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Analysis of Early Human Immunodeficiency Virus Type 1 DNA Synthesis by Use of a New Sensitive Assay for Quantifying Integrated Provirus

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A novel *Alu*-long terminal repeat (LTR)-based real-time nested-PCR assay was developed to quantify integrated human immunodeficiency virus type 1 (HIV-1) DNA in infected cells with both accuracy and high sensitivity (six proviruses within 50,000 cell equivalents). Parallel assays for total HIV-1 DNA and two-LTR HIV-1 DNA circles allowed the synthesis and fate of the different HIV-1 DNA species to be monitored upon a single round of viral replication.

The RNA genome of human immunodeficiency virus type 1 (HIV-1) is, like those of other retroviruses, reverse transcribed in the cytoplasm into a double-stranded linear DNA containing a copy of the long terminal repeats (LTR) at each terminus. The resulting linear DNA molecule moves into the nucleus as a component of the preintegration complex, where it may integrate into the host cell genome (17). This process is dependent on viral integrase activity, which is essential for a productive infection (7, 8, 13, 16, 19, 21). In addition to linear DNA species, HIV-1 DNA circles with one or two LTR are detected in the nucleus (1, 11).

While the unintegrated forms of HIV-1 DNA can be readily detected by using standard PCR or Southern hybridization techniques, quantitative analysis of integrated HIV-1 DNA has been hampered by the lack of a reliable assay. Early studies have attempted to quantify integrated viral DNA by PCR using one primer annealing in the HIV-1 LTR and a second in the highly repeated chromosomal *Alu* element (*Alu*-LTR PCR) (6, 18). *Alu* repeated sequences are interspersed in the human genome and number at least 900,000 copies per haploid genome, giving an average distance of 4 kb between *Alu* elements (2). However, previous *Alu*-LTR PCR methods have limited sensitivity and do not take into account that the distance between an integrated viral DNA and its nearest *Alu* repeat is variable.

To detect and accurately quantify integrated HIV-1 DNA with high sensitivity, we have developed a new method, based on *Alu*-LTR real-time nested PCR. In conjunction with the study of HIV-1 DNA integration, real-time quantitative PCR was used to analyze the synthesis and fate of total HIV-1 DNA and two-LTR HIV-1 circles during a single-round of viral replication.

Generation of an integrated HIV-1 DNA standard. To accurately determine the integrated HIV-1 DNA copy number within a sample, the standard should be representative of a natural viral infection and contain numerous integration sites with a wide distribution of distances between the provirus LTR and the nearest *Alu* sequence. To generate a proper integrated HIV-1 DNA standard, HeLa cells were infected with a Δ*env* HIV-1 R7 Neo virus pseudotyped with the G glycoprotein from vesicular stomatitis virus (VSV-G). VSV-G-pseudotyped HIV-1 R7 Neo virus stocks were prepared by cotransfection into 293T cells of the pR7 Neo Δ*env* vector (gift from U. Hazan) with an expression vector encoding VSV-G. The pR7 Neo Δ*env* vector was constructed by deleting the envelope coding sequence of the HIV-1 R7 Neo genome (9), in which a neomycin resistance gene replaces the HIV-1 *nef* coding sequence. Infected cells were then cultured for several weeks in the presence of G418 (500 μg/ml) to select cells that contained integrated viral DNA and to allow the loss of all unintegrated forms. Ten days after initiation of the G418 selection, neomycin-resistant cell clones were counted; they numbered approximately 5,000. For our standard cell line, designated HeLa R7 Neo, the copy number of integrated viral DNA matched the total HIV-1 DNA copy number. Thus, based on total HIV-1 DNA and β-globin quantifications (see below), we estimated that the standard cell line contained 1.24 ± 0.03 proviruses per cell (data not shown).

