

## Establishment of a Stable, Inducible Form of Human Immunodeficiency Virus Type 1 DNA in Quiescent CD4 Lymphocytes In Vitro

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**Human immunodeficiency virus type 1 (HIV-1) possesses the ability to establish a complete infection in nondividing host cells. The capacity of HIV-1 to infect nondividing cells probably contributes significantly to its pathology in vivo, as reflected by infection of peripheral T lymphocytes, tissue macrophages, and microglial cells. However, the in vitro demonstration of the establishment of stable HIV-1 infection in quiescent T cells remains controversial. We have developed a primary T-cell model of acute HIV-1 infection of quiescent CD4 lymphocytes that demonstrates the development of a complete, reverse-transcribed form of virus that is stable for over 10 days in culture. To ensure that our primary cell culture was representative of a quiescent population, the CD4 lymphocyte targets were monitored for membrane expression of activation antigens and for shifts in cell cycle from G<sub>0</sub>/G<sub>1</sub> to S/G<sub>2</sub> phase. The presence of viral DNA fragments reflecting progressive reverse transcription was determined by PCR analysis. HIV entered primary CD4 cells rapidly, but viral DNA accumulated slowly in the resting cell cultures. DNA species containing regions of full-length reverse transcription were not detected until 3 to 5 days after infection. In parallel with the appearance of complete viral DNA, spliced RNA transcripts, predominantly of the *nef* species, were detected by reverse transcriptase PCR amplification. When infected CD4 cells were sorted on the basis of cell cycle analysis of DNA content, the accumulation of a complete viral DNA form was found to occur in both the purified G<sub>0</sub>/G<sub>1</sub>-phase cell subset and the cell fraction enriched for the minor S-phase subset. In contrast, spliced viral RNA products could be detected only in the enriched S-phase cell fraction. These results demonstrate that HIV-1 can infect and establish a complete, stable form of viral DNA in primary CD4 lymphocytes in vitro but is blocked from transcription in the absence of cell activation. The findings are consistent with in vivo data from HIV-infected individuals that show the existence of viral DNA predominantly as a stable, extrachromosomal form in T cells of the peripheral circulation.**

Retroviruses generate a DNA reverse transcript from genomic RNA; this viral DNA integrates into cellular DNA to establish infection (36). Oncogenic retroviruses, represented typically by the avian sarcoma and murine leukemia viruses, require a dividing host cell to establish a productive infection. In contrast, lentiviruses can establish infection in nondividing, terminally differentiated cells. The human lentiviruses, human immunodeficiency virus types 1 and 2 (HIV-1 and -2), possess the ability to stably infect nondividing host cells, including macrophages and brain microglial cells (38, 39). A critical determinant in HIV infection of nondividing cells is the viral capacity to direct transport of the proviral preintegration complex to the cell nucleus independently of cell division (4, 30). Recent studies indicate that the p17 matrix protein contains a domain required to target nuclear localization and that the Vpr accessory protein may participate in a complementary way to facilitate nuclear entry of the preintegration complex (3, 4, 37). The ability of HIV to infect nondividing cells is undoubtedly important to viral pathology in vivo. HIV infection in lymphoid organs appears to be maintained in tissue macrophages (30, 39), and infection in the brain occurs predominantly in macrophages and microglial cells (17, 38). Additionally, the massive seeding and spread of HIV within the CD4

lymphocyte compartment (2) is reconciled more easily with the current perception that the virus persists in T cells in vivo, even though the vast majority of these cells are found in a quiescent, nondividing state (6). Data from several studies indicate that HIV found in CD4 lymphocytes of the peripheral circulation and lymph nodes is maintained predominantly in a nonproductive viral form either as extrachromosomal viral DNA (4, 5) or in a state of restricted transcription (2, 8, 24, 25, 28).

The demonstration of HIV-1 infection of primary, quiescent T cells in vitro has remained controversial. Although several reports have shown that HIV can readily enter quiescent T cells in culture and subsequently be induced to replicate upon exogenous stimulation (20, 31, 41, 43), the state of viral DNA and the stability of infection in the absence of cell activation have not been resolved clearly. Published data in this research area have been contradictory. Some investigators have demonstrated that HIV-1 infection of quiescent T cells results in partially reverse-transcribed viral DNA that is labile and rapidly degraded (41, 42), while other investigators have reported the establishment of a complete infectious cycle with the detectable production of early mRNA transcription (31). Our own studies using a primary CD4 cell model have indicated that HIV is capable of achieving an infection that is stable for an extended time in culture (29). We have pursued these observations by performing experiments to characterize both the molecular form of the stable virus infection and the cellular state of the infected target. Our results demonstrate that in

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CD4 lymphocytes, which are truly representative of a quiescent cell population, HIV-1 infection in vitro progresses to a stable, fully reverse-transcribed form of viral DNA that is transcriptionally inactive.

## MATERIALS AND METHODS

**Primary CD4 lymphocyte cultures.** Highly enriched preparations of CD4 lymphocytes were isolated from peripheral blood samples from healthy, HIV-seronegative donors as described previously (29). Briefly, a lymphocyte fraction was obtained by Ficoll-Hypaque density gradient centrifugation, and monocytes were removed. CD4 lymphocytes were purified by negative selection with the panning method (40) with a mixture of murine monoclonal antibodies specific for CD8, CD16, and CD22 (OKT8 [Ortho Diagnostics] and Leu-11b and Leu-14 [Becton-Dickinson Immunocytometry], respectively). The resulting cell preparations were at least 95% viable as determined by trypan blue dye exclusion and contained 90 to 98% CD4-positive lymphocytes as monitored by automated flow cytometry with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to CD4 (Leu 3a+b; Becton-Dickinson). The isolated CD4 cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with L-glutamine (1 mM), penicillin (50 U/ml), streptomycin (50 µg/ml), and human AB serum (5%, vol/vol) that was negative for HIV and hepatitis B virus antibodies (Gemini Bio-Products, Calabasas, Calif.). To ensure that the separation procedure or in vitro culture conditions did not induce detectable cell activation, CD4 cells were analyzed by flow cytometry at various times following isolation for the expression of membrane activation markers and for shifts in cell cycle from G<sub>0</sub>/G<sub>1</sub> to S phase.

**Virus infection.** An infectious virus stock of the NL4-3 clone of HIV-1 (1) was prepared by transfecting plasmid DNA into the CEM T lymphoblastoid cell line (10) with lipofectin (9). Working preparations of virus were derived from a single passage of the stock through the CEM line. A high-titer stock of the LAI (LAV<sub>BRU</sub>) strain of HIV-1 (21) was prepared from infection of CEM cells. Infectivity titers based on the 50% tissue culture infectious dose (TCID<sub>50</sub>) were assessed by a terminal dilution microassay with the MT-2 cell line (14). End point titers were determined by a p24 antigen assay (Abbott Laboratories, North Chicago, Ill.).

For infection of primary cells, virus stocks were diluted in RPMI 1640 medium without serum and without Polybrene. Aliquots of  $4 \times 10^6$  to  $6 \times 10^6$  quiescent CD4 lymphocytes in 0.5 to 1.0 ml were incubated with virus for 2 to 4 h at 37°C at multiplicities of infection (MOI) of 0.1 to 1.0 TCID<sub>50</sub> per cell. After infection, excess virus was removed by extensive washing with phosphate-buffered saline (PBS) (Dulbecco A). Cell recovery was monitored by viable cell counts with trypan blue dye exclusion. In experiments requiring early-time-point kinetics, virus infection was synchronized by a 1-h adsorption at 4°C followed by a 30-min incubation at 37°C to allow virus entry. The cells were then cooled and maintained at 4 to 8°C during PBS washes, and aliquots of cells and supernatant were taken as baseline determinants (time zero) for subsequent PCR and p24 antigen analyses. In experiments using PCR amplification to detect HIV DNA formation, virus preparations of the NL4-3 clone were filtered through a 0.45-µm-pore-size membrane prior to CD4 cell infection to minimize potential inoculum contamination from viral DNA associated with cell membrane fragments. Initial studies demonstrated that further treatment of these filtered preparations with DNase had little effect on the remaining detectable, virion-associated DNA; therefore, this treatment was not applied routinely.

Following infection, the CD4 lymphocytes were cultured in RPMI medium with 5% human AB serum at  $4 \times 10^6$  to  $5 \times 10^6$  cells in 1 ml in polystyrene culture tubes (15 ml; Corning Plastics). Levels of infection were determined at sequential time points by taking cell samples for PCR analysis and by inducing productive virus replication with T-cell mitogen stimulation. Cells were distributed into round-bottom microtiter plates (Linbro Plastics) at  $10^5$  cells per 200 µl of medium per well in triplicate, and 3 µg of phytohemagglutinin (PHA) (Sigma Chemicals, St. Louis, Mo.) per ml plus 5 U of recombinant interleukin-2 (rIL-2) (DuPont, NEN Research Products, Boston, Mass.) per ml was added to induce cell proliferation and virus replication. Unstimulated, infected CD4 cells and uninfected-cell controls were included for culture. To minimize experimental variation due to individual cell donor differences, a given experimental design was repeated two to four times with primary cells from different donors. HIV production was assessed by measuring the amount of soluble p24 antigen released into culture supernatants. Samples of 100 µl were taken from each microculture well, stored at -70°C, and batch assayed for p24 by enzyme-linked immunosorbent assay (Abbott Laboratories). The determination of p24 values was based on the average of results for triplicate culture wells.

