Residual HIV-1 DNA Flap-independent nuclear import of cPPT/CTS double mutant viruses does not support spreading infection

Candela Iglesias, Mathieu Ringeard, Francesca Di Nunzio, Juliette Fernandez, Raphael Gaudin, Philippe Souque, Pierre Charneau, Nathalie Arhel

To cite this version:
Candela Iglesias, Mathieu Ringeard, Francesca Di Nunzio, Juliette Fernandez, Raphael Gaudin, et al.. Residual HIV-1 DNA Flap-independent nuclear import of cPPT/CTS double mutant viruses does not support spreading infection. Retrovirology, BioMed Central, 2011, 8 (1), pp.92. 10.1186/1742-4690-8-92. pasteur-00646599

HAL Id: pasteur-00646599
https://hal-pasteur.archives-ouvertes.fr/pasteur-00646599
Submitted on 30 Nov 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Residual HIV-1 DNA Flap-independent nuclear import of cPPT/CTS double mutant viruses does not support spreading infection

Candela Iglesias¹,²†, Mathieu Ringeard¹,³†, Francesca Di Nunzio¹, Juliette Fernandez¹, Raphael Gaudin¹,⁴, Philippe Souque¹, Pierre Charneau*¹ and Nathalie Arhel¹*

Abstract

Background: The human immunodeficiency virus type 1 (HIV-1) central DNA Flap is generated during reverse transcription as a result of (+) strand initiation at the central polypurine tract (cPPT) and termination after a ca. 100 bp strand displacement at the central termination sequence (CTS). The central DNA Flap is a determinant of HIV-1 nuclear import, however, neither cPPT nor CTS mutations entirely abolish nuclear import and infection. Therefore, to determine whether or not the DNA Flap is essential for HIV-1 nuclear import, we generated double mutant (DM) viruses, combining cPPT and CTS mutations to abolish DNA Flap formation.

Results: The combination of cPPT and CTS mutations reduced the proportion of viruses forming the central DNA Flap at the end of reverse transcription and further decreased virus infectivity in one-cycle titration assays. The most affected DM viruses were unable to establish a spreading infection in the highly permissive MT4 cell line, nor in human primary peripheral blood mononuclear cells (PBMCs), indicating that the DNA Flap is required for virus replication. Surprisingly, we found that DM viruses still maintained residual nuclear import levels, amounting to 5-15% of wild-type virus, as assessed by viral DNA circle quantification. Alu-PCR quantification of integrated viral genome also indicated 5-10% residual integration levels compared to wild-type virus.

Conclusion: This work establishes that the central DNA Flap is required for HIV-1 spreading infection but points to a residual DNA Flap independent nuclear import, whose functional significance remains unclear since it is not sufficient to support viral replication.

Background

During infection, the reverse transcriptase (RT) of retroviruses converts the (+) strand RNA genome into double-stranded DNA prior to nuclear import. Similar to all DNA polymerases, the retroviral RT requires the 3’ OH of a primer to initiate polymerisation. The primer used for (-) strand synthesis is a cellular tRNA already present in the retroviral particles prior to infection and anneals to the 5’ region of the genome at the primer binding site (PBS). The primer used for (+) strand synthesis is a polypurine tract (PPT) present in the 3’ region of the RNA genome that resists RNase H degradation concomitant with (-) strand synthesis. The genome of lentiviruses contains two such cis-acting purine-rich sequences: the 3’ PPT common to all retroviruses and an additional central PPT present in the coding sequence of the integrase. The resulting initiation of (+) strand synthesis at the cPPT as well as at the 3’PPT leads to a (+) strand discontinuity at the centre of lentiviral genomes [1-5].

Similar to central (+) strand initiation, the termination of reverse transcription is a further feature that distinguishes lentiviruses from other orthoretroviruses. For most retroviruses, termination occurs when the RT reaches the 5’ end of the template. In the case of lentiviruses however, such as HIV-1 [6], EIAV [4], and FIV [5], reverse transcription terminates ca. 100 nt downstream of the cPPT at stretches of A and T nucleotides (the central termination sequence, CTS) whose
conformation disfavours binding to RT enzyme and thus halts reverse transcription. As a result of the cPPT and CTS cis-acting sequences, the final product of lentiviral reverse transcription contains a ca. 100 nt overlap, or “DNA Flap”, at the centre of the genome.

Mutations introduced either in the cPPT [7-9] or in the CTS [6] of HIV-1 lead to a loss of HIV-1 infectivity that is proportional to the number and positional impact of introduced mutations. The conversion of cPPT purines into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.

