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A quantitative assay for HIV DNA integration *in vivo*

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Early steps of infection by HIV-1 involve entry of the viral core into cells, reverse transcription to form the linear viral DNA, and integration of that DNA into a chromosome of the host. The unintegrated DNA can also follow non-productive pathways, in which it is circularized by recombination between DNA long-terminal repeats (LTRs), circularized by ligation of the DNA ends or degraded. Here we report quantitative methods that monitor formation of reverse transcription products, two-LTR circles and integrated proviruses. The integration assay employs a novel quantitative form of Alu-PCR that should be generally applicable to studies of integrating viruses and gene transfer vectors.

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To quantify the early steps of HIV infection, we assayed different forms of the unintegrated HIV cDNA using fluorescence-monitored Taqman PCR (Fig. 1*a*). In this method, the DNAs to be analyzed were amplified as in conventional PCR, but a third oligonucleotide probe DNA was designed to anneal between the amplification primers. The probe DNA contained a fluorescent reporter-quencher pair that was separated by the exonuclease activity of *Taq* polymerase during amplification[1]. The target sequence was then quantified during each PCR cycle by reading the fluorescence of the released reporter.

Quantification by fluorescence-monitored PCR

To assay reverse transcription, we used primers that amplify the region of the HIV cDNA between the left LTR sequence and the 5′ end of the *gag* gene ('late reverse transcriptase (RT) amplicon' henceforth). This assay detects only DNA forms that have completed the two template switches of reverse transcription, a relatively late stage. The 2-LTR-circle assay used primers that cross the junction generated by ligation of the DNA ends[2,3]. Standard curves for quantification of the late RT amplicon and 2-LTR circles were prepared by serial dilution of matching cloned DNAs of known concentrations.

Provirus quantification by Alu PCR

Detection of integrated proviruses was accomplished by amplification with primers complementary to the HIV LTR and chromosomal Alu repeats[4–9]. A complication for quantification is that retroviral integration can occur at many locations in the human genome (Fig. 1*a*, bottom). Consequently, each provirus has a unique distance to the nearest Alu sequence and so generates amplification products of different lengths. A PCR reaction using a mixed population of proviruses as template will yield many different PCR products, each amplifying with a different efficiency. In order to generate a standard curve for quantification, a DNA standard must be used that represents the full distribution of distances between the HIV LTRs and flanking Alu elements.

To generate a standard curve for Alu-PCR, we prepared cellular DNA containing proviruses integrated at many locations as a result of viral infection. We infected human cells (293T or SupT1) with high-titer stocks of HIV-based vectors, then grew them for 30 days to insure that all the extra-chromsomal forms of viral DNA were lost. We then collected chromosomal DNA and assayed dilutions. Amplification of the infected cell DNA with the Alu-PCR primers yielded a signal derived from the heterogeneous population of proviruses. The number of proviruses per cellular genome was then quantified using the late RT primer set, which also detects full-length HIV cDNAs. The resulting standard curve relates the Alu-PCR signal to the number of HIV proviruses present (Fig. 1*b*). Thus for a sample of infected cell DNA with an unknown number of proviruses, the Alu-PCR signal could be measured and the number of integrated proviruses read off the standard curve.

To confine HIV replication to a single cycle, we used HIV-based vectors instead of wild-type HIV. The vector particles used contained HIV-derived genomes transducing a *gfp* marker gene. These genomes were packaged with HIV proteins and the vesicular stomatitis virus G (VSV-G) protein envelope, allowing efficient infection of diverse cell types[10–13].

Analysis of viral DNA metabolism after infection

We first infected 293T cells with HIV-based vectors[10,11], then collected aliquots over three days and analyzed total DNA by fluorescence-monitored PCR (Fig. 2). Late RT products reached a maximum of about 20 copies per cell 12 hours after infection, then declined in abundance over the next 50–60 hours (Fig. 2*a* and Table 1). The 2-LTR circles peaked in abundance at 24 hours after infection and declined thereafter (Fig. 2*b*). The later peak for 2-LTR circles is as expected as the late RT DNA is the precursor of the 2-LTR circle.