**PCR amplification of viral nucleic acids.** Cell samples were maintained at -70°C until the completion of an experiment. Total nucleic acids were extracted (13) by lysing cells with incubation at 50°C for 45 min in a buffer of sodium dodecyl sulfate (SDS)-NaCl-EDTA-Tris containing 200 µg of proteinase K per ml (13). Nucleic acids were extracted from lysates with phenol-chloroform, precipitated with ethanol, and resuspended in 5 mM Tris (pH 8.3).

PCR amplification of HIV-1 DNA was based on a modification of published methods (13, 42). Reaction conditions were first optimized for all oligonucleotide primers by using a range of target amounts (0.1 to 100 amol) for linearized

pNL4-3 plasmid DNA (Invitrogen PCR Optimizer Kit). Aliquots of cell lysates were diluted to 100 µl in a buffer containing 50 mM Tris (pH 8.5), 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 10 µg of bovine serum albumin (BSA), 0.2 mM deoxynucleoside triphosphates, and 0.25 µg of each oligonucleotide primer. Reaction mixtures were boiled for 2 min, cooled, and held at 61°C, and *Taq* DNA polymerase (2.5 U; Perkin-Elmer Corp., Norwalk, Conn.) was added. PCR amplification was performed for 30 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 3 min. Four sets of primer pairs were used to amplify different regions of viral DNA (42), on the basis of sequence homology to NL4-3 (23). The region spanning R to U5 within the 5' long terminal repeat (LTR) (U5/LTR) was detected by using primers R11 (5'-GTTAGACCAGATTTGAGCCTG-3'; sense) and AA55 (42). The *env* region was amplified with primers 033 and 272 (18, 22), the *gag* region was amplified with primers LA8 and LA9 (42), and the region spanning R and *gag* (LTR/*gag*) was amplified with primers M667 and M661 (42). The intersample variation was monitored by parallel PCRs measuring total cellular DNA with primers specific for human β-actin (7) with 20 cycles of amplification.

Reverse transcriptase PCR (RT-PCR) amplification of HIV-1 spliced RNA products was based on a published strategy of detecting alternative splice events (13, 26). Spliced RNA transcripts were detected with a primer pair (primers A and B) that flanked the major 5' splice donor and the acceptors used for *tat*, *rev*, and *nef* mRNA products. Reactions were performed in 100 µl containing 50 mM Tris (pH 8.3), 10 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 µg of BSA, 0.2 mM deoxynucleoside triphosphates, and 0.25 µg of each primer. The nucleic acid preparations were heated to 65°C for 90 s to denature secondary structures, cooled to 42°C for 2 min to anneal primers, and treated with murine leukemia virus reverse transcriptase (100 U; Bethesda Research Laboratories, Gaithersburg, Md.) for 30 min at 42°C prior to boiling and the addition of *Taq* polymerase. PCR amplification was performed for 30 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 3 min. cDNA fragments of 290, 118, and 100 bp, which corresponded to sequences for *tat*, *rev*, and *nef*, respectively, were obtained. This strategy is depicted in Results (see Fig. 3); the nucleotide sequences of the primers and resulting cDNA fragments have been published previously (13). To monitor the intersample variation, a parallel PCR for total cellular RNA was performed with primers specific for the β subunit of pyruvate dehydrogenase (G3PDH) (35) with 25 cycles of amplification at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. A cDNA product of 102 bp was formed with the primer pair PDH 129 (5'-GGTATGGATGAGGAGCTGGA-3'; sense) and PDH 230 (5'-CTTCCACTAAGCCCTCGACTAA-3'; antisense).

**Southern blot analysis.** Amplification products (5 to 8% of the reaction mixture) were resolved by electrophoresis on 5 to 6% nondenaturing polyacrylamide gels (Novex, Encinitas, Calif.) and electroblotted onto Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.). The transferred products were denatured on the membrane with a 15-min treatment with 0.4 N NaOH followed by a brief neutralization with 1 M Tris buffer (pH 7.4). Blots were hybridized with [<sup>32</sup>P]ATP-end-labeled oligonucleotide probes specific for the DNA or cDNA regions amplified. To detect cDNA products from amplifications with the R11-AA55 and M667-M661 primer pairs, probe LTR/520 spanning positions 520 to 539 was used (5'-AAGCCTCAATAAAGCTTGCC-3'). The probe *gag*/736, spanning positions 736 to 754 (5'-GGCGACTGGTGTAGTACGCC-3') was used to detect the cDNA product from amplification with the LA8-LA9 primer pair. Probe 88-297 (7, 22) was used with the *env* primers 033 and 272, and probe 88-318 (7) was used with primers 317 and 319 to detect cellular β-actin sequence. To detect cDNA amplified from mRNA products, probe C (13) was used with primers A and B for HIV spliced RNA sequences, and end-labeled oligonucleotide PDH 129 was used with primers PDH 129 and PDH 230 to detect cellular β-G3PDH sequence. Hybridization with 25 ng of each probe was performed for 1 h at 55°C in a buffer containing 1% SDS with SSPE (0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA [pH 7.7]), BSA, and polyvinylpyrrolidone and was followed by two washes at 55°C with 1% SDS in SSPE. Autoradiography was done with one intensifying screen at -70°C for 1 to 6 h.

**Flow cytometry analysis.** The purity of CD4 lymphocyte preparations was monitored by direct staining with an FITC-conjugated murine monoclonal antibody to CD4 (Leu 3a+b; Becton-Dickinson) as described previously (15). The detection of membrane antigens associated with cell activation states was performed by dual-antigen analysis with FITC-conjugated anti-CD4 and phycoerythrin-conjugated monoclonal antibodies to CD25, CD71, DR, and CD45RO (antigens IL-2 receptor, transferrin receptor, major histocompatibility complex II and CD45RO isoform, respectively; Becton-Dickinson). The detection of HIV-infected lymphocytes was performed by using an indirect fluorescence assay with a monoclonal antibody to viral gp120 antigen (anti-LAV<sub>BRU</sub> 110.4, a gift from Elaine K. Thomas, Oncogen, Bristol-Myers) and an FITC-conjugated, affinity-purified, goat anti-mouse secondary antibody (Tago, Inc., Burlingame, Calif.). Stained cells were fixed with a 1% solution of paraformaldehyde. Antibody specificity, isotype controls of murine immunoglobulin G1-FITC and immunoglobulin G2-phycoerythrin were included in all experiments. Flow cytometric analysis was performed on an Ortho Cytofluorograf 50H or a Coulter Elite cytometer. Cell cycle analysis based on DNA content per cell was done by two different methods (6). For subsequent PCR analysis of HIV DNA, cell aliquots were permeabilized and fixed with 33% cold ethanol and then stained by a standard procedure with propidium iodide in the presence of RNase (32). For PCR analysis of HIV RNA transcripts, cells were fixed with 0.25% paraformal-

dehydrate for 1 h at 4°C, permeabilized with 0.2% Tween 20 for 15 min at 37°C, and stained with 7-aminoactinomycin D (27). Cell sorting and recovery of a purified G<sub>0</sub>/G<sub>1</sub>-phase population and an enriched S-phase subpopulation were performed on a FACStar-plus cytometer (Becton-Dickinson).

## RESULTS

**Primary CD4 cell model of HIV-1 infection.** To reflect virus-cell interactions as they are proposed to occur in vivo, a primary cell model was developed to study the regulation of HIV-1 infection and replication (29). Acute virus infection of quiescent CD4 lymphocytes was used to establish a nonproductive infection. The use of purified CD4 lymphocytes eliminated potential confounding influences of accessory cells and cytokines and allowed a more quantitative assessment of virus-cell interactions on a per-target-cell basis. Such preparations of isolated CD4 lymphocytes could be maintained in a resting state in culture for 10 to 14 days without the addition of exogenous lymphokines and could be induced to proliferate in response to T-cell mitogens. The LAI strain (LAV<sub>BRU</sub>) (21) and the NL4-3 clone (1) of HIV-1 were used to infect resting cells at an MOI of 0.5 TCID<sub>50</sub> per cell. At various times after infection, PHA mitogen and rIL-2 (29) were added to microcultures to induce lymphocyte proliferation and productive virus replication. At each time point tested, up to 10 days after infection, mitogen stimulation induced high levels of HIV replication with 50- to 2,000-fold increases in the production of soluble p24 antigen (Fig. 1A). Cells infected with either the LAI strain or the NL4-3 clone achieved peak levels of virus production, at 7 days following each mitogen induction, that equaled the levels of virus produced during parallel infections of fully proliferating CD4 lymphocytes stimulated with mitogen 3 days prior to infection (Fig. 1B). These results were in general agreement with those of previously reported studies (31, 42) and showed that HIV-1 can readily infect quiescent CD4 cells. However, in contrast to studies that indicated that HIV infection is incomplete and unstable in primary T cells (41, 42), our data demonstrated that HIV existed in a stable, inducible form for up to 10 days following infection of primary CD4 cells. Similar levels of virus production were achieved at all time points. The ability to induce high levels of virus replication did not decrease with extended times postinfection. Instead, greater increases in virus production occurred earlier following cell stimulation (day 3) in cultures induced at 6 and 10 days after infection than in cultures induced immediately after infection (day 0).

