Cell cycle regulation of human immunodeficiency virus type 1 integration in T cells: antagonistic effects of nuclear envelope breakdown and chromatin condensation

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Received 2 June 2004; returned to author for revision 12 July 2004; accepted 3 August 2004
Available online 16 September 2004

Abstract

We examined the influence of mitosis on the kinetics of human immunodeficiency virus type 1 integration in T cells. Single-round infection of cells arrested in G1b or allowed to synchronously proceed through division showed that mitosis delays virus integration until 18–24 h postinfection, whereas integration reaches maximum levels by 15 h in G1b-arrested cells. Subcellular fractionation of metaphase-arrested cells indicated that, while nuclear envelope disassembly facilitates docking of viral DNA to chromatin, chromosome condensation directly antagonizes and therefore delays integration. As a result of the balance between the two effects, virus integration efficiency is eventually up to threefold greater in dividing cells. At the single-cell level, using a green fluorescent protein-expressing reporter virus, we found that passage through mitosis leads to prominent asymmetric segregation of the viral genome in daughter cells without interfering with provirus expression.

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Keywords: HIV-1; T cells; Virus integration; Cell cycle; Mitosis; Nuclear envelope; Chromosome condensation

Introduction

The afferent phase of human immunodeficiency virus type 1 (HIV-1) replication cycle in T cells is highly dependent on activation and proliferation signals (Steven-

son et al., 1990; Zack et al., 1990, 1992). T cell activation through ligation of immune system receptors not only stimulates HIV-1 reverse transcription by increasing the pool of intracellular nucleotides, but also promotes subsequent nuclear import and integration, both of which can occur even in the absence of cell division (Eckstein et al., 2001a; Unutmaz et al., 1999). Although HIV-1 reverse transcription can proceed to completion in quiescent blood CD4+ T cells, the process is less efficient and much slower than in activated T cells, often leading to pre-integration latency (Persaud et al., 2003; Pierson et al., 2002). During the last decade, much effort has been dedicated to characterize the mechanisms by which HIV-1 infects such non-dividing T cells (Eckstein et al., 2001a, 2001b), or macrophages and dendritic cells (Canque et al., 1999;
Gartner et al., 1986; Patterson and Knight, 1987). Indeed, at variance with murine leukemia virus (MLV), which preferentially replicates in dividing cells because it needs nuclear envelope (NE) prophasic disorganization to access host cell DNA (Lewis and Emerman, 1994; Lewis et al., 1992; Roe et al., 1993), lentiviruses such as HIV-1 have also the capacity to cross the NE of interphasic cells. In this case, it is assumed that nuclear import of reverse transcription complexes (RTCs), which present as loosely organized filamentous structures (Nermut and Fassati, 2003), is mediated by interactions with shuttling transport receptors and components of the nuclear pore complex, and involves redundant viral nuclear localization signals displayed by viral proteins (Vpr, integrase, p17 matrix) or a DNA flap (central polypurine tract) located at the center of the viral DNA (Sherman and Greene, 2002). In contrast, the impact of mitotic events on HIV-1 replication cycle in T cells is still poorly documented. Although it is admitted that NE disassembly frees access of HIV-1 to host cell DNA and promotes subsequent integration, whether cell division exerts some influence on either the kinetics or the efficiency of HIV-1 nuclear import and integration in T cells remains elusive.

In this report, we demonstrate that dividing T cells display increased permissiveness to HIV-1 infection, and we show that NE disassembly allows for more efficient docking of viral DNA to chromatin while chromosome condensation delays integration until late stages of mitosis.

**Results**

**Passage through mitosis promotes virus integration in T cells**

To investigate the impact of cell division on HIV-1 infection of T cells, we established an experimental system using H9 cells, which are CD3+CD4+TCRα/β+ clonal derivatives of HUT78 cells selected for high permissiveness to HIV-1 (Popovic et al., 1984). These cells can be readily synchronized in G1 by double-thymidine block (see Materials and methods) and, upon thymidine removal, they immediately enter into the S-phase, progress to the G2/M-phase in 8–12 h, and have already completed one round of cell division by 24 h (Fig. 1A). In a first series of experiments, G1-synchronized H9 cells were exposed to...
replication-competent HIV-1NL4-3 for the last 2 h of the second thymidine block. A high 1 μg p24/3 × 10^6 cells virus concentration was used because lower (10–100 ng p24) concentrations did not allow to precisely quantify integrated viral DNA in single-round infections (data not shown). Cells were then released to synchronously progress through the cell cycle or they were kept arrested in G1b with Aphidicolin, a known inhibitor of DNA polymerase alpha (Ikegami et al., 1978). Cultures were maintained in the presence of protease Saquinavir to limit analysis to single-round infection; real-time PCR to quantify viral DNA species was performed 24 h postinfection (PI). Total and linear viral DNA were assessed by LTR U3–U5 and LTR U3-gag PCR, respectively. Virus integration was examined using a reported Alu-LTR nested PCR assay (Brussel and Sonigo, 2003). However, although very sensitive (20 copies proviral DNA/10⁴ cells), this assay generates important background signal due to linear amplification of single-stranded nonintegrated LTR-containing viral DNA during the first 12 amplification cycles. To circumvent this fact, the assay was modified by treating the samples at the end of this first PCR round with exonuclease I, before performing the second amplification round. This procedure resulted in eliminating >95% of the background (data not shown) without affecting the assay sensitivity, which remained at 20 copies viral DNA/10⁶ cells.