***Alu*-LTR-based real-time nested-PCR procedure.** Amplification reactions were performed with the Light Cycler instrument (Roche Diagnostics, Meylan, France). In a first round of PCR, integrated HIV-1 sequences were amplified with two outward-facing *Alu* primers that anneal within conserved regions of the *Alu* repeat element together with an HIV-1 LTR-specific primer (Fig. 1a). As previously proposed (18), the use of two outward-facing *Alu* primers optimizes the probability of amplifying an LTR sequence, as *Alu* elements are present in either orientation relative to the integrated provirus (Fig. 1a). In addition, we used an LTR primer extended with a lambda phage-specific heel sequence at the 5′ end of the oligonucleotide (L-M667) in this first amplification step (Fig. 1a). *Alu*-LTR sequences were amplified in duplicate from 1/50 of total

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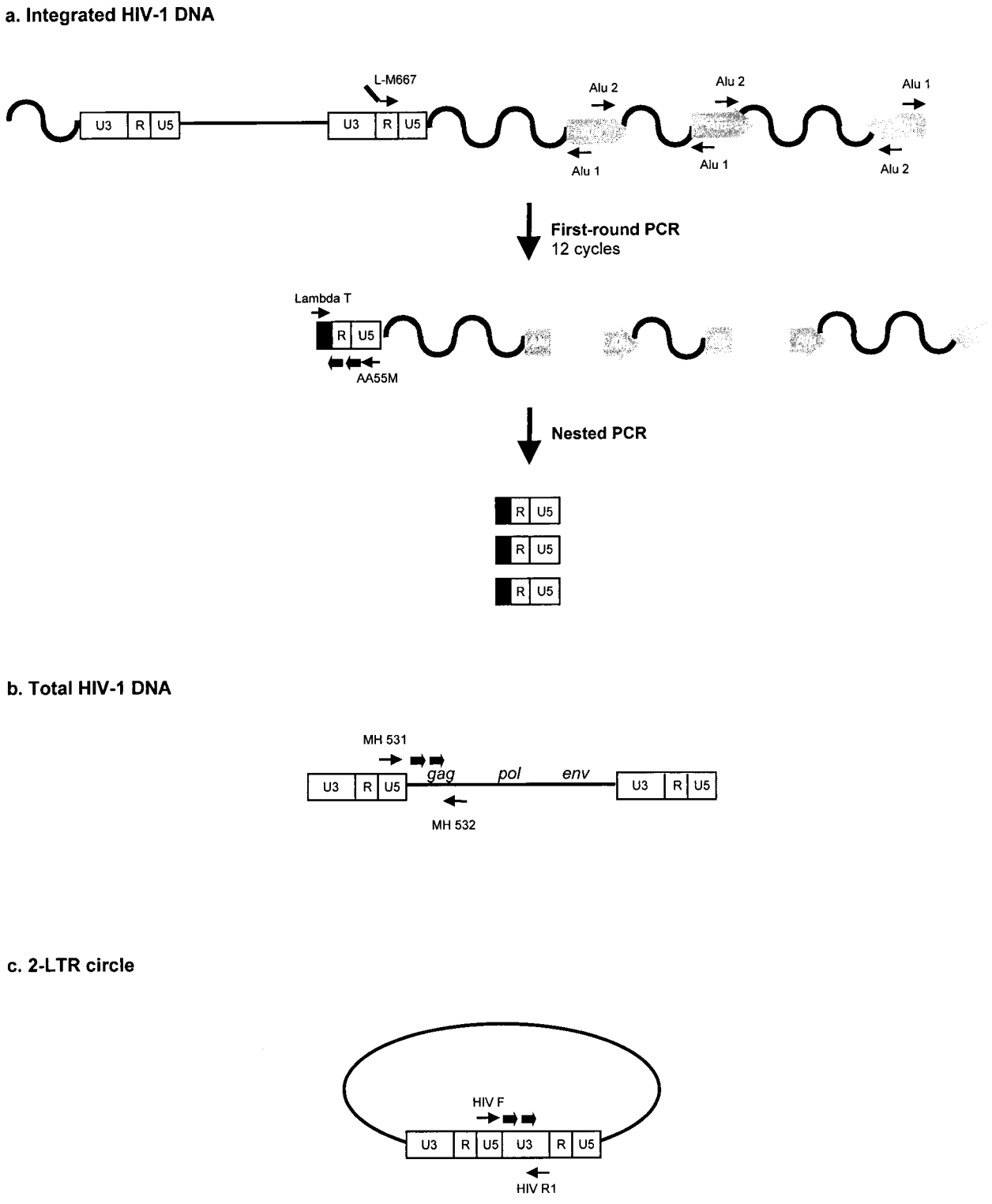

