

# Intracytoplasmic Maturation of the HIV-1 Preintegration Complexes Determines Their Capacity to Integrate into Chromatin

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## Abstract

Maturation of HIV-1 preintegration complexes (PIC) is one of the key events in the early phase of HIV-1 replication. However, little is known about the transformation of PIC structure during reverse transcription and nuclear translocation. Here, we focus on comparative analysis of protein composition, reverse transcription and integrative capacity of cytoplasmic (cPIC) and nuclear PIC (nPIC). To quantitatively evaluate maturation and nuclear translocation of the complexes, nPICs and cPICs were isolated from HeLa cells, infected by spinoculation with MMLV Env-pseudotyped HIV-1, and analyzed by real-time PCR using primers specific for early (strong-stop) and late (complete) HIV-1 DNA. While most PICs completed reverse transcription

in the cytoplasm, some complexes got into the nucleus before completing DNA synthesis. Immunoprecipitation of PICs and subsequent PCR analysis identified viral proteins IN, Vpr, MA, and RT in both cPICs and nPICs, whereas CA was representatively detected only in cPICs. Both cPICs and nPICs carried out reverse transcription reaction *in vitro*. However, in contrast to cPICs, *in vitro* completion of reverse transcription in nPICs did not increase their integration into immunoprecipitated chromatin maintaining nucleosomal organization. These results suggest that PIC maturation occurs predominantly in the cytoplasm and nPICs containing RT and immature DNA are defective for integration.

## Introduction

The early events of the HIV-1 life cycle include entry of the viral core into target cell, assembly of the reverse transcription complex (RTC), its transformation into pre-integration complex (PIC) upon completion of reverse transcription, trafficking into the nucleus, and finally integration of the viral DNA into chromatin (reviewed by Nisole & Saib, 2004). Molecular details and temporal organization of these processes remain among the least investigated and most controversial problems in the biology of HIV. Reverse transcription is generally completed in 8 to 12 h, whereas virus-specific DNA can be detected in the nuclei of infected cells as early as 4 h post-infection (Fassati & Goff, 2001). This and the finding that nuclear PICs may contain RT (Bukrinsky et al., 1993) suggest that the distinction between RTCs and PICs is somewhat arbitrary.

HIV-1 PICs isolated from the cytoplasm of infected cells is known to contain reverse-transcriptase (RT), integrase (IN), matrix protein (MA) and Vpr (Bukrinsky et al., 1993; Heinzinger et al., 1994; Miller et al., 1997). The capsid protein (CA) is detectable in RTCs early after infection, but it is absent

## Results

### Infection of cells and isolation of RTC/PICs.

**Infection.** HeLa cells were spinoculated in 6-well plates with MLV Env-pseudotyped NL4-3 HIV-1 at 3,600 x g, 18°C for 2 h (to allow viral adhesion but delay viral internalization). Viral titers were normalized by p24 ELISA to 0.5 pg of p24 per cell. After spinoculation cells were washed by culture media at room temperature and incubated in fresh medium at 37°C for 2 or 5 h.

**Harvesting of cells.** After incubation cells were washed by PBS with 0.5 mM EDTA twice, harvested by trypsinization and washed again with PBS.

**Extraction of RTC/PICs from cytoplasm.** All subsequent manipulations were performed at 4°C according to Fassati & Goff (2001). Briefly, cells were resuspended in 5 volumes of hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 5 mM DTT, and EDTA-Free protease inhibitor cocktail (Roche)] and incubated on ice for 20 min. Then the cells were pelleted by centrifugation, resuspended in hypotonic buffer containing 0.025% Brij-96