The defect in viral replication observed for cPPT mutant viruses [7,10,11] is not accounted for by defects in viral DNA synthesis, integration, or virus production [9] but by a defect in nuclear import [9,10,12]. The importance of the central DNA Flap for nuclear import was harnessed by the gene therapy field when it was shown that re-insertion of the cPPT and CTS sequences into pyrimidines compromises central initiation of (+) strand synthesis and thus central DNA Flap formation [7]. Similarly, the mutation of dA/DT tracts in the CTS leads to aberrant (+) strand termination events spread over a distance of over 500 nt downstream of the cPPT [6]. Therefore, both the cPPT and CTS are required to form a functional central DNA Flap, and HIV-1 infection is impaired if either are mutated.
pyrimidines in the A-boxes, is one of the least affected mutants [7]. The 225T mutant combines mutations in all four boxes and is the most affected amongst the synonymous cPPT mutants [17]. The addition of a single non-synonymous mutation (cPPT-D mutant) has the most profound effect on viral replication [9]. Although this mutant introduces an amino acid change at position 188 of the integrase coding region (K188R), the control virus (cPPT-AG) with the same amino acid change behaved like wild-type virus in terms of virus production and viral DNA synthesis [9]. Position 188 of integrase (IN) is important for salt bridge formation between the catalytic core and the N-terminal domain, however, the presence of an arginine rather than a lysine at this position is naturally found in HIV-2 IN and does not perturb salt bridge formation [40]. The previously described CTS mutant, which was designed to disrupt the dA-dT tracts of the CTS while respecting the corresponding integrase amino acid sequence, results in DNA Flaps of abnormal length and severely impairs infectivity [6].

We generated double mutants (DM) by combining in HIV-1LAI and HIV-1LAI Δenv-vsvG viruses, the 223, 225T or cPPT-D cPPT mutants with the previously described CTS mutation [6]. There were no appreciable differences in virus p24 concentration between the wild-type virus and any of the mutant viruses (Additional File 1 Figure S1), confirming that DNA Flap mutations do not affect virus production [9].

Impact of DNA Flap mutations in single-cycle infectivity

We first tested the single and double mutant viruses for infectivity in single cycle infections in the P4-CCR5 reporter cell line, which are HeLa CD4+ CXCR4+ CCR5 + carrying the Lac Z gene under the control of the HIV-1 LTR promoter [6]. cPPT and CTS HIV-1 mutants exhibited an impairment of infection that was significant and increasingly important with increasing number of mutations (Figure 2A). Reduction was consistent and was of 2-, 14-, 16- and 20-fold for 223, CTS, cPPT-D and 225T, respectively, compared to wild-type virus (Figure 2B). The combination of cPPT and CTS mutations increased this defect significantly in the case of the DM-223 and DM-D mutants. The DM-D mutant was the most affected of all DNA Flap mutants and only retained a very residual infectivity of 0.8 +/- 0.3% of wild-type virus (Figure 2B). The pseudotyping of HIV-1 with the vesicular stomatitis virus glycoprotein (VSV-G), which confers broad tropism and increases infectivity by 10-fold, attenuated the infectivity defects observed with wild-type envelope HIV-1LAI viruses (Figure 2C). The average reduction in infectivity was between 2- and 5-fold for all single mutants. However, the combining of cPPT and CTS mutations significantly further decreased the infectivity of pseudotyped viruses. The most affected mutant, DM-D VSV-G, retained an infectivity of 9.4 +/- 0.9% of wild-type virus (Figure 2D).

Taken together, these results indicate that all cPPT single and double mutants are severely impaired in infectivity, but that the most disrupted mutant still maintains residual infection (ca. 1% for non-pseudotyped viruses and ca. 10% for VSV-G pseudotyped viruses) in a one-round titration assay.

It has recently been reported that central DNA Flap mutant HI-viruses maintain wild-type infectivity in single cycle infection assays following production in APO-BEC3G-negative 293T cells [16]. To test the possibility that the apparent discrepancy between our results and those reported by Hu et al. 2010 [16] might reflect differences between virus strains or the production protocol, the molecular clones used in Hu et al. were obtained from E. Poeschla (Mayo Clinic). The ability of these viruses to infect P4-CCR5 indicator cells was tested in parallel with our wild-type virus and DNA Flap mutants. The mutants from both studies displayed a comparable infectivity defect (up to 10-fold) at all multiplicities of infection tested (Figure 2E).