FIG. 1. Real-time PCR strategies and location of primers and probes for the quantification of integrated HIV-1 DNA (a), total HIV-1 DNA (b), and two-LTR circles (c). Thin arrows, primers; thick arrows, probes.

cell DNA in a 20-μl reaction mixture comprising 1× Light Cycler Fast Start DNA master hybridization probes (Roche), 4 mM $MgCl_2$, 100 nM L-M667 primer, and 300 nM (each) primers *Alu* 1 and *Alu* 2 (18) (Table 1). Given the high number of *Alu* elements within the human genome, abundant amplifications of inter-*Alu* sequences occurred simultaneously with the amplification of *Alu*-LTR sequences. To remain in the exponential phase, only 12 cycles of amplification were performed.

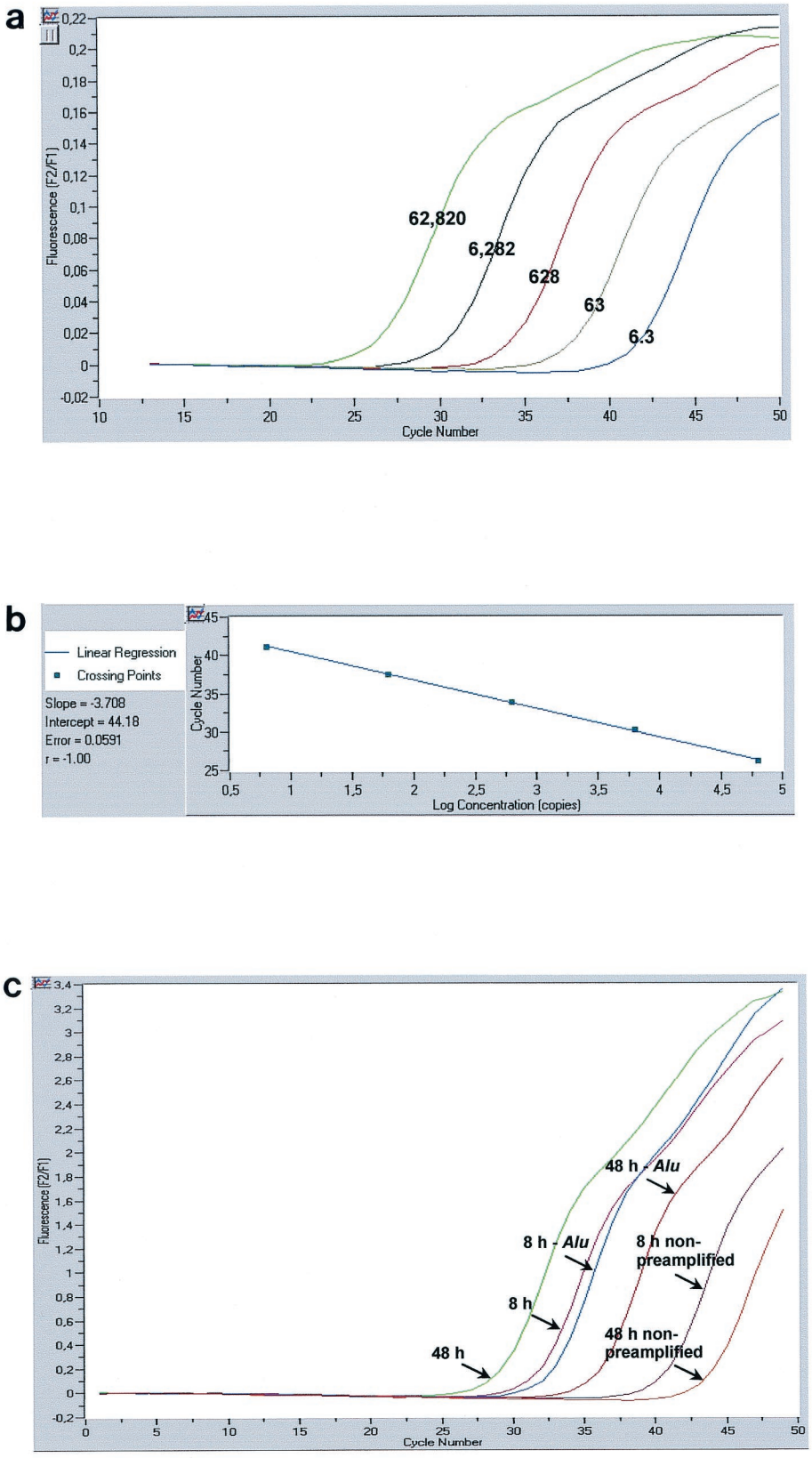

FIG. 2. Characteristics of *Alu*-LTR nested PCR. (a) Fluorescence curves generated by two-step amplification of serial dilutions of HeLa R7 Neo cell DNA. The copy numbers for each standard dilution are shown over the corresponding fluorescence curve. (b) Linear regression for quantifying integrated HIV-1 DNA. (c) Specificity of the *Alu*-LTR nested PCR. Fluorescence curves generated by amplification of viral DNA from CEM cells infected with the VSV-G-pseudotyped HIV-1 R7 Neo virus and collected in the presence of *Alu* primers (pink, 8 h p.i.; green, 48 h p.i.), in the absence of *Alu* primers (blue, 8 h p.i.; red, 48 h p.i.), or without a preamplification step (purple, 8 h p.i.; orange, 48 h p.i.) are shown.

TABLE 1. Primer and probe sequences

Primer or probe[c]	Sequence (5′–3′)	Target
MH 531	TGTGTGCCCGTCTGTTGTGT	Total HIV-1 DNA
MH 532	GAGTCCTGCGTCGAGAGAGC	
MH FL*	CCCTCAGACCCTTTTAGTCAGTGTGGAA[a]	