First, it was verified by quantifying total and linear HIV DNA that cell division did not interfere with virus entry or reverse transcription (data not shown). However, because released cells had divided during the 24 h PI, their total viral DNA copy numbers were decreased by 43 ± 6% relative to G1b-arrested cells (4575 ± 2240 vs. 8227 ± 3618/10⁴ cells; mean ± SEM, n = 6, P = 0.001, paired Student’s t test) (Fig. 1B). Therefore, to compare virus integration efficiency under the two conditions, integrated viral DNA amounts had to be normalized relative to total viral DNA rather than to total cellular DNA or cell numbers. Thus, the number of integrated viral DNA copies per 5000 copies of total viral DNA was 158 ± 12 in G1b-arrested vs. 406 ± 53 in released cells (n = 4; P = 0.02; paired Student’s t test) (Fig. 1C), corresponding to 3% (range: 2.6–3.8) and 8% (range: 6.4–11.6) of total viral DNA, respectively.

We then tested the physiological relevance of these findings by using primary T cells that were cultured for 24 h in the presence of interleukin (IL)-2, with or without tumor necrosis factor (TNF)-α, before exposure to HIV-1NL4-3, conditions that are known to trigger T cell exit from quiescence without eliciting cell division (Unutmaz et al., 1999) (Fig. 2A). T cells activated by phytohemagglutinin (PHA) and IL-2 for 48 h and then by IL-2 alone for 24 h were used as dividing cell controls. PCR for assessing viral DNA species was performed 24 h PI. In line with another report (Pierson et al., 2002), reverse transcription efficiency, assessed by relating linear to total viral DNA amounts, was reduced to 50–60% under the IL-2 ± TNF-α condition vs. >90% for the PHA/IL-2 control condition. Thus, integrated viral DNA amounts had to be normalized relative to linear viral DNA to actually compare the three culture conditions. Integrated viral DNA levels were then found to be 2.5- to 4-fold higher in dividing than in activated T cells (Fig. 2B), confirming the data obtained with H9 cells.

Altogether, these data indicate that passage through mitosis leads to increased permissiveness of primary and transformed T cells to HIV-1 infection.

**Chromosome condensation delays virus integration in mitotic T cells**

We then examined the impact of the cell division process on the kinetics of virus integration in T cells. Hence,
synchronized H9 cells that had been exposed to HIV-1<sub>NL4-3</sub> and either released or arrested in G1b as above (see Fig. 1A) were sequentially analyzed by Alu-LTR and 2-LTR circle PCRs. Copy numbers of integrated viral DNA were again normalized relative to total viral DNA. Twelve hours PI, integrated viral DNA copy numbers were 2- to 6-fold higher in G1b-arrested than in released cells, reaching a maximum 24 h PI in both cases (Fig. 3A). Two-LTR circles followed a similar kinetics. When assessing the ratio of integrated viral DNA copy numbers in released cells vs. G1b-arrested cells, values ranged from 0.1 to 0.7 before 15 h PI but increased thereafter to reach 2.9 at 24 h PI (Fig. 3B), indicating that integration mainly occurred during the last 6 h PI in dividing cells. No integration events could be detected before 10 h PI under either condition, which contrasts with reports that HIV-1 integration occurs as early as 4 h PI in exponentially growing T cell lines (Brussel and Sonigo, 2003; Vandegraaff et al., 2001). This discrepancy most probably relates to the fact that, for assessing integrated viral DNA, we modified the Alu-LTR nested PCR assay to reduce to negligible levels background amplification of nonintegrated linear viral DNA.