Because HIV infection of quiescent T cells has been a point of controversy (12, 31, 41, 42), experimental conditions were designed to ensure that our CD4 cell model was truly representative of the quiescent state present in vivo. To eliminate in vitro variables known to contribute to cell activation, a negative rather than a positive selection method was used for CD4 lymphocyte isolation, and prescreened human AB serum instead of fetal bovine serum was used to supplement the culture medium. To monitor whether the cell separation procedure induced detectable activation, CD4 cells taken after separation were compared with the unfractionated mononuclear cells by direct-staining flow cytometry for membrane expression of antigens associated with cell activation (Table 1). With six different donor cell preparations, no changes were observed in the levels of expression for CD25, CD71, DR, and CD38 antigens. During culture in the absence of exogenous stimuli, isolated CD4 cells did not exhibit levels of [<sup>3</sup>H]thymidine incorporation above the expected baseline level for periods of 8 days (data not shown). Because total cellular DNA synthesis is an insensitive indicator of minor population changes, additional parameters were monitored for possible alterations induced by

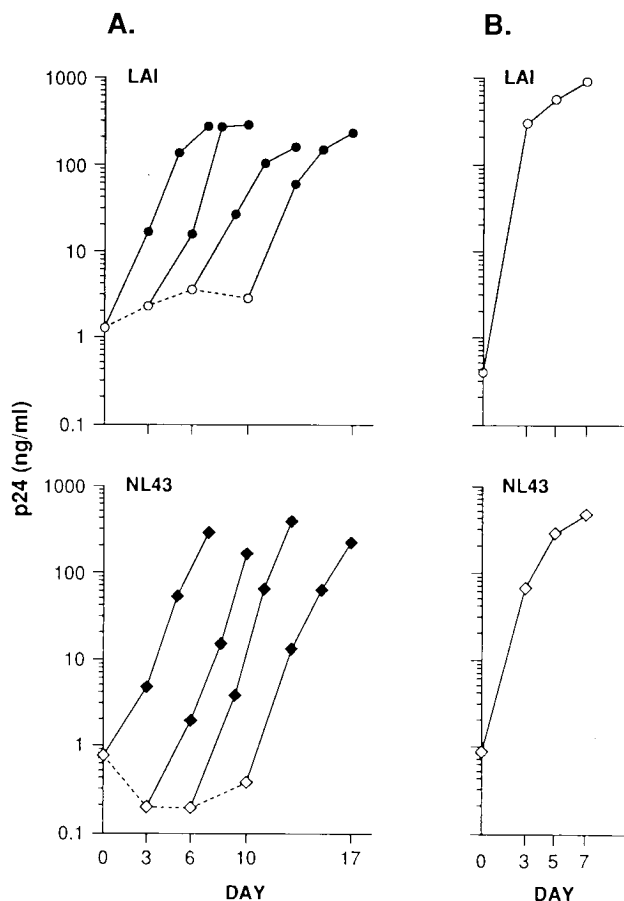


FIG. 1. Cell model of HIV infection. (A) Primary, quiescent CD4 cells were infected with the LAI strain (●) and the NL4-3 clone (◆) of HIV-1 on day 0 at an MOI of 0.5 TCID<sub>50</sub> per cell. PHA (3 μg/ml) and rIL-2 (5 U/ml) were added to microcultures immediately after infection (day 0) and on days 3, 6, and 10 after infection to induce virus replication. Supernatants were tested for soluble p24 on days 3, 5, and 7 following each induction cycle. (B) For comparison, virus replication was measured in proliferating primary CD4 cells stimulated with PHA plus rIL-2 for 3 days prior to infection. The figure depicts the combined, averaged results from two repeated experiments with different cell donors.

long-term culture, in either the presence or absence of virus infection. It is known that a very minor portion of peripheral blood lymphocytes are found normally to be in S phase (6); cell cycle analysis was performed on aliquots of the cultured CD4 cells to determine if shifts in cell cycle from the predominant G<sub>0</sub>/G<sub>1</sub>-phase population to the minor S/G<sub>2</sub>-phase subset occurred with increasing time in culture. No significant shifts in cell cycle from G<sub>0</sub>/G<sub>1</sub> to S/G<sub>2</sub> phase were detected in experiments using cells from five different donors (Table 2). Only 1% of the G<sub>0</sub>/G<sub>1</sub> cells moved into S phase during 8 to 10 days of culture, and no increase in the relative proportion of G<sub>2</sub>-phase cells could be detected. In addition, HIV infection alone did not cause any change in the resting state of the infected CD4 cell culture (Table 2). Cell activation antigens were also monitored in two experiments, and no changes in the levels of expression were observed for CD45RO, CD25, and DR antigens (Table 2). Taken together, these results indicated that our primary cell model of isolated CD4 lymphocytes is truly representative of a quiescent T-cell population similar to that found in the peripheral circulation in vivo.

**Detection of viral nucleic acids following HIV infection.** To investigate the viral state after infection of quiescent CD4 cells

TABLE 1. Lack of cell perturbation during CD4 cell isolation

Cells <sup>a</sup>	% (mean $\pm$ SD) of CD4 cells coexpressing <sup>b</sup> :			
	CD38	DR	CD25	CD71
PBL	33 $\pm$ 11	7 $\pm$ 6	7 $\pm$ 4	ND <sup>c</sup>
CD4	30 $\pm$ 9	5 $\pm$ 4	7 $\pm$ 5	3 $\pm$ 2
CD4/ON	33 $\pm$ 11	5 $\pm$ 3	8 $\pm$ 5	3 $\pm$ 2

<sup>a</sup> PBL, peripheral blood lymphocytes freshly isolated by Ficoll-Hypaque density gradient centrifugation; CD4, CD4 lymphocytes isolated by negative selection panning; CD4/ON, isolated CD4 cells held for 16 to 18 h in medium at 37°C prior to infection.

<sup>b</sup> Values are derived from those for six different blood donors used for in vitro infection experiments. FITC-conjugated antibodies were used to detect cell surface antigens CD38 (Leu-17/T10 antigen), DR (major histocompatibility complex II matrix), CD25 (IL-2 receptor), and CD71 (transferrin receptor).

<sup>c</sup> ND, not determined.

in culture, aliquots of cells were taken at several times for PCR analysis of HIV DNA formation. The PCR method was based on a published strategy (42) that uses a series of primer pairs to detect different regions of HIV DNA representing initial to nearly complete negative-strand DNA (Fig. 2A). To determine the relative efficiencies of amplification and detection for the different primer pairs and their corresponding oligonucleotide probes, a viral DNA standard curve was generated. Four- to fivefold serial dilutions of the 8E5 T cell line, containing one integrated copy of HIV DNA per cell (11), were made into a constant background of  $5 \times 10^4$  primary, uninfected CD4 lymphocytes (Fig. 2C). Amplification of the DNA regions representing initial reverse transcription (U5/LTR) and nearly complete reverse transcription (LTR/gag) demonstrated similar efficiencies of detection, equalling one to five DNA copies per  $10^3$  cells, or approximately 0.1 to 0.5% of the initial cell population. The sensitivity of detection for the *env* region approximated that for LTR/gag; for the *gag* region, it was approximately fivefold lower (Fig. 2C). Aliquots of  $5 \times 10^4$  primary CD4 cells were processed for PCR amplification of HIV DNA. Uninfected cells and cells infected in the presence of 3  $\mu$ M zidovudine (AZT) were compared with cells taken after infection with NL4-3 at 4 h (time zero) and 1, 3, 5, and 8 days (Fig. 2B). In agreement with other reports (19, 34), initiated segments of viral DNA (U5/LTR) were found in the virion inoculum. The viral DNA region corresponding to U5/LTR was detected immediately following infection, even in the presence of RT inhibition with AZT. However, the presence of initiated sequences of HIV DNA did not appear to reflect gross DNA contamination of the virion preparations, because regions representing late reverse-transcription events (*gag* and LTR/gag) were not seen on day 0. Progression to full-length minus-strand HIV DNA (LTR/gag) was not detected until 3 days following infection. cDNA products corresponding to the intermediate regions of *env* and *gag* were seen at 1 and 3 days after infection, respectively. All regions of the viral DNA showed relative quantitative increases occurring between 3 and 8 days following infection (Fig. 2B). PCR analysis was repeated in a second experiment and showed the same pattern of viral DNA formation. The results demonstrated that full-length viral DNA formed and accumulated in quiescent CD4 cell cultures following HIV infection.

To determine if any viral mRNA transcripts were made in infected resting cell cultures, an RT-PCR method with primers bracketing alternate splice sites (primers A and B) was used to detect HIV-specific, spliced mRNA transcripts (13) (Fig. 3A). A predominant cDNA product corresponding to a *nef* transcript was found to accumulate in the cells (Fig. 3B). The

TABLE 2. Quiescent characteristics of primary CD4 lymphocyte cultures

Expt	CD4 cells <sup>a</sup> (n)	Cell cycle <sup>b</sup>	Antigen <sup>c</sup>	% Positive cells on day postinfection:				
				0	3	5 or 6	8 or 10	
1	Control (5)	G <sub>0</sub> /G <sub>1</sub>	S	98.2	98.0	97.7	96.8	
				1.0	1.3	1.7	2.2	
				0.8	0.7	0.7	1.0	
		+LAI (2)	G <sub>0</sub> /G <sub>1</sub>	S	97.8	97.5	96.9	
					1.7	2.1	2.5	
					0.6	0.5	0.6	
	+NL4-3 (3)	G <sub>0</sub> /G <sub>1</sub>	S	98.1	96.9	96.9		
				0.9	1.7	2.0		
				1.0	1.5	1.1		
	2	Control (2)	CD45RO	DR	49	51	48	47
					12	14	14	18
					8	9	9	10
+NL4-3 (2)			CD45RO	DR	50	50	46	
					13	13	15	
					10	8	10	

<sup>a</sup> Isolated CD4 lymphocytes were infected on day 0 with the LAI strain (MOI = 0.5) or the NL4-3 clone (MOI = 0.1) of HIV-1. Cell aliquots were taken for FACS analysis prior to infection (day 0) and on days 3, 5 or 6, and 8 or 10 postinfection and compared with the uninfected-cell control.

