCCT 3′; nt 1 to 40). Hybridization was performed at 42°C for 16 h in 1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% SDS, 1% polyethylene glycol, and 0.5% skim milk powder, after which the membranes were washed as described above, except that the final two washes were at 42°C, and exposed to film. As stated below, some reactions were performed with ^{32}P-labelled U3 primer at 0.4 μM and the PCR products were separated on 6% acrylamide minigels (Mini-PROTEAN II cell; Bio-Rad, Hercules, Calif.) before the gels were dried and autoradiographed.

Cloning and sequencing of *Alu*-PCR products. Nested *Alu*-PCR was performed on lysates of two different donor monocyte cultures which had been infected with HIV-1_{Ba-L} after 5 to 7 days in culture and lysed at 5 to 7 days postinfection. First-round PCR with 5 μl of lysates (equivalent to 5×10^4 cells) was performed with *Alu* 278 and PBS primers, while the second round was done with the same *Alu* primer together with an antisense primer to the R region (5′ AGGCTCAGATCTGGTCTAA 3′; nt 484 to 466) on 2 μl of the first-round product. The PCR conditions and cycling for each round were as described above for single-round *Alu*-PCR.

Amplified DNA fragments (300 to 1,000 bp) from the second-round reaction were then purified from a 1% low-melting-temperature agarose gel with a Prep-A-Gene matrix (Bio-Rad), ligated into pGEM-T vector (Promega), and transformed into competent JM109 cells according to the manufacturer's protocol. Clones containing inserts were then screened for LTR sequences by colony hybridization with the LTR oligonucleotide probe, and positive clones were further checked by PCR with *Alu* 278 and R primers. For sequencing, miniprep DNA from clones with inserts with sizes of 500 to 700 bp was amplified with M13 forward and reverse primers, and the products were purified and then sequenced by PRISM Ready Reaction Dye Primer Cycle Sequencing with Dye-labelled T7 and SP6 primers (Applied Biosystems Inc., Foster City, Calif.).

RESULTS

Reverse transcription in HIV-1-infected fresh and cultured monocytes. To first determine how soon after infection cDNA synthesis could be detected in fresh monocytes and cultured MDM, monocytes from the same donor culture were infected with HIV-1_{Ba-L} on the day of isolation or after 7 days in culture and then were lysed at 12 h, 48 h, and 14 days postinfection. The cell lysates were analyzed by PCR with primers to the *gag* region (SK38 and -39), which will only detect reverse transcripts which are almost full length. Complete transcripts were detectable within infected MDM by 12 h, with the copy number increasing with the time of infection (Fig. 1A). In contrast,