In as much as these data indicate that virus integration mainly occurs at a late stage of cell division in T cells, we next specifically examined the influence of chromosome condensation/decondensation, on virus integration. As before, synchronized H9 cells were exposed to HIV-1<sub>NL4-3</sub> for the last 2 h of the second thymidine block, and they were either released or arrested in metaphase by adding Taxol or Nocodazole (Fig. 4A). Nocodazole treatment disrupts the microtubule network, while Taxol induces their polymerization and stabilization (Yvon et al., 1999). NE disassembly and chromatin condensation of metaphase-arrested cells were assessed by labeling with an anti-LAP2 monoclonal antibody (mAb) followed by counterstaining with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Fig. 4B). Real-time PCR analysis, 24 h PI, showed that integrated viral DNA copy numbers in cells that had been released were on average 3.8-fold (range: 2.2–5.7) greater than in metaphase-arrested cells (Fig. 4C). Of note, none of the drugs interfered with virus entry or reverse transcription (data not shown). Because these data suggested that chromosome condensation antagonized virus integration, we examined whether chromosome relaxation induced by protein kinase inhibitor Staurosporine affected integration. Adding Staurosporine to cells already arrested in metaphase by Nocodazole is known to induce concomitant nucleophosmin/B23 dephosphorylation and chromosome decondensation (Lu et al., 1996; Th’ng et al., 1994) (Fig. 4D).
Synchronized H9 cells, which had been exposed as before to HIV-1<sub>NL4-3</sub>, were arrested in metaphase by Nocodazole and treated or not by Staurosporine for the last 4 h of culture. Alu-LTR nested PCR analysis, 24 h PI, confirmed that Staurosporine-induced chromosome relaxation restored integration to normal levels in metaphase-arrested cells (Fig. 4E). Staurosporine did not interfere with virus integration in either released or G1b-arrested cells nor did it induce detectable polyploidy in treated cells (data not shown) (Hall et al., 1996).
Altogether, these data indicate that the kinetics of viral integration is determined by the chromosome condensation/decondensation cycle in mitotic cells.

**NE disassembly promotes viral DNA docking to chromatin**

This led us to examine the impact of NE disassembly on both the efficiency and kinetics of HIV-1 RTC docking to chromatin. Hence, synchronized H9 cells were arrested in G1b or in metaphase by 24-h culture with Aphidicolin or Nocodazole before exposure to HIV-1NL4-3/IND64A (Fig. 5A), a nonintegrative mutant chosen to formally exclude the integration step from analysis. At the indicated time points, cells were fractionated to isolate postnuclear chromatin fractions. Interphasic chromatin was obtained by a two-step procedure based on sequential solubilization of the plasma membrane and NE (see Materials and methods), while metaphase-arrested cells were submitted to hypotonic lysis before mitotic chromosome purification by centrifugation over a sucrose cushion (Collas et al., 1999). Real-time PCR, used to detect mitochondrial cytochrome b (Simon and Malim, 1996), showed that the chromatin fractions were depleted by ≈95% in cytoplasmic mitochondria (data not shown), which stresses the accuracy of the purification procedures. To compare data from the different conditions, chromatin-associated viral DNA copy numbers determined by LTR U3–U5 PCR were normalized relative to total viral DNA detected in the cells before fractionation. Results were therefore expressed as percentages of chromatin-bound viral DNA. This showed that chromatin-bound viral DNA already represented 95% of total viral DNA in metaphase-arrested cells as early as 4 h PI, relative to only 20% in G1b-arrested cells (Fig. 5B). Because the NE of interphasic nuclei was solubilized by 1% deoxycholate before chromatin isolation (see Materials and methods), we verified that this did not account for the difference noted between interphasic and mitotic cells: Indeed, incubating mitotic chromosomes with 1% deoxycholate did not modify chromatin-associated viral DNA copy numbers (data not shown).

Altogether, these data suggest that NE disassembly leads to more efficient docking of HIV-1 RTCs to chromatin, thereby accounting for increased permissiveness of dividing cells to the virus.