MH LC*	TCTCTAGCAGTGGCGCCCGAACAG[b]	
HIV F	GTGCCCGTCTGTTGTGTGACT	Two-LTR circle
HIV R1	ACTGGTACTAGCTTGTAGCACCATCCA	
HIV FL*	CCACACACAAGGCTACTTCCCTGA[a]	
HIV LC*	TGGCAGAACTACACACCAGGGC[b]	
L-M667	ATGCCACGTAAGCGAAACTCTGGCTAACTAGGGAACCCACTG	Integrated HIV-1 DNA (first-round PCR)
Alu 1	TCCCAGCTACTGGGGAGGCTGAGG	
Alu 2	GCCTCCCAAAGTGCTGGGATTACAG	
Lambda T	ATGCCACGTAAGCGAAACT	Integrated HIV-1 DNA (second-round PCR)
AA55M	GCTAGAGATTTTCCACACTGACTAA	
LTR FL*	CACAACAGACGGGCACACACTACTTGA[a]	
LTR LC*	CACTCAAGGCAAGCTTTATTGAGGC[b]	

[a] Modified with fluorescein at the 3′ end.
[b] Phosphorylated at the 3′ end and modified with LC red 640 dye at the 5′ end.
[c] Primers and probes were purchased from TIB MOLBIOL (Berlin, Germany). *, probe sequence.

Thus, the first-round PCR cycle conditions were as follows: a denaturation step of 8 min at 95°C and then 12 cycles of amplification (95°C for 10 s, 60°C for 10 s, and 72°C for 170 s).

In a second round of PCR using the lambda-specific primer (Lambda T) and an LTR primer (AA55M), only products from the first-round PCR could be amplified (Fig. 1a). In contrast to the first-round PCR, which generated fragments of various lengths, the nested amplification resulted in discrete DNA fragments. Nested PCR was performed on 1/10 of the first-round PCR product in a mixture comprising 1× Light Cycler Fast Start DNA master hybridization probes, 4 mM $MgCl_2$, 300 nM Lambda T primer, 300 nM AA55M primer, and 200 nM (each) hybridization probes LTR FL and LTR LC (Table 1). The nested-PCR cycling profile began with a denaturation step (95°C for 8 min), followed by 50 cycles of amplification (95°C for 10 s, 60°C for 10 s, and 72°C for 9 s).