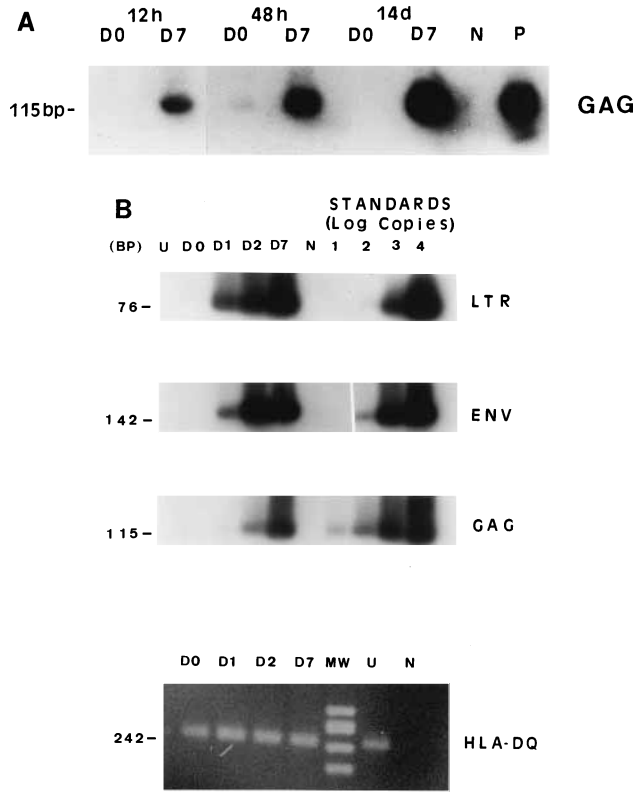


FIG. 1. (A) Effect of culture duration on susceptibility of monocytes to HIV-1 infection, as determined by PCR for full-length reverse transcripts. Lysates of monocytes and MDM exposed to HIV-1_{Ba-L} either on the day of isolation (D0) or after 7 days in culture (D7) were prepared as detailed in the text. DNA from 10^5 cells at 12 h, 48 h, and 14 days postinfection was amplified for 35 cycles with SK38 and -39 primers which amplify a 115-bp region of the *gag* gene. Amplicons were separated in agarose and detected by Southern hybridization. N, negative control containing no DNA; P, positive plasmid control. The figure is representative of four replicate experiments. (B) Effect of culture duration on the extent of cDNA synthesis in infected monocytes and MDM. DNA from 24-h lysates of a monocyte culture (representative of eight replicate experiments) exposed to HIV-1_{Ba-L} either immediately upon isolation (D0) or after 1, 2, and 7 days in culture (D1, D2, and D7) was amplified by PCR with the primer pairs U5-PBS (which amplifies a 76-bp region of LTR [early transcripts]), SK68 and -69 (a 142-bp region of *env* [intermediate transcripts]), and SK38 and -39 (a 115-bp region of *gag* [late (full-length) transcripts]). Cell equivalents (10^5) were used in each reaction, and known HIV-1 proviral copy number equivalents from 8E5 cells (10^1 to 10^4) were amplified concurrently to serve as standards. Amplified products were detected by Southern blotting. To control for input DNA in the samples analyzed for HIV-1 cDNA, PCR with HLA-DQα primers (20) was performed on 5×10^4 cell equivalents. The 242-bp products were electrophoresed through 2% agarose and stained with ethidium bromide. U, uninfected cells; N, no DNA control; MW, molecular weight markers (ϕX174 *Hae*III bands with sizes of 310, 281 to 271, 234, and 194 bp).

freshly isolated monocytes exposed to virus generated no detectable cDNA, even up to 14 days later. This result concurs with our previous finding that although monocytes isolated from peripheral blood express reasonably high levels of CD4, as assessed by flow cytometry, they are resistant to productive infection with HIV-1 (36).

Since completion of cDNA synthesis could not be detected in fresh monocytes but was evident within 12 h of infection of macrophages cultured for 7 days, we next used PCR with three sets of primers to determine whether reverse transcription was initiated in monocytes exposed to virus on the day of isolation but terminated before completion. In addition, by also infecting monocytes from the same donor culture after 1, 2, or 7 days in culture, we could determine when initiation and completion of cDNA synthesis could be detected in these cells. Lysates were analyzed with U5-PBS, SK68-SK69, and SK38-SK39 primer pairs to detect all transcripts initiated, those which had proceeded to intermediate length, and those almost completed, respectively, within the first 24 h of infection. In six replicate experiments, no product was found in day 0 samples with any of the three sets of primers, indicating that reverse transcription is not initiated in these cells (Fig. 1B, top panels). This result was not due to a lack of, or a difference in the amount of, amplifiable DNA within these samples, as the HLA primers gave similar levels of amplification with all samples (Fig. 1B, bottom panel). If, however, the cells were maintained in culture for a day or more prior to infection, reverse transcription was clearly initiated (Fig. 1B, LTR panel) and proceeded to completion (*gag* panel) within 24 h of infection. Increasing the time in culture before exposure to virus generally resulted in higher copy numbers of initiated, intermediate, and full-length transcripts detectable at 24 h (Fig. 1B, LTR, *env*, and *gag* panels, respectively).

The levels of cDNA detected in monocyte cultures infected after increasing times in culture correlated with the proportion of cells from these cultures which were productively infected, as determined by intracellular p24 antigen detection by flow cytometry at 14 days postinfection. As was expected from the results described above, day 0 cultures did not contain productively infected cells. For monocytes cultured 1 to 7 days prior to infection, the proportion of productively infected cells increased with time in culture, from an average of 22% for 1-day-old to 47% for 7-day-old cells (average of six experiments).

Since several groups have reported that freshly isolated monocytes can be productively infected with primary macrophage-tropic isolates (17, 42) rather than laboratory strains, we repeated this analysis with three patient isolates (HIV-1_{17}, HIV-1_{19}, and HIV-1_{676}) which were initially isolated in MDM then grown for a single passage in PBMCs to obtain sufficient virus. Even when nested PCR for the *gag* region was used to improve sensitivity to <10 copies per 10^5 cells, again no evidence was found for cDNA synthesis in fresh monocytes. On the other hand, complete reverse transcripts were detected in day 1 and 2 cultures at 24 h postinfection (Fig. 2), confirming the results found with the HIV-1_{Ba-L} strain. As before, detection of cDNA correlated with infection as measured by the detection of p24 antigen (data not shown).