**Passage through mitosis limitedly affects the number of integration sites per HIV-1-infected cell**

We finally examined, at the clonal level, the impact of cell division on viral DNA integration, distribution, and expression (Fig. 6A). Synchronized H9 cells were exposed to a vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped NL4-3-GFP reporter virus, which encodes the enhanced green fluorescent protein (GFP), after which they were immediately cloned and released from the thymidine block. Alternatively, infected cells were further kept arrested for 24 h in G1b by Aphidicolin or in metaphase by Nocodazole, to allow completion of the viral cycle before cloning. After 3-week amplification, 160 clones were recovered from the 768 G1b-arrested cells seeded (cloning efficiency: 21%), from which only 10 (6%) comprised GFP-expressing cells (Fig. 6B). Comparatively, 25 of 157 clones (16%) recovered from the 749 released cells seeded comprised GFP(+) cells, which, with the same overall 21% cloning efficiency, corresponded to 2.7-fold increase in GFP(+) clones \( (P = 0.006; \chi^2\) test vs. released cells). Intermediate results were obtained with metaphase-arrested cells, since 12 of 118 clones (10%) recovered from 768 seeded cells comprised GFP(+) cells. The clones recovered from cells infected under each condition also differed as to GFP(+) cell percentages per positive clone (Fig. 6C), with 100% GFP(+) cells in 7 of the 10 clones recovered from G1b-arrested cells, and median values of 36% and 19% for the released and

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**Fig. 5. Effect of mitosis on HIV-1 RTC docking to chromatin.** (A) Experimental design: G1b- or metaphase-arrested (MET) H9 cells were exposed to nonintegrative HIV-1NL4-3/IND64A; subcellular fractionation and LTR U3–U5 PCR analysis were performed at the indicated time points. (B) Determination of chromatin-bound viral DNA: Data are normalized relative to total viral DNA copy numbers detected in the cells before fractionation. Data are from one experiment out of two.
metaphase-arrested cells, respectively, the differences being statistically significant \((P = 0.025\) to \(P = 0.003\); Mann and Whitney \(U\) test).

To determine whether passage through mitosis also affected the number of integration events per infected cell, individual GFP(+) clones obtained under each of the three conditions were analyzed for total viral DNA content. However, the \(Alu\)-LTR nested PCR could not be used here due to the limited number of integration events per initially infected cell and to the variable distance between integrated viral DNA 3' LTRs and the nearest \(Alu\) repeat. Therefore, linear viral DNA content was quantified instead, assuming that after 3–4 weeks PI, it equaled that of integrated virus. The median viral DNA copy numbers per GFP-expressing cell was then 1.2 and 2 in clones derived from G1b-arrested and released cells \((P = 0.004\); Mann and Whitney \(U\) test), respectively (Fig. 6D).

These data indicate that cell division leads to an increased proportion of GFP(+) clones while affecting in a more limited manner the number of integration sites per GFP-expressing cell.

Because heterogeneous GFP expression in clones recovered from released or metaphase-arrested cells could reflect either asymmetric segregation of the viral genome or a variegation effect due to virus integration near heterochromatin, the GFP(+) and GFP(−) cell subsets sorted from clones recovered from REL- or MET-infected cells: Data are expressed as viral DNA copy numbers per 100 cells. In C, D, and E, bars indicate median values. Data are obtained from three experiments.

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*Fig. 6. Effect of cell division on the segregation and expression of the viral genome. (A) Experimental design: Synchronized H9 cells were exposed to a GFP-expressing reporter virus for the last 2 h of a thymidine block; they were then either cloned immediately and released (REL) or cultured further for 24 h with Aphidicolin (G1b) or Nocodazole (MET) before cloning; FACS analysis and real-time PCR were performed after 3-week amplification. (B) Percentages of GFP(+) clones among clones recovered under each of the three conditions. (C) Percentages of GFP-expressing cells among the GFP(+) clones. (D) LTR \(U3\)-gag PCR analysis of the GFP(+) clones: viral DNA copy numbers were normalized relative to the percentage of GFP(+) cells in the corresponding clone (copy number = \(\text{[copies / 100 cells]} / \text{[GFP(+) cells / 100 cells]}\)). (E) LTR \(U3\)-gag PCR analysis of GFP(+) and GFP(−) cell subsets sorted from clones recovered from REL- or MET-infected cells: Data are expressed as viral DNA copy numbers per 100 cells. In C, D, and E, bars indicate median values. Data are obtained from three experiments.*
asymmetric segregation of HIV-1 genome in daughter cells.

**Discussion**

The influence of cell proliferation on retrovirus replication has been extensively studied as it was shown that cellular division influenced Rous sarcoma virus (RSV) replication (Humphries and Temin, 1974; Humphries et al., 1981) and it is now admitted that it promotes retroviral nuclear entry and subsequent integration (Fouchier and Malim, 1999). Interestingly, there is accumulating evidence that differences in this respect between lentiviruses and other retroviruses were overestimated, and that requirement for mitosis depends on the capacity of each virus to cross the NE of interphasic cells (Katz et al., 2002, 2003). In this regard, it has also been shown that pharmacological arrest of target cells in the G1/S phase only marginally affects HIV-1 nuclear import and integration whereas it reduces by approximately 50- and 500-fold RSV and MLV infectious titers, respectively (Hatzioannou and Goff, 2001).