The copy number of integrated HIV-1 DNA was determined in reference to a standard curve generated by concomitant two-stage PCR amplification of a serial dilution of the standard HeLa R7 Neo cell DNA (Fig. 2a) mixed with uninfected-cell DNA to yield 50,000 cell equivalents. Quantification was achieved by the second-derivative maximum method provided by the Light Cycler quantification software, version 3.5 (Roche Diagnostics). In our system, the linear regression obtained from amplification of serial dilutions of the integrated HIV-1 DNA standard was linear over a 5-$\log_{10}$-unit range, and our *Alu*-LTR nested-PCR procedure allowed the detection of approximately six proviruses within 50,000 cell equivalents (Fig. 2b).

Specificity of the *Alu*-LTR nested-PCR assay. During the first-round PCR, the L-M667 oligonucleotide can prime the formation of a single-stranded DNA from all LTR containing HIV-1 DNA, leading to an overestimation of the actual integrated HIV-1 DNA copy number. To control these linear amplifications, we performed a nested-PCR procedure omitting or not *Alu* primers in the first-round PCR. Figure 2c displays fluorescence curves obtained during amplification of cell DNA from CEM lymphoid cells infected with the VSV-G-pseudotyped HIV-1 R7 Neo virus and collected 8 or 48 h postinfection (p.i.). In the presence of *Alu* primers, the fluorescence curves shifted, moderately but reproductively, to the left, compared to an amplification performed in the absence of *Alu* primers (Fig. 2c). The shifts were about 1 cycle and 7 cycles, corresponding to 2-fold and 50-fold increases in copy numbers, for the 8- and 48-h time points, respectively. Furthermore, to determine the extent of second-round amplification of nonpreamplified viral DNA, we performed an additional control where the first-round PCR was replaced by a 4°C incubation of the PCR mixture. As expected, the amplification curves shifted dramatically to the left when a preamplification step was performed. The shift corresponded to approximately 2.3- (9 cycles) and 3.5-$\log_{10}$-unit (14 cycles) increases in copy numbers for the 8- and 48-h time points, respectively. Taken together, these findings indicate that the PCR signal obtained in the absence of *Alu* primers must be subtracted from the total signal, especially at early times following infection, and that the second-round amplification of nonpreamplified viral DNA is efficiently prevented.

Synthesis and fate of HIV-1 DNA species in a single-round infection assay. To validate our integration assay, we quantified integrated HIV-1 DNA, together with total HIV-1 DNA and two-LTR circles in a single round of viral replication. Eighteen million CEM cells were infected by VSV-G-pseudotyped HIV-1 R7 Neo virus by using 35 ng of $p24^{gag}$ antigen (measured by enzyme-linked immunosorbent assay; Perkin-Elmer Life Sciences, Paris, France) per 10^6 cells. One hour p.i., cells were washed in phosphate-buffered saline (PBS), exposed to trypsin (25 μg/ml) for 1 min at 37°C, and washed once with medium and twice with PBS. The cells were then cultured in RPMI 1640 supplemented with 10% fetal calf serum. At each time point, 1×10^6 to 3×10^6 infected cells were collected. To eliminate residual pR7 Neo Δ*env* vector

DNA, the samples were washed in PBS and incubated with 750 to 1,500 U of DNase I (Invitrogen, Cergy Pontoise, France) in a buffer comprising 20 mM Tris-Cl, pH 8.3, 50 mM KCl, and 2 mM $MgCl_2$ for 1 h at room temperature. Cells were washed in PBS, and dry cell pellets were frozen at −80°C until use. Total cell DNA was extracted with a QIAamp blood DNA minikit (Qiagen, Courtaboeuf, France).