Integration in fresh and cultured monocytes. Whether HIV-1 cDNA became integrated in infected monocytes and MDM and the timing of this event relative to reverse transcription were investigated by *Alu*-PCR. This method specifically detects the presence of integrated viral sequences as opposed to episomal viral DNA forms by the use of primers directed to *Alu* repeat elements, of which their are estimated to be approximately 10^6 copies within the human genome (39), and the use of an antisense primer to the LTR-U3 region of HIV-1. Analysis of HIV-1_{Ba-L}-infected PBMC cultures revealed the method to be specific for DNA from infected cells. DNA from uninfected cells that was amplified to a similar extent by *Alu*-PCR failed to hybridize with the LTR probe (data not shown). Hybridization of amplified DNA from infected PBMCs showed the expected smear (~0.2 to 2 kb) corresponding to multiple integration events, with different distances between the proviral LTR and adjacent *Alu* sequences (data not shown). To ensure that this signal did not arise from episomal forms of viral DNA trapped within cellular material, uninfected PBMC lysate was spiked with linearized HIV-1 containing plasmid DNA (pHXB2) and amplified by *Alu*-PCR. Again,

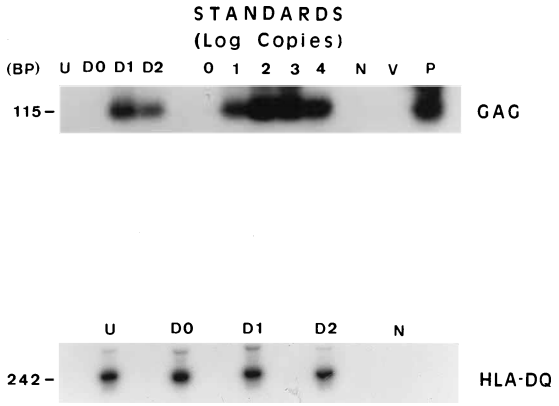


FIG. 2. Detection by nested PCR of full-length reverse transcripts in monocytes infected with primary isolates of HIV-1. Monocytes from the same donor culture were infected with a macrophage-tropic patient isolate (HIV-1_{19}) either on the day of isolation (D0) or after 1 or 2 days in culture (D1 and D2) and were lysed 24 h after infection. Cell equivalents (10^5) were amplified in a nested PCR with *gag* primers. Tenfold dilutions of 8E5 cells (10^4 to 10^0) were amplified concurrently to serve as standards for HIV-1 copy number. The 115-bp product from the second round of PCR was detected by Southern hybridization. N, no DNA control; V, input virus control to check for contaminating viral DNA in the virus stock used for infection; P, positive plasmid control; U, uninfected cells. Similar results were obtained with primary isolates HIV-1_{17} and HIV-1_{676}. Cell equivalents (10^4) were also amplified with HLA-DQα primers GH26 and -27 to standardize the amount of DNA in the lysates. The 242-bp product was detected by liquid hybridization with ^{32}P-labelled oligonucleotide probe GH64 as described by Lee et al. (20).

the amplified DNA smear failed to hybridize with the LTR probe (data not shown).

Since *Alu*-PCR appeared to be a specific technique for detecting integration, it was used to determine the kinetics of integration following the infection of susceptible MDM. Macrophages infected with HIV-$1_{Ba\text{-}L}$ after 7 days in culture were lysed at various times (1 to 15 days) postinfection. *Alu*-PCR analysis of these samples revealed integrated LTR sequences detectable by day 1 in some cultures and by day 3 in all cultures (data not shown). Additional controls, in which 7-day-postinfection lysates were amplified with *Alu* primers alone or with the U3 primer alone, were negative by hybridization (data not shown).

To examine integration in monocytes exposed to virus when first isolated (nonpermissive) or after only a day in culture (permissive), a more sensitive version of *Alu*-PCR was employed. Rather than hybridization of Southern blots, a method in which the U3 primer was end labelled with ^{32}P and used in PCR and the products were then separated by polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography was employed. Integration could be detected in susceptible monocytes as early as 24 h after virus exposure, with greater levels of integrated proviral DNA evident by 7 days postinfection (Fig. 3A). As expected, fresh monocytes in which replication is blocked prior to the initiation of reverse transcription (Fig. 1 and 2) showed no integration signal even after 7 days.