Although it is currently assumed that dividing T cells display increased permissiveness to HIV-1 infection, the influence of major mitotic events such as NE breakdown and chromosome condensation/decondensation on viral DNA docking and subsequent integration remains poorly characterized. To address the issue, we developed a model in which integrated viral DNA is quantified over the course of a single-round infection of H9 T cells that had been either arrested in G1b or released from a thymidine block to synchronously proceed through cell division. We first found that, 24 h PI, released cells harbored significantly more integrated viral DNA than G1b-arrested cells. However, because integrated provirus copy numbers had to be normalized relative to total viral DNA detected in G1b-arrested relative to released cells, our data indeed supports this hypothesis. Our findings are consistent with a report that Nocodazole removal promotes Moloney leukemia virus integration into metaphase-arrested cells (Roe et al., 1993), and they indicate that telophase chromosome decondensation should trigger integration of the viral DNA from chromatin-bound RTCs in dividing cells.

Nucleo-cytoplasmic fractionation has often been used to follow the kinetics of HIV-1 nuclearization in its target cells (Ao et al., 2004; Gallay et al., 1995a, 1995b; Popov et al., 1998; Simon and Malim, 1996). However, the classical methods do not allow specific assessment of intranuclear viral DNA, most of which remain associated with nuclear pore complex components on the NE external face. Therefore, here, interphasic nuclei were treated by deoxycholate to solubilize the NE before the postnuclear chromatin fraction was subjected to PCR analysis. This led to the finding that chromatin-associated viral DNA represented only 20–40% of total viral DNA detected in G1b-arrested relative to ≥95% in metaphase-arrested cells. Although one cannot formally rule out that viral DNA could complex with chromatin only after cell lysis, our data strongly suggest that NE fragmentation actually enhances HIV-1 RTC docking to chromatin, thereby accounting for the subsequent increased viral DNA integration in mitotic cells. An important finding is here the discrepancy noted between the timing of HIV-1 nuclear entry and integration: conversely to nuclear import, which proceeds co-linearly with virus reverse transcription, integration appears thus as a time-consuming process that could well represent the limiting step of the afferent phase of the virus replication cycle in T cells. Based on comparative analysis of the kinetics of virus nuclear import and integration, it can be inferred that the time for chromatin-bound HIV-1 RTCs to integrate interphasic chromatin varies from 6 to 8 h in T cells, an estimation that fits with the kinetics of 2-LTR circles formation. However, whether 2-LTR circles represent a reliable marker for assessing the kinetics of virus nuclear entry remains unclear even though it is widely admitted that linear viral DNA synthesized in the cytoplasm is the precursor of the circular DNA species found in the nucleus (Shank and Varmus, 1978). Our data that formation of 2-LTR junctions follows a kinetics parallel to that of integrated provirus argues for the postnuclear-entry circularization of viral DNA and, thus, the use of this marker. That integrated
At the single-cell level, analysis of clones derived from T cells that were released immediately after exposure to a GFP reporter virus confirmed the increased permissiveness of dividing cells to the virus. Indeed, passage through mitosis before virus integration was associated with greater than 2-fold increased frequency of GFP(+) clones relative to clones recovered from cells that had been infected while arrested in G1b. Analysis by real-time PCR of the GFP(+) and GFP(−) cell subsets of these clones confirmed that cell division leads to asymmetric segregation of the viral genome (Hajhosseini et al., 1993; Katz et al., 2003) and affects in a more limited manner the number of integration sites per infected cell. Although one cannot exclude that some asymmetry could also result from virus integrating into host cell DNA before entry into mitosis, these data are consistent with our finding that virus integration mainly proceeds at late stages of mitosis in dividing cells. The unexpected finding that percentages of GFP(+) cells in clones derived from metaphase-arrested cells were approximately 2-fold lower than in clones recovered from released cells, is indicative of a shift in viral genome segregation (Katz et al., 2003). Such observation that metaphase-arrested cells undergo more than one division before integration occurs suggests that at least part of integration-competent viral DNA has the capacity to persist as episomes closely associated to host-cell DNA. A similar mechanism of virus persistence has been reported for double-stranded DNA viruses, such as herpesviridae or papillomaviruses, for which a single protein most often acts as molecular linker between the viral genome and host-cell chromatin (Abroi et al., 2004; Ballestas and Kaye, 2001; Ballestas et al., 1999; Barbera et al., 2004). Because it has been shown that HIV-1 preferentially integrates into transcriptionally active genes in exponentially growing T cells (Schröder et al., 2002), we also considered the hypothesis that access of viral DNA to condensed mitotic chromatin could elicit redistribution of integration sites towards heterochromatin, resulting in positional silencing of the viral genome. Analysis by real-time PCR of GFP(−) and GFP(+) clones recovered from metaphase-arrested or released cells showed an absolute correlation between GFP expression and presence of the viral genome. This finding, which is consistent with the report that, in the presence of Tat, HIV-1 transcription is efficient regardless of the integration sites (Jordan et al., 2001), argues against such possibility and suggests that HIV-1 positional silencing should be at most a rare event in this case.