<sup>b</sup> Cell cycle analysis based on DNA content per cell with propidium iodide stain.

<sup>c</sup> Cell surface antigen expression determined with phycoerythrin-conjugated antibodies.

appearance of detectable mRNA occurred late in the infection, after 3 to 6 days. In contrast, many different species of HIV spliced RNA, including *rev* and *tat*, were detected readily during productive viral replication in CD4 cells activated 3 days after infection by the addition of PHA (Fig. 3B). Because PCR with primers A and B also detects structural *vpu-env* transcripts, a second set of primers overlapping the major 3' intron (primers A and D [Fig. 3A]) was used to amplify selectively singly spliced *vpu-env* RNA to rule out the presence of this mRNA and confirm the identity of the *nef* transcript in the samples. The PCR product corresponding to *vpu-env* mRNA could not be detected at any time point during infection of the quiescent CD4 cells but could be seen following PHA mitogen stimulation, as predicted (data not shown). RT-PCR detection of specific viral mRNA transcripts was performed in repeated experiments using different cell donors, once with LAI infection and three times with NL4-3 infection. Spliced HIV mRNA representing the *nef* species was detected consistently between 4 and 8 days following infection of quiescent cell cultures. When probed blots were exposed for an extended time (16 to 20 h), higher-molecular-weight cDNA products in addition to the *nef* species could be seen in samples from infected quiescent cells. However, the additional RNA transcripts were found only in cells infected for 8 days, and *nef* RNA remained vastly predominant (data not shown).

**Formation of a stable, inducible provirus.** The findings of virus-specific, full-length DNA and limited mRNA production in infected quiescent cells indicated that HIV-1 was capable of establishing a biologically stable DNA form in the absence of cell activation. To test this premise, experiments were designed to detect the presence of fully reverse-transcribed viral DNA that was functional and inducible. The RT inhibitor AZT was

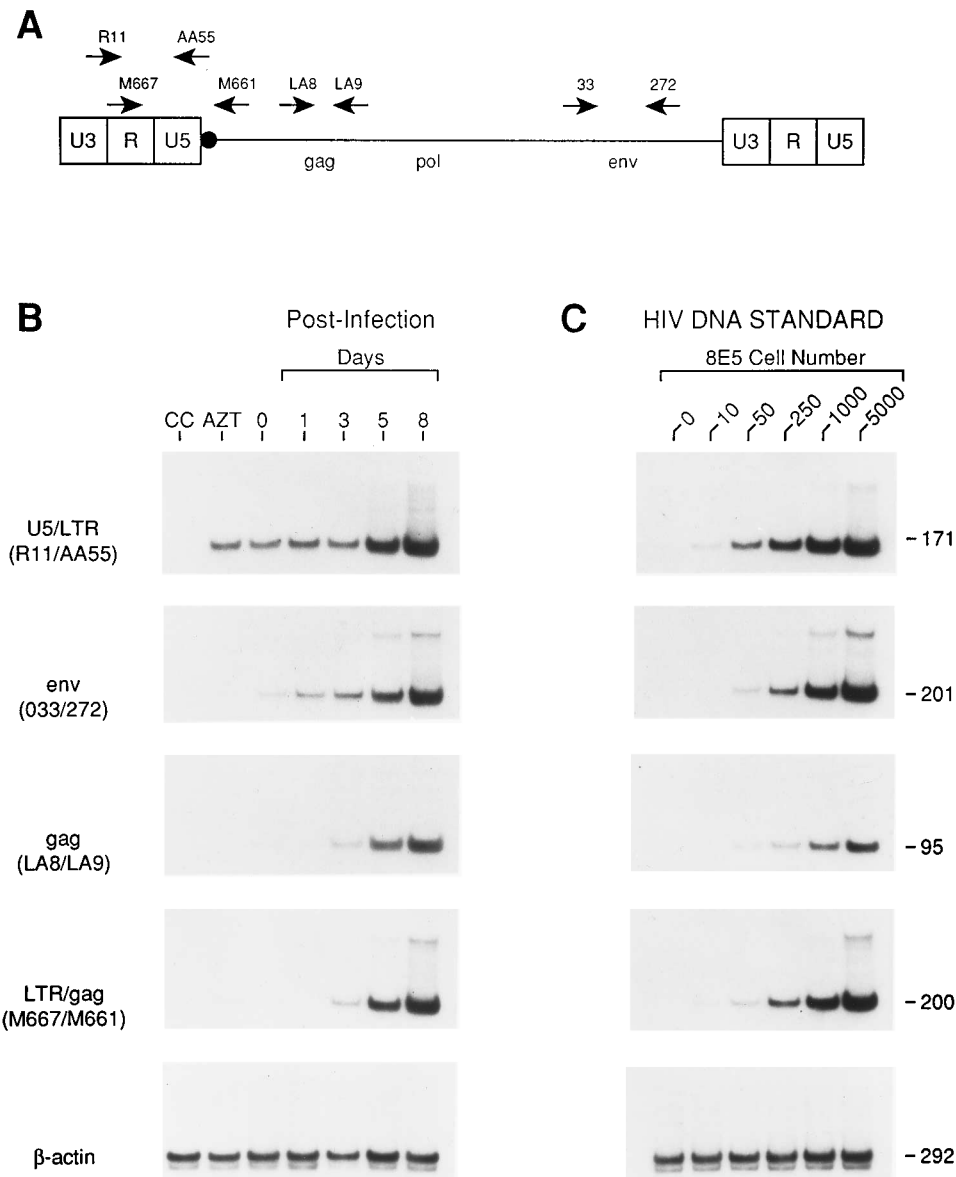


FIG. 2. HIV DNA formation in primary CD4 cells. (A) Schematic representation of HIV-1 DNA and the relative locations of oligonucleotide primer pairs used to detect different DNA regions. Primers R11 and AA55 are directed to the initial region of DNA formed by reverse transcription (U5/LTR). After the first template switching event, the regions of *env* (33 and 272) and *gag* (LA8 and LA9) are reverse transcribed sequentially. Primers M667 and M661 detect the region spanning the primer binding site and the 5' LTR (LTR/*gag*) which represents reverse transcription to full-length, minus-strand viral DNA. (B) Unstimulated, resting CD4 cells were infected with a filtered preparation of the NL4-3 clone (MOI = 0.1) for 4 h, and cell samples were taken immediately following infection (time 0) and at 1, 3, 5, and 8 days after infection (experiment of Fig. 4B). PCR amplification was performed on nucleic acid extracts from aliquots of  $5 \times 10^4$  cells. An uninfected-cell control (CC) and cells infected in the presence of  $3 \mu\text{M}$  AZT (AZT) were tested in parallel. The AZT control was included for primer pairs R11-AA55 and M667-M661 only. (C) Serial dilutions of the HIV-infected T-cell line 8E5 were made in a cell background of  $5 \times 10^4$  primary CD4 cells, nucleic acids were extracted, and PCR amplification was performed. Five percent of each reaction product was resolved by 6% polyacrylamide gel electrophoresis and hybridized with labeled probe. Autoradiography was done at  $-70^\circ\text{C}$  for 3 h for HIV DNA and 6 h for cellular  $\beta$ -actin. Numbers on the right indicate lengths of products in base pairs.

used to block the initiation and/or progressive reverse transcription of viral DNA. AZT ( $3 \mu\text{M}$ ) was added to CD4 cell aliquots either 1 h before infection or at serial time points after infection and 2 h prior to the addition of PHA plus rIL-2. At each time point, the induction of virus replication in the absence of AZT was compared with virus induction with the addition of AZT at 2 h prior to cell stimulation or at initiation of infection (Fig. 4). The addition of AZT at 3 to 4 h, 1 day, or 3 days after infection blocked the subsequent induction of virus replication. However, at times of  $\geq 5$  days, complete virus rep-

lication with soluble p24 production was induced from infected cells in the presence of AZT. Escape from AZT inhibition of RT was found with infection at both high and moderate inoculum doses, using the LAI strain at an MOI of 1 (Fig. 4A) and using filtered preparations of the NL4-3 clone at an MOI of 0.1 (Fig. 4B). Apparent differences in the relative effects of initial AZT treatment between infections with LAI and NL4-3 were due to differences in residual amounts of p24 that were present following the infection procedure. When the experimental design was repeated with two cell donors with LAI infection and