Alu-PCR was also performed on lysates of 7-day MDM infected with primary patient isolate HIV-1_{676}. Since this isolate generally infects a smaller proportion of cells than HIV-$1_{Ba\text{-}L}$, nested *Alu*-PCR was used for enhanced sensitivity. Proviral DNA was detected in infected cultures from two different donors at 4 days postinfection (Fig. 3B). The signal intensity from an equivalent number of cells from one culture (donor 1, 1-week exposure time) was much less than that from the other (donor 2, 1-day exposure time), reflecting the donor variability often observed in susceptibility of MDM to infection with HIV-1. The different-sized products found with the two donor cell populations presumably also reflect donor variability in the sites of integration.

As conclusive evidence that *Alu*-PCR specifically detects proviral DNA and to see whether any specific cellular integration sequences could be identified in HIV-1-infected MDM (28), amplicons from nested *Alu*-PCR of 5- to 7-day-postinfection lysates were cloned and sequenced. The sequence information obtained from four clones (three from one culture and one from another) is given in Fig. 4 and shows definitively the occurrence of integration events in HIV-1-infected MDM. Each clone contained *Alu* repeat sequences separated from a common viral sequence, corresponding to the start of the 5′ LTR-U3 region, by various lengths (16, 79, 118, and 194 nt) of nonviral sequence. The *Alu* sequences showed some diversity, as would be expected, since the repeat elements are not homogeneous, but the HIV-1 sequences were highly conserved and corresponded to that for HIVBAL1 from the Human Retroviruses and AIDS Sequence Database (24). The few changes from the sequence in the database that were found were all either purine-to-purine or pyrimidine-to-pyrimidine changes often associated with reverse transcriptase activity. Each clone contained a distinct sequence immediately 5′ to the HIV-1 sequence.

The nonviral, non-*Alu* sequences found in the four clones showed no homology with the HIVNL43 sequence immediately upstream of the 3′ LTR region and, therefore, do not represent fragments of HIV-1 DNA amplified because of nonspecific annealing of the *Alu* primer to the viral sequence in this region. The presumably cellular sequence of clone 6-25 has 75% homology with a region of the *pol* gene (3578 to 3593 of the HIVNL43 sequence), but this homology is probably a consequence of the clone's short length (16 nt). It showed much greater homology (87 to 94%) with a number of human sequences in the EMBL and GenBank data banks, again presumably because of its length. The remaining three non-*Alu*

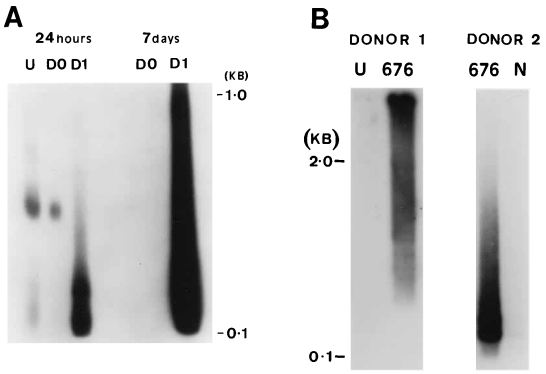
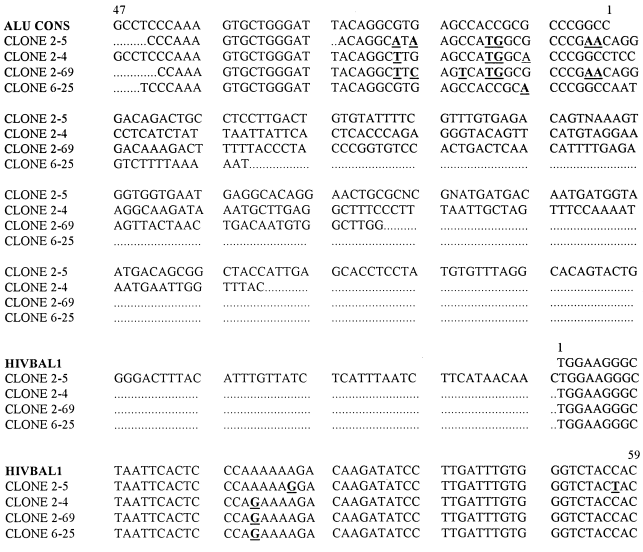