In conclusion, our findings indicate that, whereas nuclear import of HIV-1 DNA in T cells is a rapid process, integration into the host cell DNA is time-consuming, likely to be the limiting step of the virus replication cycle afferent phase. Our results also indicate that cell division influences the efficiency of viral DNA association with chromatin, which may well account for the increased permissiveness of dividing T cells to the virus. Finally, the chronology of virus integration in dividing T cells appears as the balance between the effect of the NE disassembly that frees access to the host cell genome and chromosome condensation that hampers virus integration.

Materials and methods

Cell culture and synchronization

H9 lymphoid cells were grown exponentially at 37 °C in humidified 5% CO₂, in RPMI 1640, 10% fetal calf serum (FCS), 1% glutamine, 1% antibiotics (GIBCO BRL, Life Technologies, Cergy-Pontoise, France). Cells were synchronized in G1 by double-thymidine block (2 mM for 18 h; Sigma-Aldrich, St Quentin Fallavier, France) as reported (Chaudhary and Courvalin, 1993). At the end of the second block, cells were washed and released to synchronously progress through the cell cycle. Alternatively, cells were kept arrested in G1b by 5 μg/ml Aphidicoline (Ikegami et al., 1978) or they were blocked at the metaphase/anaphase transition by 1 μM Taxol or 500 ng/ml Nocodazole (all from Sigma-Aldrich) (Yvon et al., 1999; Zieve et al., 1980). Primary T cells (>85% pure) were separated from blood mononuclear cells by sheep erythrocyte rosetting (Ghuckman and Degoulet, 1979). They were grown for 48 h in RPMI 1640, 10% FCS, 1% glutamine, 1% antibiotics, supplemented with 10 μg/ml PHA (Sigma-Aldrich) and 200 U/ml IL-2 (Tebu, Le Perray en Yvelines, France), and then with IL-2 alone. Alternatively, they were activated before virus exposure by 24-h culture in the presence of 200 U/ml IL-2 with or without 50 ng/ml TNF-α (R&D Systems, Abingdon, UK) (Unutmaz et al., 1999).

Cell DNA content was controlled by flow cytometry (FACScalibur®, Becton-Dickinson, Mountain View, CA) after labeling with the SYTOX cell-permeant nucleic acid stain according to the manufacturer’s instructions (Molecular Probes Inc, Eugene, Or). Chromosome condensation and nuclear envelope integrity were assessed by confocal microscopy after labeling with LAP-2 (Transduction Laboratories, Lexington, KY) mAb, followed by counterstaining with DAPI (Molecular Probes). Labeling was performed essentially as described (Canque et al., 2000). Cells were cytospun, fixed, and permeabilized for 10 min in phosphate-buffered saline, 3% Triton X-100, 3.7% formaldehyde, before coverslips were incubated with the first antibody at room temperature for 30 min. Labeling was developed by incubation with Alexa Fluor 594-conjugated goat anti-mouse IgG mAb (Molecular Probes). Cells were counterstained with 2 μg/ml DAPI before mounting in DAKO fluorescent...
mounting medium (DAKO, Carpinteria, CA). Confocal microscopy was performed with a MRC 1024 confocal laser scanning (BioRad, Marnes la Coquette, France).