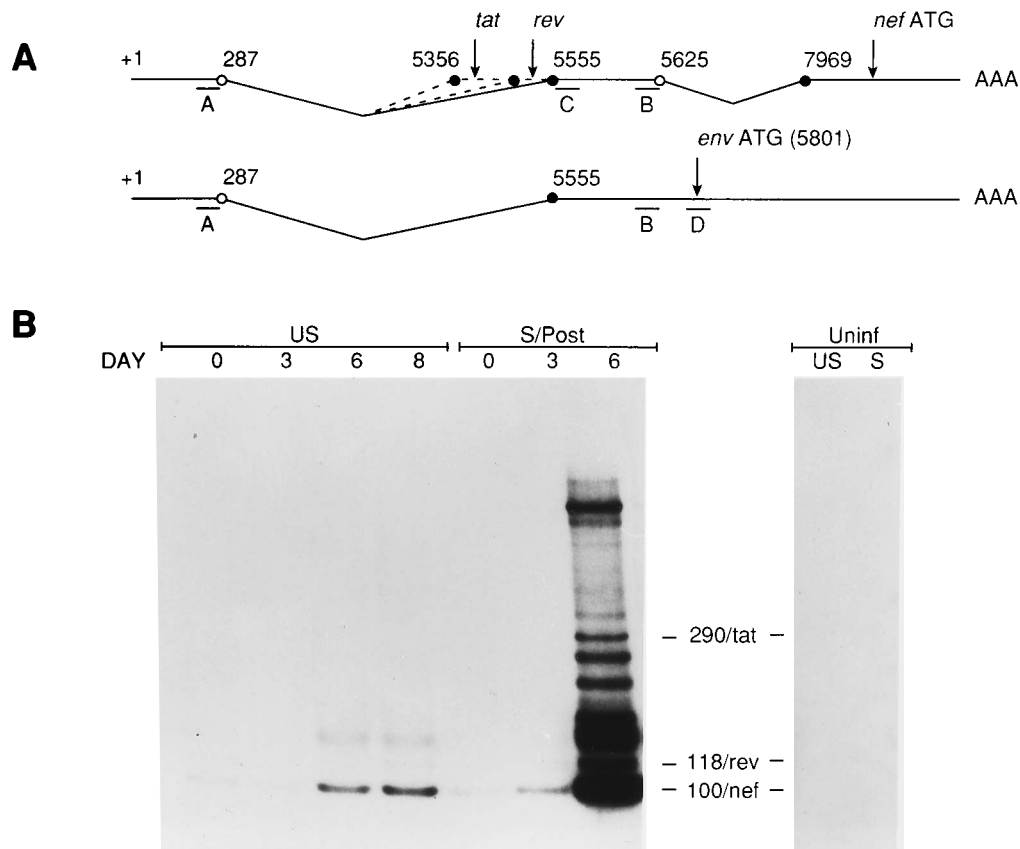


FIG. 3. Detection of HIV-1 spliced RNA transcripts. (A) Schematic representation of RT-PCR assay to detect specific RNA transcripts that utilize alternative splice sites. Nucleotide +1 is the cap site; open circles represent splice donors, and closed circles represent splice acceptors. cDNA fragments ranging from 872 to 100 bp result from reactions with primers A and B; a 285-bp fragment results from reactions with primers A and D. Oligonucleotide C is the probe used to detect a common sequence shared by products from reactions with primers A and B. Fragments F290, F118, and F100 are generated with primers A and B and correspond to spliced *tat*, *rev*, and *nef/vpu-env* transcripts, respectively. The F285 fragment is generated with primers A and D and corresponds to a singly spliced RNA transcript encoding the late genes *vpu* and *env*. (B) Quiescent CD4 cells were infected with the LAI strain at an MOI of 1 TCID<sub>50</sub> per cell. Infected cells were cultured in the absence of stimulation (US) or with the addition of PHA plus rIL-2 at 3 days postinfection (S/Post). Cell samples were taken on days 0, 3, 6, and 8 after infection; PCR analysis with primers A and B was performed on nucleic acid extracts from  $5 \times 10^4$  cells. Ten percent of each reaction product was electrophoresed on a 5% nondenaturing acrylamide gel, blot transferred, hybridized to <sup>32</sup>P-probe C, and exposed at  $-70^\circ\text{C}$  for 3 h.

with three cell donors with NL4-3, similar results were found. These biologic data were in agreement with the PCR-derived findings and showed that HIV DNA existed in an incomplete form early after infection but that reverse transcription to full-length viral DNA proceeded at a delayed or retarded pace in the absence of cell activation. Two additional types of experiments have supported this interpretation of the data and have argued against the possibility that these results are due to an extreme delay of viral entry into quiescent cells or to a slow spread of infectious virus between cells with increased time in culture. The enumeration of infected cells by detection of gp120 antigen expression following mitogen induction demonstrated that similar percentages of infected CD4 cells were found early and late after inoculation of the culture (Fig. 5). Second, the addition of a HIV-specific neutralizing antibody 4 h after infection with LAI did not diminish the levels of virus replication in CD4 cell cultures that were subsequently induced at 3, 5, and 8 days following infection (data not shown).

**Flow cytometry cell sorting of G<sub>0</sub>/G<sub>1</sub>- and S-phase cell subpopulations for PCR analysis.** Because a very minor portion (1 to 3%) of CD4 lymphocytes isolated from peripheral blood are found to be in S phase at any given time, these studies were pursued further to determine if complete viral DNA formation

was occurring only in this minor subset of cycling cells. Quiescent cultures of CD4 cells were infected with a filtered preparation of NL4-3. On days 4 and 8 following infection, cells were sorted with a fluorescence-activated cell sorter (FACS) on the basis of cell cycle analysis to obtain a G<sub>0</sub>/G<sub>1</sub>-phase subset purified to >99% and an S-phase subset enriched to >65% (Fig. 6A). PCR amplification for the most complete viral DNA region (LTR/*gag*) was performed on the total, unsorted CD4 cell population and the sorted cell subsets. As noted before, the relative amount of viral DNA increased in the total CD4 cells between days 4 and 8 postinfection (Fig. 6B). In the sorted cell subsets, viral DNA accumulated more rapidly in the cycling S-phase cell fraction. After 4 days of infection, the majority of full-length HIV DNA detected appeared to reside within the enriched S-phase cell fraction; however, completed viral DNA formation was also clearly present in the purified G<sub>0</sub>/G<sub>1</sub>-phase cell fraction. By day 8 of infection, a significant level of completed reverse transcription to viral DNA was found in the purified G<sub>0</sub>/G<sub>1</sub>-phase cell fraction, approaching that seen within the enriched S-phase subset (Fig. 6B). On the basis of a parallel analysis of the HIV DNA standard curve, it was estimated that the G<sub>0</sub>-phase sample extracted from  $2.5 \times 10^4$  cells contained 5,000 copies of the

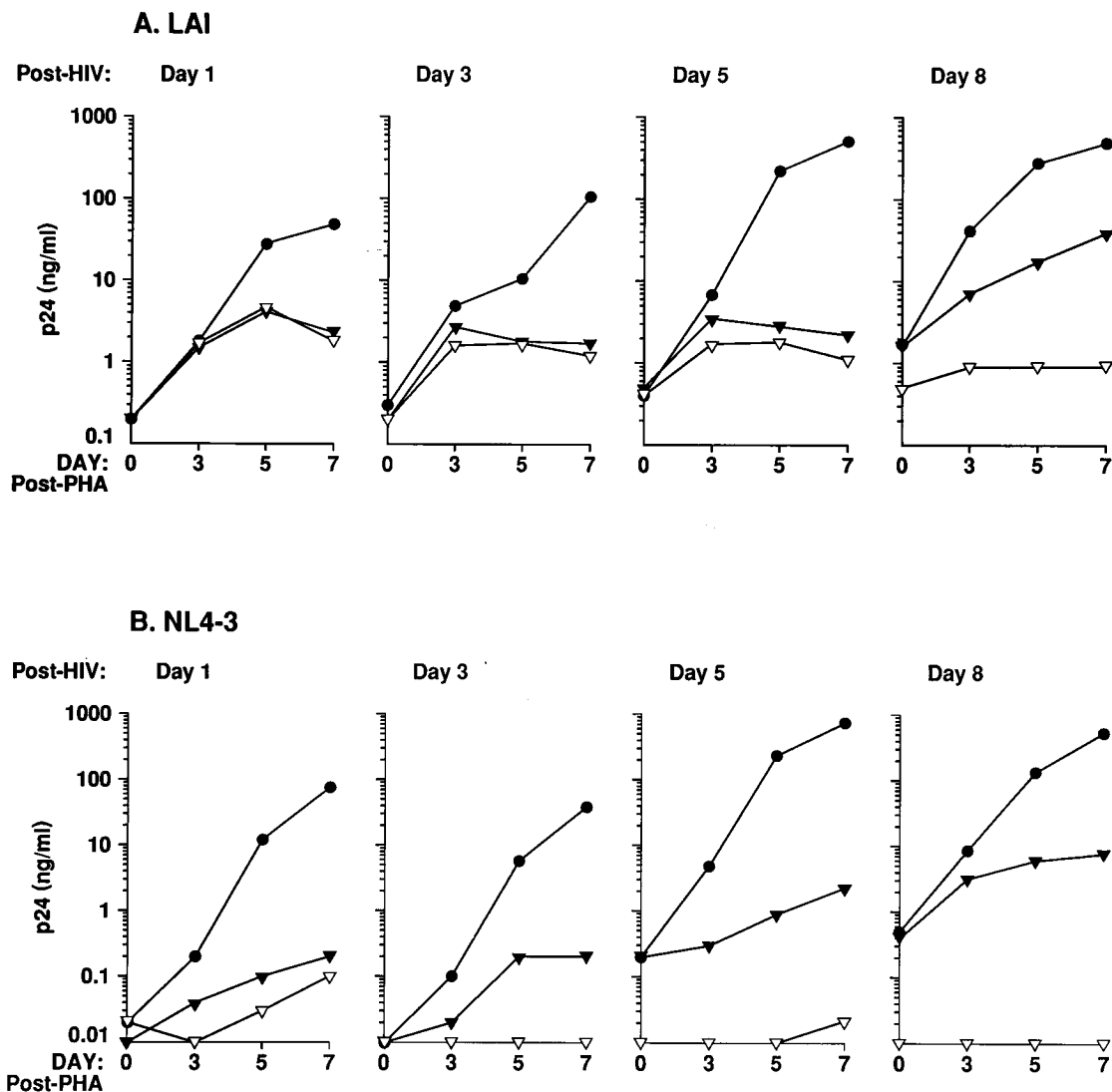


FIG. 4. Detection of a replication-competent form of HIV DNA. Resting CD4 cells were infected on day 0 with either the LAI strain at an MOI of 1 (A) or a filtered preparation of the NL4-3 clone at an MOI of 0.1 (B). On days 1, 3, 5, and 8 following infection, PHA and rIL-2 were added to cell aliquots in the presence or absence of 3  $\mu$ M AZT. Induction of complete virus replication was assessed by monitoring production of soluble p24 from infected cells without AZT ( $\bullet$ ), with AZT added 2 h prior to stimulation ( $\blacktriangledown$ ), and with AZT added prior to infection ( $\nabla$ ). Representative experiments, using both high (A) and moderate (B) levels of virus inoculum, are depicted.