FIG. 3. (A) Time course of HIV-$1_{Ba\text{-}L}$ integration in fresh and cultured monocytes as assessed by *Alu*-PCR. Monocytes from the same donor were pulsed with virus for 2 h either immediately upon isolation (D0) or after 1 day in culture (D1). Cells were then lysed 24 h and 7 days later, and cell equivalents (10^5) were amplified by *Alu*-PCR with ^{32}P-labelled U3 primer. Products were analyzed by 6% PAGE and autoradiography. U, uninfected monocytes from the same donor culture. The results shown are representative of three replicate experiments. (B) Detection of HIV-1 provirus in 7-day cultured MDM from two donors infected with a primary isolate (HIV-1_{676}). Cells were lysed 4 days after infection, and cell equivalents (10^5) were amplified by two rounds of *Alu*-PCR with nested LTR primers. Products from the second round were detected by Southern hybridization. Autoradiography was performed for 1 week for the left-hand panel (donor 1) and for 1 day for the right-hand panel (donor 2). U, uninfected MDM; 676, MDM from two different donor cultures infected with patient isolate HIV-1_{676}; N, no DNA control.



	47				1
ALU CONS	GCCTCCCAAA	GTGCTGGGAT	TACAGGCGTG	AGCCACCGCG	CCCGGCC
CLONE 2-5	.....CCCAAA	GTGCTGGGAT	..ACAGGC**A**T**A**	AGCCA**TG**GCG	CCCG**AA**CAGG
CLONE 2-4	GCCTCCCAAA	GTGCTGGGAT	TACAGGC**T**TG	AGCCA**TG**GC**A**	CCCGGCCTCC
CLONE 2-69	.....CCAAA	GTGCTGGGAT	TACAGGC**TT**C	AG**T**CA**TG**GCG	CCCG**AA**CAGG
CLONE 6-25	.....TCCCAAA	GTGCTGGGAT	TACAGGCGTG	AGCCACCGC**A**	CCCGGCCAAT
CLONE 2-5	GACAGACTGC	CTCCTTGACT	GTGTATTTTC	GTTTGTGAGA	CAGTNAAAGT
CLONE 2-4	CCTCATCTAT	TAATTATTCA	CTCACCCAGA	GGGTACAGTT	CATGTAGGAA
CLONE 2-69	GACAAAGACT	TTTTACCCTA	CCCGGTGTCC	ACTGACTCAA	CATTTTGAGA
CLONE 6-25	GTCTTTTAAA	AAT.....	.....	.....	.....
CLONE 2-5	GGTGGTGAAT	GAGGCACAGG	AACTGCGCNC	GNATGATGAC	AATGATGGTA
CLONE 2-4	AGGCAAGATA	AATGCTTGAG	GCTTTCCCTT	TAATTGCTAG	TTTCCAAAAT
CLONE 2-69	AGTTACTAAC	TGACAATGTG	GCTTGG.....	.....	.....
CLONE 6-25	.....	.....	.....	.....	.....
CLONE 2-5	ATGACAGCGG	CTACCATTGA	GCACCTCCTA	TGTGTTTAGG	CACAGTACTG
CLONE 2-4	AATGAATTGG	TTTAC.....	.....	.....	.....
CLONE 2-69	.....	.....	.....	.....	.....
CLONE 6-25	.....	.....	.....	.....	.....
					1
HIVBAL1					TGGAAGGGC
CLONE 2-5	GGGACTTTAC	ATTTGTTATC	TCATTTAATC	TTCATAACAA	CTGGAAGGGC
CLONE 2-4	.....	.....	.....	.....	..TGGAAGGGC
CLONE 2-69	.....	.....	.....	.....	..TGGAAGGGC
CLONE 6-25	.....	.....	.....	.....	..TGGAAGGGC
					59
HIVBAL1	TAATTCACTC	CCAAAAAAGA	CAAGATATCC	TTGATTTGTG	GGTCTACCAC
CLONE 2-5	TAATTCACTC	CCAAAAA**G**GA	CAAGATATCC	TTGATTTGTG	GGTCTAC**T**AC
CLONE 2-4	TAATTCACTC	CCA**G**AAAAGA	CAAGATATCC	TTGATTTGTG	GGTCTACCAC
CLONE 2-69	TAATTCACTC	CCA**G**AAAAGA	CAAGATATCC	TTGATTTGTG	GGTCTACCAC
CLONE 6-25	TAATTCACTC	CCA**G**AAAAGA	CAAGATATCC	TTGATTTGTG	GGTCTACCAC