**Viruses and infections**

The pNL4-3 molecular clone (HIV-1NL4-3) was a gift of the NIH AIDS Research and Reference Reagent Program (Rockville, MD). A PCR-based mutagenesis strategy (XL-Site-Directed Mutagenesis kit, Stratagene) was used to introduce site-directed mutations in the IN gene (IN<sub>64A</sub>) of this clone. Plasmid DNA of clone pNL4-3 was digested with KpnI and EcoR1, generating a 1.3-kb fragment (4158–5743) that contained the IN coding sequence. This fragment was subcloned into the pUC-18 vector and mutations were introduced between base pairs 4413 and 4443, changing IN sequence 5'-GGCAGCTGATTGACACATTAGAAGG into 5'-GGCGAGCTGATTGACACATTAGAAGG where codon 5'-GCT encode for Alanin 64 (mutation D64A). Following digestion with endonucleases, the mutated fragment was ligated with the appropriately digested gel-purified pNL4-3 DNA backbone, and the region of the resulting plasmid was sequenced. The pNL4-3-GFP reporter virus was kindly provided by R.F. Siliciano (John Hopkins University School of Medicine, Baltimore, MD) (Pierson et al., 2002). The 293T cells were grown in Dulbecco’s Modified Eagle Medium, 10% FCS, 1% glutamine and 1% antibiotics. Cells (4 × 10⁶ to 5 × 10⁷ cells) were plated in 150-cm² plates (Becton Dickinson, Le Pont de Claix, France) 18 h before transfection with 60 μg of wild type or mutant pNL4-3, or with 60 μg pNL4-3-GFP and 30 μg pVSV-env. Culture supernatants were harvested 48 and 72 h later, centrifuged (300 × g), and normalized based on p24 content (Coulter HIV-1 p24 Antigen Assay, Beckman Coulter, Villepinte, France).

For single-round infection, H9 cells were exposed in spinoculation (1200 g, 25 °C) to wild type or mutant virus (1 μg p24 equivalent/3 × 10⁶ cells) for the last 2 h of the double-thymidine block or after further 24-h culture with Aphidicolin, Taxol, or Nocodazole. They were then washed, cultured for 6–36 h with Saquinavir® (1 μg/ml; Roche Pharmaceuticals, Hertfordshire, UK), and treated with DNase (20 U/ml), 2 mM EGTA and 10 mM MgCl₂ for the last 2 h of infection (Koh et al., 2000) to remove contaminant plasmids in virus supernatants.

**Quantification of HIV-1 DNA species by real-time PCR**

DNA was extracted using the DNAeasy kit as recommended by the manufacturer (Qiagen, Hilden, Germany). DNA concentration was measured by spectrophotometry, and equal DNA amounts (40–250 ng) were analyzed by PCR with a LightCycler (Roche Diagnostics). Total (LTR U3–U5) and linear (LTR U3-gag) viral DNA was quantified with primers (numbering positions correspond to the pNL4-3 DNA sequence) LTR U3–U5 sense primer 5'-CTAAGGGAACCCACG (M667, nt 498–516), LTR U3–U5 anti-sense primer 5'-CTGCTAGAGATTCCA-CAC (AA55, nt 616–635); LTR U3-gag sense primer M667, LTR U3-gag anti-sense primer 5'-TCCTGCGTCGAG AGATC (M661B, nt 678–696). The corresponding PCR products were detected with SyberGreen dye (Roche), while plasmid pNL4-3 was used to generate standard curves (range: 0.6 × 10⁶ to 6 × 10⁷ copies). Two-LTR circles were amplified with M677 sense primer 5'-GAAACCCTCATGTTAACGCC (nt 506–524) and 2-LTR anti-sense primers (2-LTRB: 5'-TGTG-TAGTTCTGCAATCGAG, nt 75–95), with subsequent detection of the corresponding amplicons by hybridization probes 2-LTRFL (5'-CCCTGCTTGTGGTGCTCTGGA-TACTAG, nt 563–591), and 2-LTRLC (5'-GATCTCAGAC CCTTATTGCTAGTTG, nt 593–620). Standard curves (range: 2 to 2 × 10⁶ copies) were generated with the 2-LTRA plasmid, which contains the 2-LTR U3–U5 junction cloned into pT-Adv (TA-cloning Kit, BD Biosciences Clontech, Palo Alto, CA). Results were normalized according to β-globin copy numbers detected with primer sense PCO4 (5'-CAACTTCATCCAGTTCTAC) and anti-sense GH2O (5'-GAAGAGCCAAGGACGG-TAC). Standard curves were generated using the Control DNA kit (Roche Diagnostics).