The total HIV-1 DNA copy number was determined with previously described primers (4) that annealed in the U5 region of the LTR (MH 531) and in the 5′ end of the *gag* gene (MH 532) (Fig. 1b). The two-LTR circles were amplified with primers spanning the LTR-LTR junction (HIV F and HIV R1), as described elsewhere (3) (Fig. 1c). U5-*gag* sequences and two-LTR junctions were amplified in duplicate from 1/50 of total cell DNA. Reaction mixtures contained 1× Light Cycler Fast Start DNA master hybridization probes (Roche Diagnostics), 4 mM $MgCl_2$, 300 nM (each) forward and reverse primers, and 200 nM (each) fluorogenic hybridization probes in a final volume of 20 μl. After an initial denaturation step (95°C for 8 min), the cycling profile for total HIV-1 DNA was 50 cycles consisting of 95°C for 10 s, 60°C for 10 s, and 72°C for 6 s and that for two-LTR circles was 15 cycles consisting of 95°C for 10 s, 66°C for 10 s, and 72°C for 10 s, followed by 35 cycles at the beginning of which the annealing temperature was decreased by 0.5°C per cycle to the secondary target temperature (59°C). The copy numbers of total HIV-1 DNA and two-LTR circles were determined in reference to a standard curve prepared by amplification of quantities ranging from 10 to 10^5 copies of cloned DNA with matching sequences. The cell equivalents in sample DNA were calculated based on the amplification of the β-globin gene (two copies per diploid cell) with the Light Cycler instrument and commercially available materials (Control kit DNA; Roche Diagnostics). The quantification results for two-LTR circle, total HIV-1 DNA, and integrated HIV-1 DNA were expressed as copy numbers per 10^6 cells.

Viral DNA was detected by 3 h p.i. The level of total HIV-1 DNA increased rapidly until 9 h p.i., reaching a maximum of 734,996 copies per 10^6 cells (Fig. 3a). Then the level of total HIV-1 DNA underwent a steep decrease until 72 h p.i., followed by a slow decay phase. In previous single-round infection assays, the initial steep decrease in the level of total HIV-1 DNA was reported to represent the proteasome-mediated degradation of unintegrated linear HIV-1 DNA (5). We detected integrated HIV-1 DNA by 3 h p.i., attesting to the rapid nuclear import of reverse transcription products and emphasizing the sensitivity of our integration assay. The level of integrated HIV-1 DNA increased until 48 h p.i to reach a maximum of 316,578 copies per 10^6 cells (Fig. 3b). By 72 h p.i., the level of integrated HIV-1 DNA matched that of total HIV-1 DNA, as expected in a single-round of viral replication. This confirmed the accuracy of our quantification method. The slow decay in the level of integrated HIV-1 DNA may reflect the death of infected cells and/or the slow division rate of infected cells carrying an integrated provirus compared to that of uninfected ones. Two-LTR circles, as detected by the presence of LTR-LTR junctions, were observed as early as 3 h p.i. The level of two-LTR circles reached 44,253 copies per 10^6 cells by 24 h p.i. and declined thereafter, reaching a quasisteady level from 96 h p.i. until the end of the culture (Fig. 3c). Two-LTR circles were