Integrated viral DNA was detectable by 24 hours and reached a plateau by 48 hours (Fig. 2*c*). By 72 hours, integrated copies accounted for the entire viral DNA detected by the late RT primers. The convergence of late RT signal and Alu-PCR signal at 72 hours provided an internal check on the accuracy of the quantification. Most of the late RT product was not converted into integrated proviruses (note the difference in *y*-axes, Fig. 2*a*–*c*). The rate of fall of the amount of late RT products was greater than could be accounted for by dilution during cell growth, indicating that the viral DNA was probably degraded by the host cell. Results from infections of 293T cells and the lymphoid-derived line SupT1 are summarized in Table 1.

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Table 1 Maximum copy number of each viral DNA form

Cells	M.O.I.	Copies per cell (time of maximum, hours)		
		Max. viral genomes	Max. 2-LTR circles	Max. integrated
293T	0.40	20.05 ± 2.43 (12)	0.15 ± 0.02 (24)	1.09 ± 0.26
SupT1	0.25	13.75 ± 0.40 (6)	0.72 ± 0.08 (24)	2.16 ± 0.58

Based on observed ratio of 1-LTR to 2-LTR circles of 9:1

We independently checked some of the results of quantitative PCR by Southern blot analysis. DNAs isolated from infected 293T cells were digested with restriction enzymes, separated on native agarose gels, transferred to nylon membranes and probed with a [^{32}P]-labeled viral DNA probe. Parallel lanes contained copy number standards generated using cloned HIV DNA. Cleavage of the HIV DNA once allows the unintegrated DNA to be visualized, but integrated DNA forms a weak smear. The unintegrated forms accumulated to a peak at 12 hours (Fig. 2*d* and *e*, lanes 1 & 2), then declined to below the level of detection by 5 days (Fig. 2*e*, lanes 3–7).

Cleavage of the HIV DNA at two locations yielded a single internal DNA fragment derived from both integrated and unintegrated forms. This signal also reached a maximum at about 12 hours and declined thereafter (Fig. 2*f*). However, this DNA remained detectable at all time points analyzed, reaching a plateau after 3–5 days. At early times, the amount of this product matches the amount seen with both the singly cleaved DNA and the late RT-PCR assay (data not shown), corresponding to mostly unintegrated viral DNA. The viral DNA signal detected by Southern blotting after 3–8 days matched the amount of integrated proviruses measured using quantitative Alu-PCR (0.8 versus 0.7 copies per cell, respectively). Thus, Southern blotting and fluorescence-monitored PCR give similar values, although only the quantitative PCR method can distinguish integrated proviruses in the presence of the unintegrated viral DNA forms.

We carried out higher resolution Southern analysis to distinguish the 1- and 2-LTR circular forms. We found the ratio of 1-LTR to 2-LTR circles to be roughly 9:1 (Fig 2*e*, lane 8), a conclusion consistent with published studies[14]. The fluorescence-monitored PCR method and Southern method yielded similar numbers of 2-LTR circles (data not shown).

Assays of the effects of inhibitors

To verify that our methods monitor the expected steps of early retroviral replication, infections were carried out in the presence of a reverse transcriptase inhibitor (AZT) or integrase (IN) inhibitors (L-731,988 and L-708,906)[14] (Fig. 3*a*–*c*). The late RT signal was reduced in the presence of AZT but normal in the presence of IN inhibitors or no drug (Fig. 3*a*). The production of 2-LTR circles was in fact increased slightly by the IN inhibitors relative to controls, probably because blocking integration provides more substrate for ligation to form 2-LTR circles[14] (Fig. 3*b*). No detectable integration occurred in the presence of the IN inhibitors, whereas the control infection yielded signal as expected (Fig. 3*c*). In addition, infections with an integrase-mutant HIV vector also yielded late RT and 2-LTR circle signals but not Alu-PCR signal (data not shown). These tests confirm that the PCR assays monitor the expected steps in the HIV life cycle.