FIG. 4. Sequences of *Alu*-PCR clones from HIV-1_{Ba-L}-infected macrophages. The *Alu* consensus sequence (ALU CONS) is taken from Jelinek and Schmid (14), and the HIVBAL1 sequence is taken from the Human Retroviruses and AIDS Sequence Database 1993 (24). Base changes from the reference sequences are underlined.

cellular sequences have little homology (55 to 64%) over the majority of their lengths with human sequences contained within EMBL or GenBank and, therefore, probably represent regions of the human genome not currently in these databases.

DISCUSSION

We have reported previously that monocytes exposed to macrophage-tropic virus immediately upon isolation from peripheral blood do not become productively infected (36). The purpose of the present study was to determine if replication in these cells was halted before the initiation of reverse transcription, during reverse transcription (resulting in the lack of full-length cDNA), or at the integration step. We found that replication was blocked prior to reverse transcription in fresh monocytes, with no evidence of any transcripts initiated or completed within the first 24 h (Fig. 1B) or even up to 7 days after infection (Fig. 1A). As expected, no integration of viral cDNA occurred in these cells (Fig. 3A). If, however, monocytes are allowed to mature in culture for a day or more before exposure to HIV-1, there is no impediment to reverse transcription and integration, which proceed rapidly following virus entry, with the latter event also being detectable within 24 h of infection (Fig. 3A). A productive infection, as assessed by quantification of intracellular p24 antigen 14 days after infection, then ensues.

Other workers who have reported that viral DNA is detectable soon after infection of fresh monocytes have not used cells immediately after isolation, as we have done, but rather ones which have been maintained, usually on plastic, for 2 days or more before infection (4, 29). When MDM were infected with HIV-1_{Ba-L}, complete reverse transcripts were detectable within the cells by 12 h, the earliest time examined (Fig. 1A). This result is in agreement with results from other groups that found full-length viral DNA present in MDM by 8 to 24 h postinfection with other macrophage-tropic strains, HIV-1_{JR-FL}, HIV-1_{ADA}, and HIV-1_{DJV} (7, 12, 29). Quiescent CD4 lymphocytes also become infected with HIV-1, but nonproductively. In the absence of cell activation, reverse transcription to full-length viral cDNA occurs slowly in these cells and is not detected until 3 days postinfection (37). As our isolation and culture conditions alone do not activate monocytes (36), susceptible MDM, unlike quiescent lymphocytes, do not appear to require activation to become productively infected, and reverse transcription of the viral RNA genome occurs soon after virus entry.

Although reverse transcription was not initiated after the exposure of fresh monocytes to HIV-1, we found that short periods in culture were sufficient for reverse transcription to proceed (Fig. 1B) and for the cells to now be productively infected (36). In fact, as little as 24 h in suspension culture was enough for numerous reverse transcripts to be detectable a day after infection, of which 1 to 10% are full-length viral DNA. If the cells are left to mature for longer periods before exposure to HIV-1, infection is generally more efficient, with increasingly greater copies of viral DNA found at 24 h and a consequent increase in the proportion of cells productively infected.

Several groups have reported the productive infection of freshly isolated monocytes with macrophage-tropic primary patient isolates (17, 42). To ensure that the lack of reverse transcription we observed in fresh monocytes was not confined to the use of a particular laboratory strain of HIV-1, we repeated our analysis with three primary isolates and found the same block to replication prior to reverse transcription as we found with HIV-1_{Ba-L}. With the primary isolates, we used a nested PCR method able to detect fewer than 10 copies of viral DNA to be certain that we were not missing a low level of transcription in our cells. However, at no time were we able to detect the synthesis of viral DNA in freshly isolated cells, while, as before, it was readily detectable in infected monocytes cultured for a day or more (Fig. 2). It is possible that differences in culture methods and conditions can explain the discrepancy between our findings and those of other groups, although which differences contribute most to these discrepancies in infection are unknown.