LTR/gag species of HIV DNA (Fig. 6C). Assuming that there is no more than one viral DNA copy per cell in a quiescent cell population, these data indicated that approximately 20% of the  $G_0$ -phase cells contained HIV DNA after 8 days of infection. It was not possible that this amount of viral DNA product could be derived from the minor cell contaminant of 0.8% non- $G_0$ / $G_1$ -phase cells present in the sorted cell fraction, even if the contaminating cells were assumed to be exclusively in S phase. For this to be the case, each S-phase cell would have had to contain 25 copies of HIV DNA (i.e., 5,000 copies per 200 cells). However, the amount of viral DNA present in the fraction enriched to 69% S-phase cells was estimated to be in the range of one viral DNA copy per 1.8 to 3.6 cells (<10,000 to >5,000 copies per 18,000 cells). To confirm this quantitative estimation of HIV DNA formation in  $G_0$ / $G_1$ -phase cells, a serial dilution experiment was performed. An aliquot of cellular nucleic acids extracted from the  $G_0$ / $G_1$  purified fraction was diluted into a constant uninfected-cell background of  $5 \times 10^4$

primary CD4 cell equivalents. The samples were amplified in parallel with an HIV DNA standard dilution curve for the LTR/gag region of viral DNA (Fig. 7). This analysis demonstrated the presence of a relatively constant percentage of cells (10 to 20%) containing HIV DNA at the different target cell dilutions: 1,000 to 2,000 per 10,000, 500 per 2,500, and 100 per 500 HIV DNA copies per  $G_0$ -phase cells. S-phase cells were not recovered in sufficient quantity to provide for a dilution analysis. CD4 lymphocytes from two additional donors have been sorted and analyzed by PCR amplification, and similar results were obtained. In one of these repeat experiments, the purity of the  $G_0$ / $G_1$ -phase cell fraction, sorted after 8 days of infection, was 99.9%, and the dilution analysis indicated a constant infected-cell percentage of 10% (i.e., 1,000 per 10,000, 250 per 2,500, and 100 per 1,000 HIV DNA copies per  $G_0$ -phase cells).

It was not possible to do RT-PCR amplification for HIV mRNA transcription with the sorted cell fractions that had

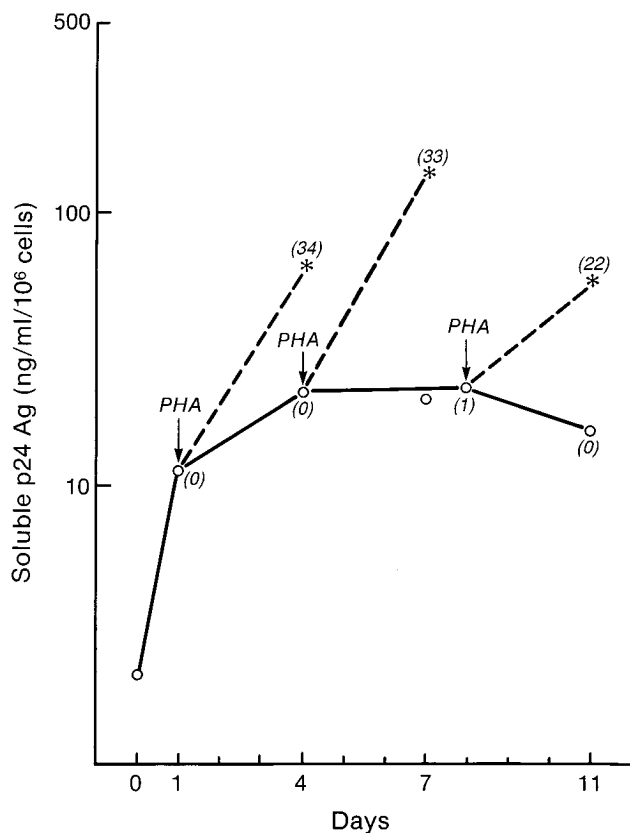


FIG. 5. Efficiency of HIV-1 infection. Cultures of quiescent CD4 lymphocytes ( $4 \times 10^6/2$  ml in 15-ml culture tubes) were infected with LAI on day 0 (MOI = 1). PHA and rIL-2 were added to cultures on days 1, 4, and 8 after infection. Virus replication was determined by p24 analysis on day 3 (\*) following cell stimulation. The percentage of cells staining positive for gp120 antigen expression, prior to and following each induction cycle, is indicated in parentheses. Cultures were not adjusted for cell viability prior to stimulation; cell viability in the HIV-infected cultures decreased from 94% on day 4 to 75% on day 11.

been prepared by the standard methodology with propidium iodide. Staining of cellular DNA with propidium iodide requires the inclusion of RNase degradation to eliminate stain interactions with RNA secondary structures (32). To enable RT-PCR analysis, a different technique, using 7-aminoactinomycin D staining of cellular DNA (27, 33), was adapted to this experimental design. Because our prior data showed the appearance of viral spliced mRNA very late in HIV infection of quiescent cells, CD4 lymphocytes were FACS sorted into  $G_0/G_1$ - and S-phase-enriched fractions for RT-PCR analysis after 8 days of infection (Fig. 8A). To minimize the amount of natural RNA degradation occurring during sample preparation, cells were sorted within 1 h of fixation and staining, harvested into iced containers, and extracted immediately after completion of the sort. Aliquots of  $5 \times 10^4$  cell equivalents were amplified by RT-PCR with primers A and B to detect cDNA corresponding to HIV spliced transcripts as described above. An HIV mRNA product corresponding to a *nef* species was detected in the total CD4 cell population after 4 days of infection and increased significantly by day 8, with the appearance of other mRNA species (Fig. 8B). In the samples from day 8, unfixed total CD4 cells were compared with fixed and stained CD4 cells and with the sorted fractions of  $G_0/G_1$ - and S-phase cells. It appeared that a substantial amount of total RNA was lost during the cell fixation and permeabilization

procedure required for the internalization of the stain and/or that cell enumeration was less accurate following fixation (Fig. 8B, day 8, unfixed versus fixed cells). Even with this technical complication, the total cellular RNA contents remained comparable for the various fixed cell samples, enabling a valid comparison of HIV mRNAs in the sorted cell fractions. In contrast to the pattern of viral DNA formation, HIV mRNA transcription was detected almost exclusively within the CD4 cell fraction enriched for S-phase cells (Fig. 8B). The experiment was repeated with cells from a different donor, and the same results were obtained. These findings demonstrated that HIV infection is capable of producing a stable, fully reverse-transcribed viral DNA form in truly quiescent CD4 lymphocytes but that viral replication is blocked from progression to transcription in the absence of cell activation.

## DISCUSSION

In T lymphocytes taken from the peripheral blood or lymph nodes of HIV-1-infected patients, the majority of HIV appears to exist as a DNA form that is unintegrated and transcriptionally inactive or that is restricted to early stages of RNA transcription (2, 5, 8, 24, 25, 28). Because the vast majority of CD4 lymphocytes in such individuals are in a quiescent state, these findings suggest that an important feature of HIV-1 infection is the ability to establish and maintain a stable infection in quiescent T cells. In contrast to the case for the *in vivo* setting, it has been difficult to demonstrate clearly *in vitro* a complete stable infection with HIV-1 in quiescent T-cell cultures. Early studies, using direct Southern blot analysis of viral DNA (12), lacked the sensitivity required to detect low-level infection in primary cell cultures. Later studies, using PCR amplification to detect viral DNA formation (31, 42), were able to show active HIV infection of resting T-cell cultures but differed in their findings of a stable provirus. One research group (41, 42) found an abortive infection resulting in partially reverse-transcribed viral DNA that was labile and rapidly degraded in the absence of induced cell activation. Another group (31) reported finding a complete infection process with stable viral DNA maintained in an unintegrated state and the early formation of viral RNA transcripts. This area of HIV research has remained controversial.