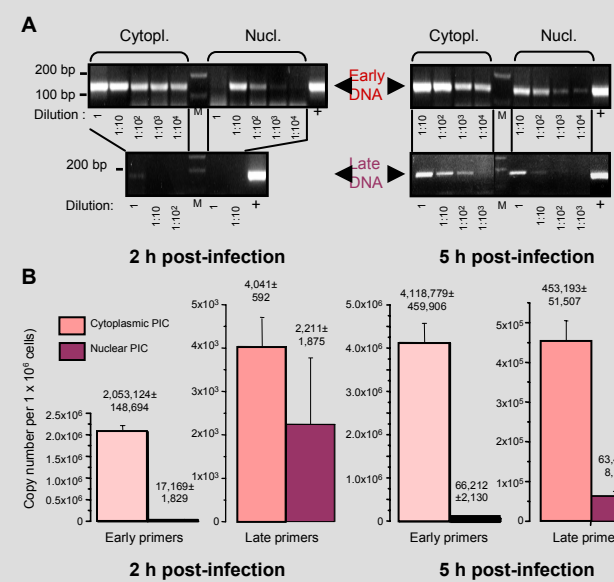
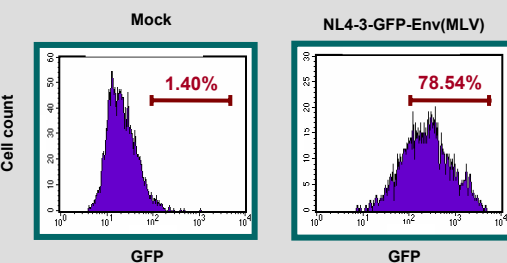
(Fluka) to detach HIV-1 preintegration complexes from cell membrane residues and cytoskeletal fragments (Bushman & Miller, 1997), and homogenized in a Dounce homogenizer type A. Nuclei were pelleted by centrifugation at 3,300 x g for 15 min, cytoplasmic extracts were clarified by centrifugation at 7,500 x g for 20 min.

**Extraction of RTC/PICs from nuclei.** Nuclear pellets were washed in 3 volumes of hypotonic buffer containing 0.5% Triton X-100 to remove fragments of cytoplasm from the nuclear surface, and then washed in 10 volumes of cold hypotonic buffer. Nuclei were resuspended in isotonic buffer [10 mM Tris HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 160 mM KCl, 1 mM DTT, and EDTA-Free protease inhibitor cocktail (Roche)] and homogenized in a ball-bearing homogenizer. Nuclear extracts were clarified by centrifugation at 8000 x g for 20 min.

**Purification of RTC/PICs.** Complexes were purified from cytoplasmic and nuclear extracts by centrifugation through 40% sucrose cushion (in hypotonic buffer for cytoplasmic and in isotonic buffer for nuclear extracts) at 34,000 RPM (100,000 x g) in a Beckman SW-60 rotor for 3 h at 4°C. Pellets of HIV-1 RTC/PICs from cytoplasmic and nuclear fractions were resuspended in buffer K [20 mM HEPES (pH 7.3), 150 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche) per 10 ml], snap-frozen in liquid nitrogen, and stored at -80°C.

### Figure 1. Analysis of infection efficiency of HeLa cells by GFP-expressing HIV-1 pseudotyped with Env (MLV).

To check efficiency of infection, we spinoculated HeLa cells with MLV Env-pseudotyped GFP-expressing NL4-3 HIV-1. Cells were analyzed by FACS 48 h after infection. Percentage of GFP-positive cells was counted using CellQuest software.



**Figure 2.** PCR analysis of nucleo-cytoplasmic distribution of HIV-1 PICs.

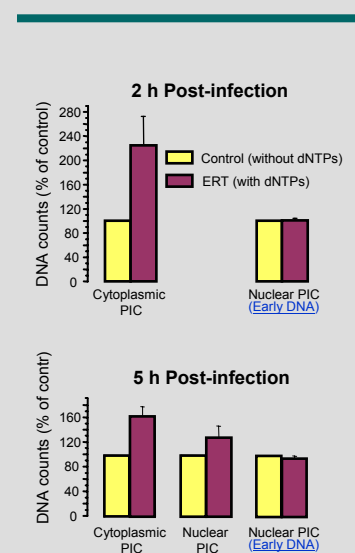
**A. PCR analysis of PICs.** Undiluted or diluted (1:10, 1:10<sup>2</sup>, 1:10<sup>3</sup>, and 1:10<sup>4</sup>) cytoplasmic and nuclear PICs (purified at 2 and 5 h post-spinoculation) were used as substrates for PCR with primers M667 (5'-GGC TAA CTA GGG AAC CCA CTG-3') and AA55 (5'-CTG CTA GAG ATT TTC CAC ACT GAC-3') specific for the negative-strand "strong-stop" DNA (the early reverse transcription product) (upper panels), and FOR-LATE (5'-TGT GTG CCC GTC TGT GTG GT-3') and REV-LATE (5'-GAG TCC TGC GTC GAG AGA GC-3') specific for the late reverse transcription products (bottom panels). Products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. M - molecular mass marker, + - positive control (NL4-3 HIV-1 DNA).