Integrated viral DNA was quantified by Alu-LTR nested PCR, essentially as described (Brussel and Sonigo, 2003), except that first-round PCR samples were treated by exonuclease I (Exonuclease I Kit, Roche Diagnostics), which avoids background amplification of single-stranded HIV-1 DNA from nonintegrated LTR-containing DNA during the second-round amplification. Primers sense L-M667 (5'-ATGCCACGTAAAGGAAACTCTGTACATGTTAACGCCAACCACGT) and anti-sense Alu-1 (5'-TCCAGCTACTGGGAGGCTGAGG) and sense Alu-2 (5'-GGCCTCCAAAAGCTGCTGGGATTAC) were used for the first 12 amplification cycles. Primers sense Lambda-T (5'-ATGCCACGTAAAGGAAACTCTGTACATGTTAACGCCAACCACGT) and anti-sense AASSM (5'-GCTAGAGATTTCACCTCACTGAA, nt 609–633) were used for the second-round PCR. Detection of the corresponding amplicons was achieved with hybridization probes LTR-FL (5'-CACAACAGAGGGCACCACCTTTTGA, nt 549–575) and LTR-LC (5'-CATCTCAAGGGAAGCTTTTATTGAGG, nt 522–546). Standard curves for integrated viral DNA were generated using Hela cells infected with Δenv HIV-1 R7 Neo virus and selected by culture with G418. Integrated viral DNA amounts were normalized relative to total or linear HIV-1 DNA copy numbers. Quantification was achieved using the second-derivative maximum methods provided by the LightCycler software v.3.5.3 (Roche Diagnostics).

**Chromatin isolation**

Cells were suspended in buffer A (20 mM HEPEs pH 7.4, 150 mM KCl, 5 mM MgCl₂, 1 mM EGTA)
supplemented with protease inhibitors and 0.025% digitonin (Sigma-Aldrich). After swelling for 10 min at room temperature, nuclei were pelleted at 1000 × g for 10 min. Supernatant was discarded and the nuclear fraction was resuspended in the same buffer supplemented with 1% deoxycholate. After 3-min incubation on ice, the chromatin fraction was pelleted (16,000 × g, 30 min, 4 °C) to eliminate NE remnants, before DNA extraction. To assess HIV-1 RT docking to mitotic chromosomes, synchronized H9 cells were further cultured for 18 h with Nocodazole before exposure to NL4-3/CD4A. Fractionation was performed as before at 4, 8 and 24 h PI, by 10-min incubation in buffer B (20 mM HEPES, pH 8.5, 5 mM MgCl₂, 10 mM EDTA, 1 mM DTT) supplemented with protease inhibitors and cytochalasin B (20 μg/ml, Alexis Biochemicals, San Diego CA). Lysates were homogenized by repeated pipetting before centrifugation at 10,000 × g for 10 min. The pellet was resuspended in 50 μl buffer C (10 mM HEPES, pH 7.5, 2 mM MgCl₂, 25 mM KCl, 1 mM DTT, 250 mM sucrose) supplemented with protease inhibitors, and mitotic chromosomes were sedimented at 1000 × g for 20 min through a 1 M sucrose cushion made in buffer C (Collas et al., 1999). Purity of the postnuclear chromatin fractions was controlled by real-time PCR to detect mitochondrial cytochrome b using primers sense 5’GGGCGCTGCT-GATCCTC C-3’ and anti-sense 5’GGGGTTGGCTAGGG-TATAATG G-3’, as reported (Simon and Malim, 1996).

Cell cloning and analysis of HIV-1-infected clones

A VSV-G-pseudotyped NL4-3-GFP reporter virus (Pier son et al., 2002) was used to analyze the effect of cell division on virus segregation and expression. H9 cells were synchronized in G1 by double-thymidine block, as described above, before exposure to the pNL4-3-GFP vector. They were then washed and seeded in 96-well culture plates at 0.3 cell/200 μl/well, or they were cultured further for 24 h with Aphidicolin or Nocodazole to allow completion of the virus cycle before cloning. Clones derived from infected cells were grown for 3 weeks in 96-well plates. Cell-containing wells were then numbered before the clones were individually picked and expanded for one more week in 25-cm² flasks. Clones recovered under each condition were tested by FACS for GFP expression, before DNA was extracted and processed for real-time PCR. The GFP(+) and GFP(−) cells of heterogeneous clones (GFP(+) cells <70% of total) were differentially sorted with a FACS Vantage™ (Becton Dickinson) before PCR analysis.

Acknowledgments

This work was supported by the Agence Nationale de Recherche sur le SIDA, by SIDACTION-Ensemble Contre le SIDA, and by the Association de Recherche sur les Deficits Immunitaires Viro-Induits (Paris, France).

We gratefully acknowledge the help of Micael Yagello and Frederic Braud for technical assistance; Dr. Ali Saib for critical discussions; Dr. Robert Siliciano for the gift of the NL4-3-GFP reporter virus; and the National Institute of Health AIDS Research and Reference Reagent Program for the gift of the pNL4-3 molecular clone and the pVSV-G expression vector.

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