In our investigations of the regulation of HIV-1 replication, we have used an *in vitro* model of acute virus infection of quiescent, primary CD4 lymphocytes isolated from the peripheral blood of HIV-seronegative donors. Studies with this cell model demonstrated that viral infection proceeded to a stage of fully reverse-transcribed viral DNA in the absence of cell activation and that this infection was stable for up to 10 days in culture. The stability of infection was shown by the similar kinetics and levels of virus replication induced at all time points after infection. Rather than decreased virus recovery with extended time in culture, there was an increase in viral replication kinetics when mitogen induction was applied 8 to 10 days after infection. These results indicated that after longer periods of infection in quiescent cells, HIV existed in a state primed for rapid virion production upon the addition of exogenous stimulus. Similar findings with a different HIV clone in peripheral blood mononuclear cell cultures have been reported recently by another group (37). Analysis of viral DNA by PCR amplification confirmed our initial observations by demonstrating the appearance and accumulation of fully reverse-transcribed HIV DNA between 3 and 8 days following infection. Coincident with the late appearance of full-length viral DNA, RT-PCR amplification detected low levels of HIV spliced RNA transcription characterized by the presence of a



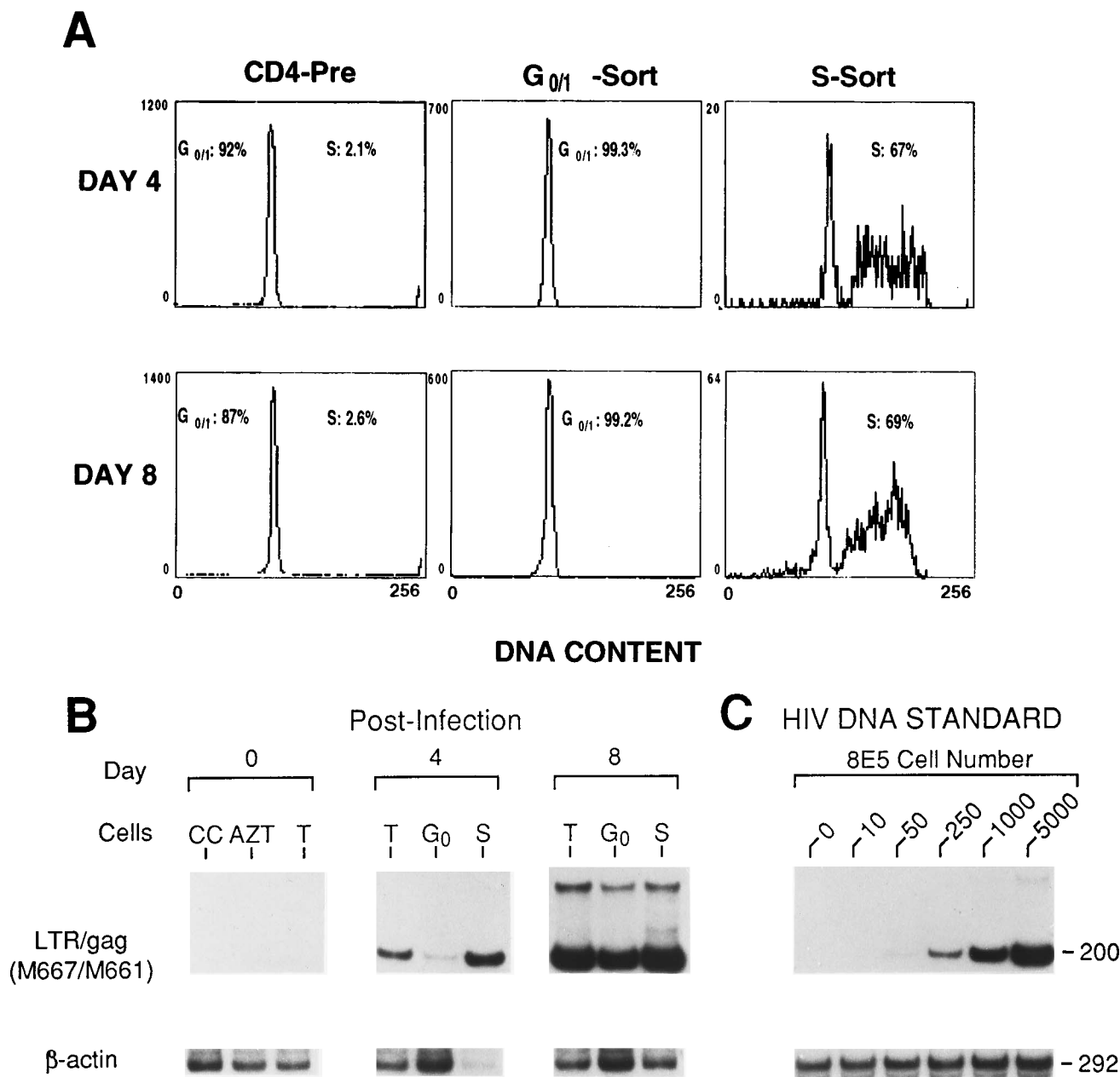


FIG. 6. HIV DNA formation in G<sub>0</sub>/G<sub>1</sub>- and S-phase cell subsets. (A) CD4 lymphocytes were infected with a filtered preparation of the NL4-3 clone (MOI = 0.1) and cultured in the absence of stimulation. On days 4 and 8 after infection, cells were FACS sorted on the basis of DNA content to recover a purified G<sub>0</sub>/G<sub>1</sub>-phase subset and an enriched S-phase subset. CD4-Pre, relative proportion of subsets in the total CD4 cell sample prior to sorting; G<sub>0/1</sub>-sort and S-sort, purities and enrichments of subsets in the postsort samples. (B) PCR amplification for the full-length fragment of HIV DNA (LTR/gag) was performed on nucleic acid extracts from aliquots of 2.5 × 10<sup>4</sup> cells from total unsorted CD4 cells (T), and sorted G<sub>0</sub>/G<sub>1</sub>-phase (G<sub>0</sub>) and S-phase (S) cell subset fractions. An uninfected-cell control (CC) and cells taken immediately following infection in the presence of AZT (AZT) were analyzed in parallel. (C) An HIV-1 DNA standard curve was analyzed in parallel. 8E5 cells were diluted into a constant background of 5 × 10<sup>4</sup> primary CD4 cells. Five percent of all reaction products was subjected to 6% polyacrylamide gel electrophoresis analysis and probing. The autoradiography exposure times were 1 h for HIV DNA and 6 h for cellular β-actin. Numbers on the right indicate lengths of products in base pairs.

predominant *nef* species. Additional experiments supported the finding of slowly accumulating, biologically functional viral DNA in quiescent cultures. A comparison of progression of infection in the presence and absence of AZT treatment to inhibit RT demonstrated that progressive reverse transcription to viral DNA occurred with increasing time after infection. After 5 days of infection, AZT treatment was no longer capable of blocking completely induced virus replication; some

portion of infected CD4 cells contained full-length viral DNA that escaped the RT block. The enumeration of productively infected cells by analysis of induced gp120 membrane expression showed similar proportions of infected cells at 1 and 4 days after virus inoculation of quiescent cultures. Taken together with the PCR-generated data showing increasing viral DNA formation in the same time frame, these findings suggest that HIV-1 rapidly establishes infection in resting primary CD4

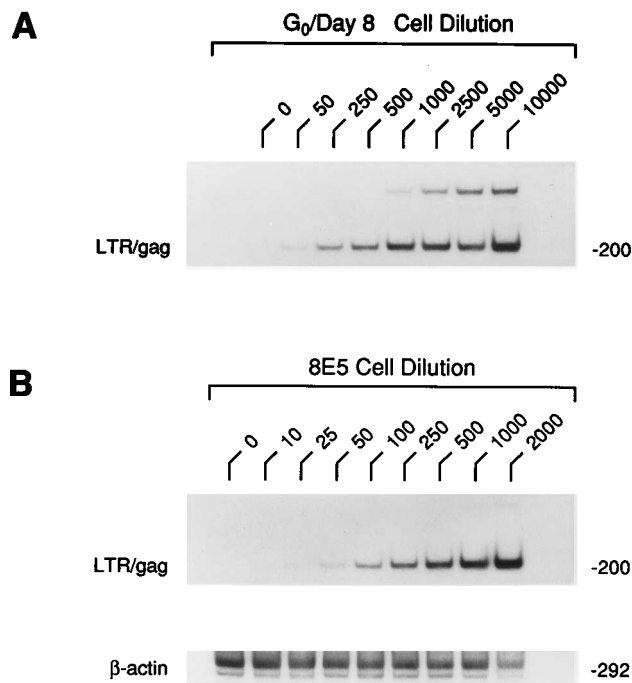


FIG. 7. Quantitative dilution analysis of HIV DNA in  $G_0/G_1$ -phase cells. (A) An extract of total nucleic acids from the purified  $G_0/G_1$ -phase cell fraction isolated after 8 days of HIV infection (experiment of Fig. 6) was diluted serially into an uninfected-cell background of  $5 \times 10^4$  primary CD4 cells. Samples were PCR amplified for the LTR/gag region of HIV DNA. (B) A dilution analysis of the HIV-infected, 8E5 cell standard in a background of  $5 \times 10^4$  primary CD4 cells was performed in parallel. Five percent of each reaction product was analyzed by polyacrylamide gel electrophoresis. Probed blots were exposed to autoradiography for 1 h for HIV DNA and 1.5 h for cellular  $\beta$ -actin. Numbers on the right indicate lengths of products in base pairs.

cells but that reverse transcription to full-length viral DNA occurs slowly in the absence of cell activation.