**B. Real-time PCR analysis of nuclear and cytoplasmic PICs.** cPICs and nPICs were analyzed in triplicate with primers specific for early or late HIV-1 DNA using DyNAmo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). Serial dilutions of DNA from 8E5 cells (cell line containing a single copy of HIV-1 LAV provirus per cell) were used as the quantitative standards. SYBR Green fluorescence was measured using CFX 3200 Opticon System. Results are presented as mean ±SD.

**C. PCR analysis of purity of cytoplasmic and nuclear extracts.** Before isolation of PICs, aliquots of cytoplasmic and nuclear extracts were normalized according to protein concentration and purity of fractionation was analyzed by PCR using primers, specific for mitochondrial DNA (Forward primer, Mito1: 5'-GAA TGT CTG CAC AGC CAC TT-3'; Reverse primer, Mito2: 5'-AGA AAG GCT AGG ACC AAA CC-3'). M-molecular mass marker, - negative control (H<sub>2</sub>O).

**Figure 3.** Analysis of protein composition of cytoplasmic and nuclear PICs (Preliminary results).

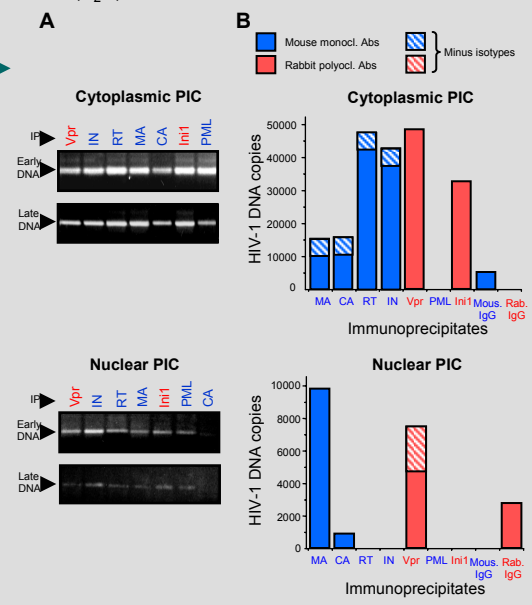
cPICs and nPICs were immunoprecipitated from complexes resuspended in buffer K using the following antibodies: mouse monoclonal antibodies to MA, RT (ABI, Columbia, MD), CA, IN (AIDS Research Reagent Program) and PML (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal antibodies to Vpr (a kind gift of Dr. Josephine Sire), and In1 (Santa Cruz Biotechnology); purified mouse and rabbit IgG (Jackson's Laboratories) - isotype controls. PIC DNA was isolated from immune complexes using IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Inc) and analyzed by PCR.



**Figure 4.** Endogenous reverse transcription (ERT) activity of cytoplasmic and nuclear PICs.

**A. PCR analysis of DNA from immune complexes.** PIC DNA from immune complexes was divided in half and analyzed by PCR using early and late HIV-1 DNA-specific primers as described for Fig. 2A. Results are shown for one experiment out of two performed.

**B. Real-time PCR analysis of DNA from immune complexes.** DNA from immunoprecipitated cPICs and nPICs was amplified with primers specific for early HIV-1 DNA and analyzed using DyNAmo SYBR Green qPCR Kit. Serial dilutions of DNA from 8E5 cells were used as the quantitative standards.



**Figure 5.** Quantitative PCR analysis of *in vitro* integration of HIV-1 PICs into isolated chromatin.

Since nucleosomal organization of the chromatin is significant for HIV-1 integration, immunoprecipitated chromatin was used as a target for integration. Chromatin was isolated from CEM cells using the following method. CEM cells were fixed with 1% formaldehyde, washed with PBS, lysed using lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), and sonicated to reduce DNA length to 200–1000 bp. The chromatin solution was precleared on protein A/G beads pre-adsorbed with sonicated salmon sperm DNA and then incubated with an antibody mixture containing histone H3 phosphorylated on serine 10 (Upstate Cell Signaling Solutions), Pol II (N-20) (Santa Cruz) and 2,2,7 trimethyl-guanosine (Ab-1) (Oncogene) or no antibody overnight at 4°C. Immune complexes were collected with protein beads pre-adsorbed with sonicated salmon sperm DNA. Following several washes, chromatin containing immunocomplexes were resuspended in 100 µl of buffer K.