Since they found no difference in the efficiencies with which reverse transcription occurred in monocytes and macrophages at different stages of differentiation, Rich and colleagues concluded that differences at the level of virus entry probably accounted for the differences in productive infection observed for these cell populations (29). This difference cannot be explained simply in terms of changes in expression of the major viral receptor, CD4, however, since freshly isolated monocytes express readily detectable surface CD4 whereas cells cultured for 1 or 2 days have levels barely detectable by flow cytometry (36). Other factors must, therefore, be involved and may include the requirement for an accessory receptor or changes in the presentation of the CD4 molecule during monocyte differentiation. Fresh monocytes were found to have a different pattern of reactivity with a panel of seven monoclonal antibodies directed against the four extracellular domains of CD4 to cells from the same donors cultured for 7 days (43). CD4 on monocytes has been reported also to have reduced binding affinity for gp120 compared with that on macrophages (8). This reduced affinity may be sufficient to prevent efficient virus attachment to fresh monocytes and thereby prevent entry and reverse transcription until the affinity increases. We are currently comparing binding and entry in freshly isolated monocytes with those in cultured monocytes in an attempt to clarify this question.

Recently, Englund and colleagues used a molecular clone of a macrophage-tropic HIV-1 isolate with a single Asp-to-Asn substitution at the invariant Asp-116 residue of integrase to show conclusively that integration is required for productive infection of both phytohemagglutinin-stimulated PBMCs and

MDM (7). PCR analysis indicated that this mutant reverse transcribed its RNA genome efficiently but failed to initiate spreading infections, presumably because the viral DNA could not integrate, although this reason was not established for these cultures. Heinzinger and coworkers (12) have reported that MDM begin to accumulate circular forms of viral DNA (markers of nuclear translocation [1]) soon after the completion of reverse transcription, but again, integration was not measured directly.

We have found previously that circular forms of viral DNA can be detected at 7 days postinfection in monocytes infected after 1 to 7 days in culture but not in monocytes pulsed with HIV-1 when first isolated (35). We have extended this work to the detection of provirus directly at various times following the exposure of fresh and cultured monocytes to HIV-1. We developed an *Alu*-PCR method to amplify sequences between the LTR of a provirus and adjacent *Alu* repeat elements, the most abundant of the numerous repeated sequences in the human genome. This technique was found to be specific for integrated viral DNA by hybridization and by cloning and sequencing of *Alu*-PCR products and was able to detect provirus within 24 h of the infection of susceptible monocytes (Fig. 3A). There appears, therefore, to be no delay in the integration of HIV-1 DNA following the infection of monocytes and MDM. As expected, no integration signal was observed in fresh monocytes up to 7 days after exposure to virus. Results with a minimally passaged primary isolate were similar to those found with multiply passaged laboratory strain HIV-$1_{Ba\text{-}L}$ (Fig. 3B). In our hands, primary isolates and laboratory isolates behave similarly in monocytes and MDM, at least at the level of reverse transcription and integration.

Overall, our findings show that in monocytes which are susceptible to infection, i.e., those which have been maintained in culture for a day or more after isolation and purification from peripheral blood, HIV-1 is able to bind, enter, reverse transcribe its genome, and integrate its provirus into the host cell's genome within the first 24 h of infection in vitro. Subsequent steps in virus replication leading to the production of progeny virions appear to occur more slowly, however, as levels of p24 antigen and virion-associated reverse transcriptase are very low or undetectable in the first few days after infection and usually do not peak until 2 to 3 weeks later in primary monocyte, MDM, and tissue macrophage cultures (4, 22, 29, 45). The reasons for this are unclear at present but could involve differences in the transcription factor milieus for lymphoid and monocytoid cells as well as other factors which await elucidation.

Our studies using freshly isolated monocytes raise the question of whether normal peripheral blood monocytes are susceptible to infection in vivo. Our in vitro data suggest that they are not and that examining this compartment alone will not lead to insights into the pathogenesis of HIV-1. Further studies are required to characterize the low proportion of blood monocytes in which viral DNA is detectable and their role in pathogenesis as well as the role played by tissue macrophages. We have shown that replication in monocytes is blocked at some point prior to the reverse transcription step, but whether this block is at the level of binding or entry or follows entry has not been determined. Regardless of where the block occurs, it is alleviated within 24 h of isolation. Studies aimed at clarifying this situation are under way, and they may also disclose novel targets for antiretroviral chemotherapy.

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