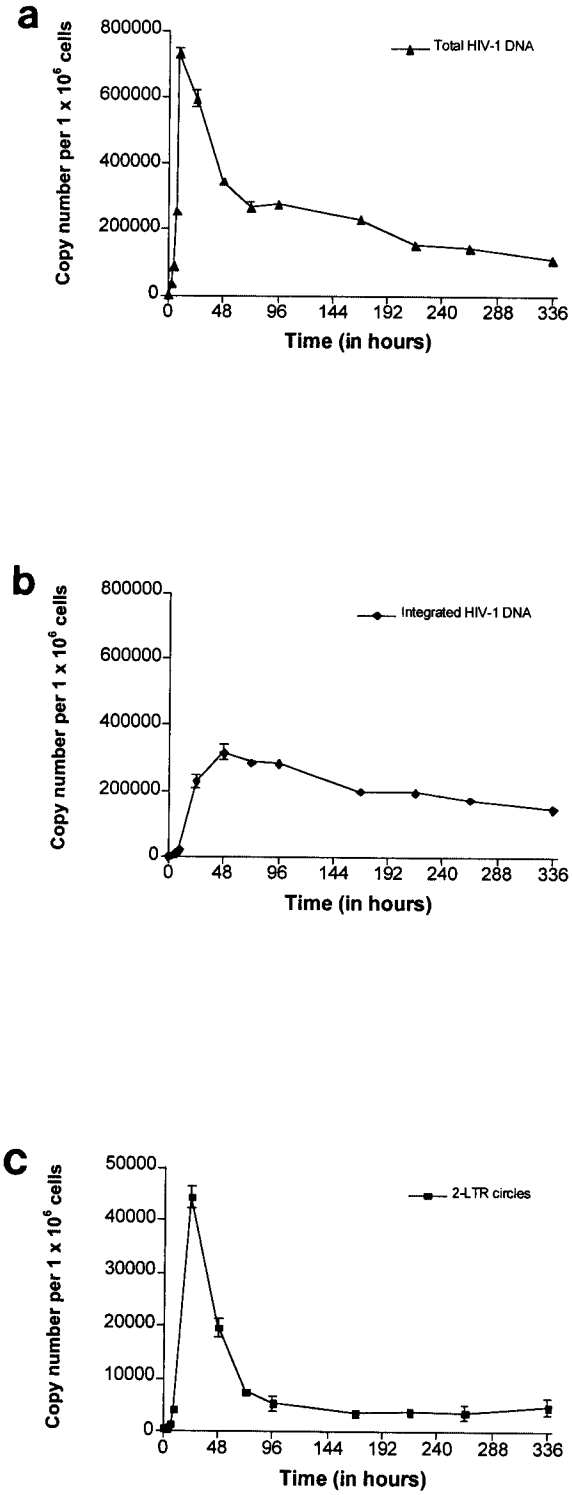

FIG. 3. Quantification of total HIV-1 DNA (a), integrated HIV-1 DNA (b), and two-LTR circles (c) during a single round of replication of the VSV-G-pseudotyped HIV-1 R7 Neo virus in CEM cells. Values are means ± standard errors of the means. The depicted results were obtained from a representative experiment.

shown to be stable DNA forms incapable of self-replication, and, consequently, they are diluted as a function of cell division (5, 15). Accordingly, a dilution effect resulting from cell division may account for the decrease in the level of two-LTR circles observed before 96 h p.i. In contrast, steady LTR-LTR junction levels from 96 h p.i. until the end of the culture were unexpected. Nevertheless, the nonspecific integration of some two-LTR circles (less than 10% in this experiment) into the host cell genome might explain the stability of LTR-LTR junction levels. Integrated LTR-LTR junctions may have been transmitted from mother to daughter cells at each division, leading to steady LTR-LTR junction levels in spite of cell proliferation. Likewise, it was previously shown that some linear HIV-1 DNA could be integrated into the cell genome by an integrase-independent process (10). This finding indicates that detection of LTR-LTR junctions does not necessarily indicate the presence of unintegrated forms of HIV-1 DNA.

Using a new sensitive assay to quantify integrated viral DNA, we analyzed the integration kinetics of viral DNA following a single cycle of replication. Our integration kinetics were similar to those obtained by Butler et al., who employed a one-step real-time *Alu*-LTR PCR-based amplification method (4). However, we were able to detect integrated HIV-1 DNA as early as 3 h p.i., while the one-step quantification method allowed the detection of proviral DNA no earlier than 12 (5) or 24 h p.i. (4). The limited sensitivity of the one-step *Alu*-LTR PCR method (1,000 copies in 100,000 cell equivalents) should account for the delayed detection of integrated HIV-1 DNA. Recently, a second method for the quantification of integrated HIV-1 DNA was devised. This method is based on a linker-primer PCR (LP-PCR) amplification (12, 20). The LP-PCR method was shown to detect four copies of proviruses in 200,000 cell equivalents (12), allowing the detection of integrated HIV-1 DNA as early as 4 h p.i. Unfortunately, the LP-PCR protocol involves many experimental steps. More recently, a real-time nested-PCR method (14) which used one primer annealing in the HIV-1 *gag* sequence and a second annealing in the *Alu* element was described. This assay has a detection limit of one provirus per 10,000 cells, i.e., comparable to ours. However, this *Alu*-LTR PCR method did not prevent the second-round amplification of nonpreamplified HIV-1 DNA, and its range of linearity was less extensive than ours (2.5 versus 5 $\log_{10}$ units).

The rapidity and sensitivity of our *Alu*-LTR-based real-time nested PCR should allow the routine analysis of HIV-1 DNA integration and may be a valuable tool to test future integrase inhibitors or to evaluate the integration efficiency of retroviral vectors designed for gene therapy.

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