Factors affecting viral DNA metabolism

We have begun to use the quantitative methods to examine factors influencing viral DNA metabolism. In one application, we compared infections using HIV-based vectors with or without the central poly-purine tract (cPPT). This sequence is known to be important for initiating reverse transcription and has recently been found to increase the nuclear localization and infectivity of HIV-based vectors[12,13]. Quantification

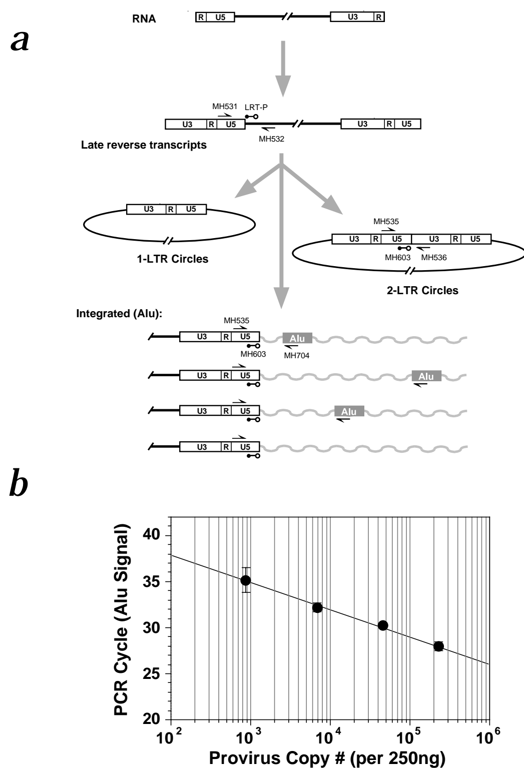

Fig. 1 Assaying early steps in HIV replication. ***a***, Early events in HIV replication and the locations of PCR primers. HIV genomic RNAs are reverse transcribed to yield linear viral DNAs. Of these, some are degraded, some circularized to 1- or 2-LTR circles, and some integrated. Arrows indicate the approximate locations of forward, reverse and probe PCR primers used to quantify HIV DNA forms. ***b***, Standard curve for quantifying integrated provirus using Alu PCR. The *y*-axis shows the PCR cycle at which the amplification signal entered the exponential range (cycle of threshold). The *x*-axis shows the known number of proviruses in the cellular DNA sample measured by quantitative PCR using the late RT primers. Repeated amplifications of the same samples yielded identical PCR signals. Titrations reveal that this method could detect one integrated provirus in about 100 cells. Comparison of standard curves generated with DNA from infection of 2 different cell lines (293T and SupT1) yielded curves that agreed within the error of the measurement (data not shown). After preparation of DNA, the yields were quantified by UV spectroscopy and in some cases by quantitative PCR using an amplicon that detects human mitochondrial DNA. This verified that the samples contained closely similar amounts of DNA (standard deviation of plus or minus 22% over 30 samples for the infection of SupT1 cells in Table 1).