To ensure that our CD4 cell cultures were truly representative of the original population of quiescent lymphocytes in the peripheral circulation, the isolated cells were monitored during culture by flow cytometry analysis for expression of membrane activation antigens and for shifts in cell cycle from  $G_0/G_1$  to  $S/G_2$  phase. No significant changes in any of the tested activation parameters were detected. Although it can always be argued that subtle alterations in the cell activation state can occur without detection, we have not found it practical to monitor early enzymatic or metabolic events following cell isolation or infection. It is unlikely that the occurrence of such early, transient signals would result in the delayed kinetics of viral DNA formation seen in our study. Because a very minor portion of peripheral blood lymphocytes are found naturally to be in S phase, it was possible that the detected reverse transcription to a full-length viral DNA species was occurring in only this cell subset. The S-phase fraction within preparations of isolated CD4 cells represented a small population of 1 to 3%; however, this fraction was well within the range of sensitivity for detection of viral DNA by our PCR method ( $\geq 0.5\%$  of the target population). The analysis of FACS-sorted cell subsets following infection by HIV demonstrated that complete viral DNA formation occurred in the purified fraction of  $G_0/G_1$ -phase cells (>99%) in addition to the S-phase-enriched cell fraction. Fully reverse-transcribed viral DNA was detected earlier after infection in the enriched S-phase cells than in the purified fraction of  $G_0/G_1$ -phase cells, which accumulated viral

DNA slowly. By day 8 of infection, the sorted cell samples showed less than a threefold difference between the S-phase and  $G_0/G_1$ -phase cell subsets in the amount of amplified viral DNA product, even though the S-phase fraction had been enriched 86-fold for cells in cycle. A dilution analysis of the infected target population demonstrated that 10 to 20% of the purified  $G_0/G_1$ -phase cell subset contained full-length viral DNA after 8 days of infection with an initial virus inoculum of 0.1 TCID<sub>50</sub> per cell. This infectivity rate for HIV in primary, quiescent CD4 cell cultures was found to be consistent within individual experiments and between several experiments using different donor cells. The PCR-derived results indicate that HIV can be highly efficient in establishing a stable DNA form in nondividing CD4 lymphocytes. The true replication competence of these DNA forms is difficult to assess, because it is not possible to induce 100% of a primary lymphocyte culture into synchronous cell division by the addition of exogenous stimuli. In contrast to the finding of progressive viral reverse transcription in quiescent cells, the appearance of detectable HIV mRNA transcription was localized to the minor subset of S-phase cells present within the total CD4 cell population. These data support the concept that proviral forms of HIV are transcriptionally inactive when present in quiescent  $G_0/G_1$ -phase lymphocytes.

The findings from our study differ in certain aspects from the published findings from previous studies using similar research techniques (31, 42). Most probably, the noted differences derive from specific differences in experimental design, especially those of cell culture conditions, virus clones, and virus inoculum. Previous studies used peripheral blood lymphocytes to study HIV infection; we have used preparations of isolated CD4 T lymphocytes to eliminate potential influences from other cells and cytokines and to achieve relatively high and consistent MOI. The initial study by Stevenson et al. (31) reported the presence of viral RNA transcripts very early after infection. We also detected HIV transcriptional activity in cultures of infected CD4 cells but in a low quantity after several days of infection. Stevenson et al. used Polybrene for virus infection and fetal bovine serum to supplement the cell culture medium. Both of these reagents can influence cell activation, and this may account for the enhanced appearance of viral RNA products in their study. Further experimental analysis, utilizing flow cytometry cell sorting, has allowed us to identify the minor subpopulation of S-phase cells as the source of the detected viral transcription. The reported findings of an abortive, labile infection in quiescent lymphocytes by Zack et al. (41, 42) differ significantly from our results. Although the culture conditions were similar, these studies used a virus inoculum significantly lower than ours, and potential outgrowth of CD8 lymphocytes in culture could have decreased further the apparent efficiency of infection. It should be noted also that although the majority of HIV DNA detected by Zack et al. represented partial RT transcripts, a minor species of full-length viral DNA was evident after 5 days of infection (42). Perhaps the major difference between these experimental models was the stability of infection achieved with the various HIV-1 clones studied. The NL4-3 clone used by ourselves and the mfA clone used by Stevenson et al. established an infection that was stable for 10 to 14 days in culture. Another group has recently reported similar results with the R7 clone of HXB2 (37). In contrast, Zack et al. found that the ability to recover infectious virus from culture diminished with time, following cell inoculation with the JR-CSF clone. It is now believed that several viral proteins, including p17 matrix, RT, Vpr, and possibly Nef, are important to the establishment of HIV-1 infection in nondividing cells (3, 4, 29, 37). It is possible that vari-

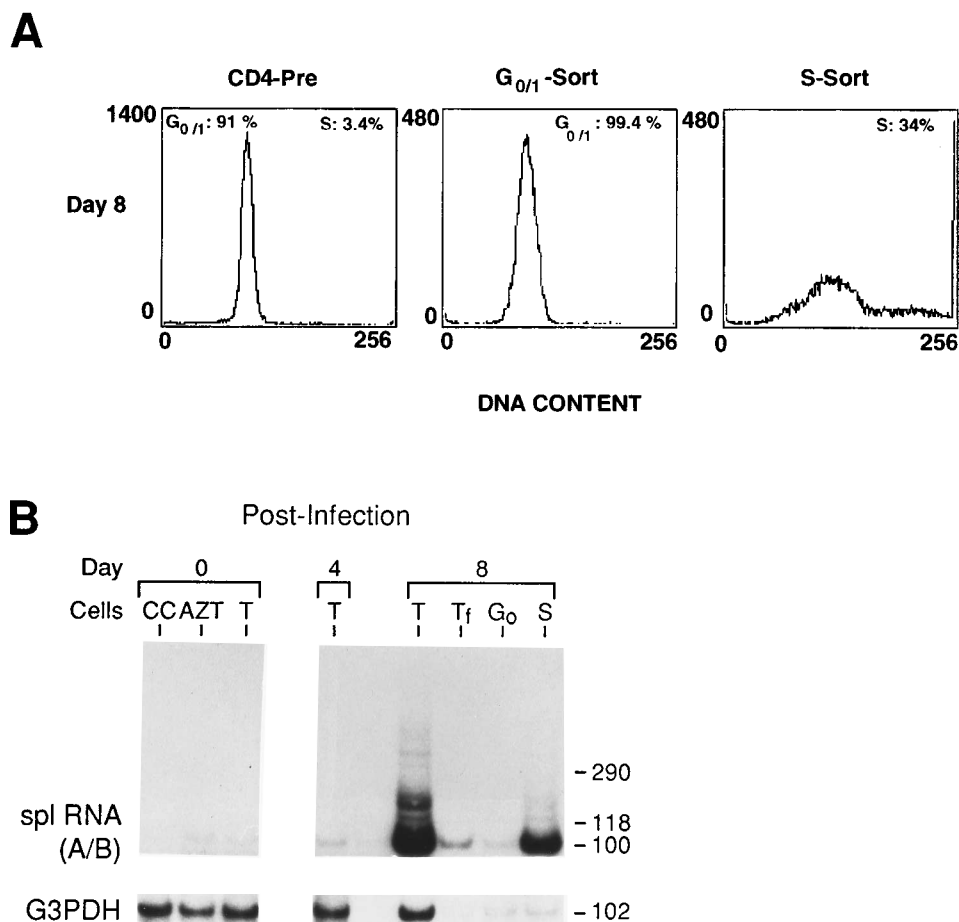


FIG. 8. Analysis of HIV transcription in  $G_0/G_1$ - and S-phase cell subsets. (A) CD4 lymphocytes were infected with NL4-3 (MOI = 0.1) and cultured in the absence of stimulation. On days 0, 4, and 8 of infection, aliquots of total CD4 cells were taken and frozen. On day 8, infected cells were fixed, stained with 7-aminoactinomycin D, and FACS sorted into a purified  $G_0/G_1$ -phase subset ( $G_0$ ) and an enriched S-phase subset (S). Histograms depict the relative subset proportions prior to sorting (CD4-Pre) and the enrichment obtained in each fraction after sorting. (B) RT-PCR amplification of HIV spliced (spl) RNA transcripts with primers A and B was performed on extracted aliquots of  $5 \times 10^4$  cells from the unfixed (T) and fixed ( $T_f$ ) total CD4 cell samples and the sorted  $G_0$ - and S-phase cell fractions. Uninfected cells (CC) and cells infected in the presence of AZT (AZT) were analyzed in parallel. The variation in total cellular RNA recovery was monitored by amplification for G3PDH gene transcription. Five percent of the reaction products was subjected to polyacrylamide gel electrophoresis analysis. The autoradiography exposure times were 18 h for HIV mRNA and 20 h for G3PDH.

ation in any, or all, of these proteins can affect the efficiency of HIV infection in primary cells. In this context, it may be relevant that the JR-CSF clone was found to be significantly less pathogenic than NL4-3 in a murine SCID-hu model of virus infection in primary T cells (16). The resolution of this question will require a more extensive evaluation and comparison of specific HIV gene sequence variations and resulting differences in protein function.

The results from our study reported here demonstrate that HIV-1 infection in vitro can progress to a fully reverse-transcribed, but transcriptionally inactive, form of viral DNA in CD4 lymphocytes that are truly representative of a quiescent cell population. These findings are consistent with in vivo data showing the existence of HIV DNA predominantly as a stable, extrachromosomal form in primary T cells from the peripheral circulation of HIV-infected individuals.

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