Cytoplasmic and nuclear PICs, subjected to ERT in the absence (control) or presence of dNTPs, were incubated with chromatin in the presence of 0.25 mM ATP for 1 h at 37°C. Integration of HIV-1 DNA was analyzed by *Alu*-LTR-based real-time nested-PCR according to Brussel & Sonigo (2003). Serial dilutions of DNA isolated from 8E5 cells were used as the quantitative standards.

**A. Fluorescence curves** generated by second round of amplification of integrated HIV-1 DNA from immune-precipitated chromatin.

**B. Quantification of integrated HIV-1 DNA** from cPICs and nPICs, isolated at 2 and 5 h post-infection. *In vitro* integration of PICs was performed in triplicate. Results are presented as mean ±SD.

## Conclusions

1. RTCs and cytoplasmic PICs formed during synchronous HIV-1 infection are characterized by significant heterogeneity in respect of reverse transcription: approximately 50% of virions begin reverse transcription later than two hours post-entry, whereas the proportion of cPICs which have completed reverse transcription increases fifty-fold during this period; only 10% of the RTCs which have begun a reverse transcription finish this process within first five hours of infection.
2. Completion of reverse transcription in cPICs precedes their nuclear translocation. Most of nPICs contain completed HIV-1 DNA. However, during the first two hours of infection a part of PICs completed reverse transcription after nuclear entry.
3. Protein composition of PICs undergoes changes coupled with their nuclear translocation. RTCs contain HIV-1 proteins MA, CA, RT, IN, and Vpr, and at least two host-cell proteins, PML and In1, whereas MA, IN, and Vpr are absently detected in nPICs.
4. Part of nPICs retains the capacity to complete reverse transcription *in vitro* suggesting that they retain active reverse transcriptase.

5. Cytoplasmic and nuclear PICs have similar integration capacity despite differences in their protein composition. Changes of protein composition of PICs coupled with their nuclear translocation and possible heterogeneity of the nPICs have no significant effect on their overall integrative capacity. Intracellular maturation of HIV-1 PICs does not appear to contribute significantly to integration.

Thus, our results suggest that PIC maturation occurs predominantly in the cytoplasm and nPICs containing RT and immature DNA may be defective for integration.

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## Literature Cited

Brussel, A. and Sonigo, P. (2003). Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. *J. Virol.* 77:10119-10124.

Bukrinsky, M. I., Sharova, N., McDonald, T. L., Pushkarskaya, T., Tarpley, W. G., and Stevenson, M. (1993). Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl. Acad. Sci. U.S.A.* 90, 6125-6129.

De Noronha, C. M., Sherman, M. P., Lin, H. W., Cavrois, M. V., Moir, R. D., Goldman, R. D., and Greene, W. C. (2001). Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. *Science* 294, 1105-1108.

Farnet, C. M. and Haseltine, W. A. (1990). Integration of human immunodeficiency virus type 1 DNA *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4164-4168.

Farnet, C. M. and Haseltine, W. A. (1991). Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J. Virol.* 65, 1910-1915.

Farnet, C. M. and Bushman, F. D. (1997). HIV-1 cDNA integration: requirement of HMG (Y) protein for function of preintegration complexes *in vitro*. *Cell* 88, 483-492.

Fassati, A. and Goff, S. P. (2001). Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. *J. Virol.* 75, 3626-3635.

Gupta, K., Ott, D., Hope, T. J., Siliciano, R. F., and Boeke, J. D. (2000). A human nuclear shuttling protein that interacts with human immunodeficiency virus type 1 matrix is packaged into virions. *J. Virol.* 74, 11811-11824.

Heinzinger, N. K., Bukrinsky, M. I., Haggerty, S. A., Ragland, A. M., Kewalramani, V., Lee, M. A., Gendelman, H. E., Ratner, L., Stevenson, M., and Emerman, M. (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7311-7315.

McDonald, D., Vodicka, M. A., Lucero, G., Svitkina, T. M., Borisy, G. G., Emerman, M., and Hope, T. J. (2002). Visualization of the intracellular behavior of HIV in living cells. *J. Cell Biol.* 159, 441-452.

Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997). Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J. Virol.* 71, 5382-5390.

Nisole, S. and Saib, A. (2004). Early steps of retrovirus replicative cycle. *Retrovirology*, 1, 9.

Turelli, P., Doucas, V., Craig, E., Mangan, B., Klages, N., Evans, R., Kalpana, G., and Trono, D. (2001). Cytoplasmic recruitment of IN1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication. *Mol. Cell* 7, 1245-1254.