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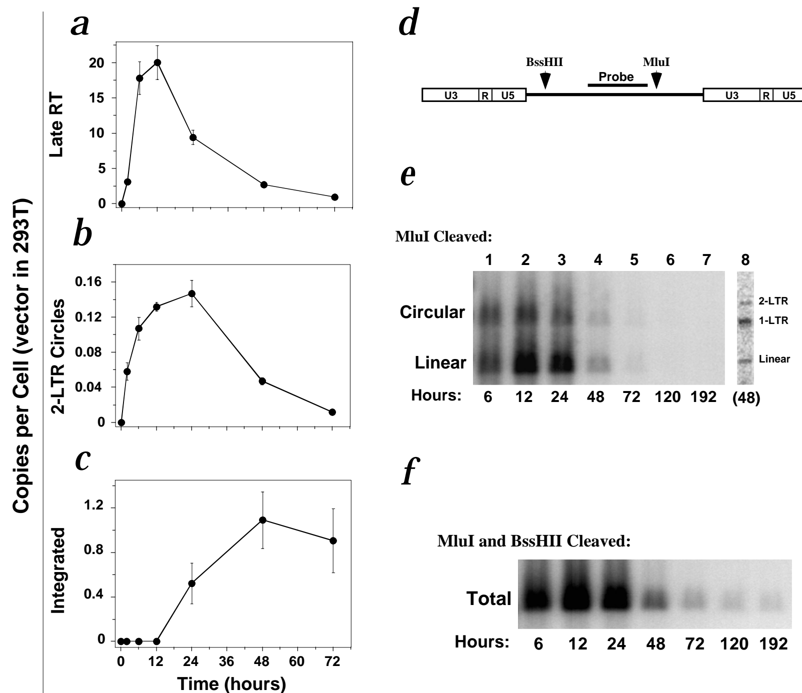
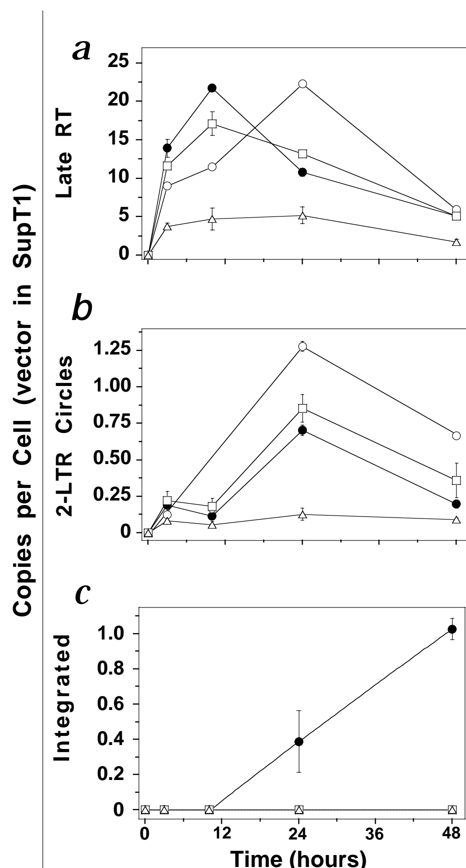


Fig. 2 DNA metabolism after infection of 293T cells with HIV-based vectors. Copies per cell of each DNA form measured are plotted as a function of time post-infection. ***a–c***, Each time point was analyzed by quantitative PCR for late reverse transcription products (*a*), 2-LTR circles (*b*) and integrated proviruses (*c*). Data represent the mean of 3 parallel infections. *y*-axes for each plot are adjusted to maximize signal. ***d***, Diagram of the HIV-based vector showing the restriction enzyme cleavage sites used and the location of sequences used for the probe on Southern blots. ***e***, Analysis of DNA forms cut once with *Mlu* I. Times are as indicated under lanes 1–7. Lane 8 illustrates a higher resolution agarose gel resolving the circular forms containing one and two LTRs. ***f***, Southern-blot analysis of DNA samples digested with *Mlu*I and *Bss*HI. Times are as indicated in hours.

of late RT products and integrated proviruses revealed that the presence of the cPPT resulted in more efficient conversion of total RT product to proviruses in some infections (unpublished data). Lack of the cPPT in the vectors used in experiments detailed in Figs. 2 and 3 might explain the observed inefficient conversion of total cDNAs to proviruses.

Our data also indicated that 2-LTR circles decline in abundance primarily due to dilution during cell growth. These findings emphasize the stability of circular cDNA forms, which is potentially at odds with a report by Sharkey and coworkers[3]. They proposed that circles decayed rapidly, with a half-life of less than 24–48 hours (although we note that the cell types, viruses, and time over which the cultures were analyzed differed in the two studies). This is an important issue, as Sharkey *et al.* inferred from the presence of circles in patients undergoing successful therapy that HIV replication nevertheless continued. We infected SupT1 cells with an HIV-based vector in the presence of the cell-cycle inhibitor aphidicolin and found that 2-LTR circles peaked in abundance by 24 hours as usual and did not change in abundance until the culture was terminated. This supports the conclusion from the data in Fig. 2 that 2-LTR circles decline in abundance (in the absence of cell-cycle arrest) primarily due to dilution by cell division. However, in infections with replication-competent HIV, 2-LTR circles declined in abundance in the presence of aphidicolin, but this was due to death of infected cells, which is an important factor to evaluate in modeling loss of viral sequences.



Discussion

The quantitative methods described here permit a detailed accounting of viral DNA metabolism after infection, including integration. These methods should be useful in analyzing alterations of the early steps of replication due to inhibitors or viral and cellular mutants. The approach used for quantification of Alu-PCR is potentially applicable to studies of any integrating genetic element.

Fig. 3 Assay of the effects of inhibitors on HIV cDNA metabolism. ***a–c***, Total DNA was prepared from the indicated time points and analyzed for copy number of late RT products (*a*), 2-LTR circles (*b*), and integrated provirus (*c*). Infections were carried out in the absence (control, ●) or presence of 25 μM AZT (△), 10 μM L-731,988 (□) and 20 μM L-708,906 (○).

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Methods

Cell infections and DNA purification. The HIV-GFP–transducing vector particles used were produced from the cell line SODk1CG2, identical to SODk1CG1 (ref. 11), but transduced with the vector a second time to increase copy number (data not shown). Multiplicity of infection was scored by count of *gfp*-expressing cells 36 h after infection for HIV vectors. For PCR time points, infections were started with ~1×10^5 cells in 12-well plates. Supernatants of vector-derived virus were filtered through 0.45-µm filters and treated with DNase I (Roche, Indianapolis, Indiana) at 10–20 U/ml for 60 min at room temperature to prevent viral DNA carryover. Virus was added to cells in minimal volume with 20 µg/ml DEAE-dextran and incubated at 37 °C. Inhibitors, if present, were 25 µM AZT, 10 µM L-731,988, and 20 µM L-708,906 (Merck Laboratories, West Point, Pennsylvania)[14]. At 2 h post-infection, supernatant was replaced with fresh DMEM medium and time points were collected by collecting cells, washing with PBS and collecting DNA with the DNeasy Tissue Kit (Qiagen, Valencia, California).

Quantitative PCR. The primer sets used to detect each sequence (purchased from GenSet or Integrated DNA Technologies, Paris, France and Coraville, Iowa) were as follows: late RT forward, MH531: 5′-TGTGTGCCCGTCTGTTGTGT-3′; late RT reverse, MH532: 5′-GAGTCCTGCGTCGAGAGAGC-3′; late RT probe, LRT-P: 5′-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3′. 2-LTR circle forward, MH535: 5′-AACTAGGGAACCCACTGCTTAAG-3′; 2-LTR reverse, MH536: 5′-TCCACAGATCAAGGATATCTTGTC-3′; 2-LTR probe, MH603: 5′-(FAM)-ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAMRA)-3′; Alu forward, MH535 (above); Alu reverse, SB704: 5′-TGCTGGGATTACAGGCGTGAG-3′; Alu probe, MH603 (above). Mitochondrial forward primer, MH533: 5′-ACCCACTCCCTCTTAGCCAATATT-3′; mitochondrial reverse primer, MH534: 5′-GTAGGGCTAGGCCCACCG-3′; mitochondrial probe, mito-probe: 5′-(TET) CTAGTCTTTGCCGCCTGCGAAGCA (TAMRA)-3′. With each experiment, a standard curve of the amplicon being measured was run in duplicate ranging from 10 to 1×10^6 copies plus a no-template control, all diluted into an equivalent amount of uninfected cell DNA (250 ng or 500 ng). Reactions contained 1× Taqman universal master mix (Perkin-Elmer, Foster City, California), 300 nM forward primer, 300 nM reverse primer, 100 nM probe primer and 100–500 ng of template DNA in a 30-µl volume. After initial incubations at 50 °C for 2 min and 95 °C for 10 min, 40 cycles of amplification were carried out at 15 s at 95 °C followed by 1 min at 60 °C (1 min 30 s for Alu PCR). Reactions were analyzed using the ABI Prism 7700 sequence detection system (PE-Applied Biosystems, Foster City, California). Detailed protocols are available at http:// www. salk.edu/LABS/idl/idlb/idlbfram.html.

Acknowledgments

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