To test the possibility that the discrepancies between our results and those from Hu et al. might result from the use of different indicator cell lines, the GHOST3
Figure 2 Single-cycle titration assay in P4-CCR5 cells indicate an increased infectivity defect of DNA Flap double mutant viruses compared with cPPT or CTS single mutants. (A) P4-CCR5 cells were infected with 4 ng or 1 ng p24 of HIV-1 wild-type envelope and infectivity was measured 48 h p.i. by tat transactivation of the beta-gal promoter (mean of triplicates +/- SD representative of two independent experiments). (B) Relative infection of the DNA Flap mutant viruses compared with wild-type LAI virus (mean of three independent experiments carried out in triplicate over a range of viral concentrations +/- SD). Statistical relevance was calculated using paired t test to compare cPPT single mutant viruses with their corresponding DM virus, *** p < 0.001. (C) P4-CCR5 cells were infected with 1 ng or 0.1 ng p24 of HIV-1 VSV-G and infectivity was measured 48 h p.i. by Tat transactivation of the beta-gal promoter (mean of triplicates +/- SD representative of three independent experiments). (D) Relative infection of the DNA Flap mutant viruses compared with wild-type LAI virus (mean of three independent experiments carried out in triplicate +/- SD). Statistical relevance was calculated using paired t test to compare cPPT single mutant viruses with their corresponding DM virus, *** p < 0.001. (E) P4-CCR5 cells were infected with wild-type env NL4-3 viruses from the Hu et al., 2010 study (WT, mcPPT, DM) in parallel with our wild-type and mutant LAI viruses (LAI, D, DM). Results show the mean beta-galactosidase activity of duplicates +/- SD and are representative of three independent experiments. (F) GHOST3 cells were infected with increasing doses of viruses from the Hu et al. study [16]. Infectivity was assessed at 48 h p.i using flow cytometry. Results show the mean percentage of GFP positive cells of three independent experiments.
cells used in the Hu et al. study were obtained also from E. Poeschla and infected in parallel with molecular clones from Hu et al. and our wild-type and mutant viruses (Figure 2F and Additional File 2 Figure S2). GHOST3 cells are HOS CD4+ CXCR4+ CCR5+ carrying the green fluorescent protein (GFP) gene under the control of the HIV-2 LTR promoter. This reporter cell line has the advantage that it does not require cell lysis or addition of substrate, and allows for percentages of infected cells to be measured. We note however that percentages of infected cells have a reduced dynamic range (0-100%, Figure 2F), compared with RLU values from P4-CCR5 cells (4-5 log10, Figure 2A, C), and do not distinguish single from multiple integration events. The percentage of infected cells was consistently less for DNA Flap mutants compared with wild-type virus (up to 6-fold), but the amplitude of the infectivity defect was reduced or lost at high multiplicities of infection (Figure 2F), where multiple integration events occur. The narrow linear dynamic range of GHOST3 cells may account for the diverging results reported by Hu et al. [16].

Impact of DNA Flap mutations on viral replication

Single cycle infection assays are sensitive to very low infectivity and successful integration events as measured by beta-gal activity, but do not guarantee that the viruses can establish a spreading infection in cell culture. We therefore carried out kinetics of replication following infection with wild-type envelope HIV-1. MT4 cells were chosen because they are highly permissible to HIV-1, allowing rapid and efficient replication. MT4 cells are APOBEC3G-negative, and therefore the role of central (+) strand initiation in protecting the genome from editing [14,16] is not a confounding factor in this assay. In 2 independent experiments, the replication of wild-type HIV-1LAI peaked at 5-6 days post infection; in contrast, the replication of 223, 225T, CTS and cPPT-D single mutants was delayed on average by 3, 5, 6 and 9 days, respectively, compared with wild-type HIV-1LAI (Figure 3). While DM-223 showed a delay similar to that of the CTS mutant, virus replication remained undetectable for DM-225T and DM-D mutants over the 24-day period, indicating that despite minimal single-cycle infection, these viruses do not replicate in the time frame of the assay.

To verify that the observed replication defect is not cell-dependent, we also tested the ability of double-mutant viruses to establish spreading infection in human primary PBMCs from two healthy donors, which were infected with 0.1 and 1 ng p24 of wild-type and double mutant viruses. The replication of wild-type HIV1LAI peaked at 7-10 days post-infection (Figure 4). In contrast, no peak of replication was detected for double mutant viruses over the 40-day period, and virus concentration was only sporadically measurable above the assay minimum detectable limit (ca. 3 ng/ml). The fact that DM 223, which replicated in MT4 cells despite some delay compared to wild-type virus (Figure 3), did not establish spreading infection in PBMCs confirms the high permissiveness of MT4 cells. Moreover, although viruses used for this study were wild-type for Vif, the stronger block in replication observed in PBMCs may also reflect the increased sensitivity of DNA Flap mutants to APOBEC3G/F-mediated deamination in PBMCs [14,16]. We conclude that DNA Flap double mutant viruses cannot establish spreading infection in
lymphocytic cell cultures despite detectable levels of infectivity in single cycle infection assays.

Impact of DNA Flap mutations on nuclear import
Once reverse transcribed in the cytoplasm, the HIV-1 linear DNA is imported into the nucleus where it either integrates or circularizes. Circular forms of non-integrated viral DNA contain one or two long terminal repeats (LTR) and are thought to be dead-end products of infection. Since they are found exclusively in the nucleus of infected cells, they constitute convenient markers of nuclear import. We therefore used quantitative PCR to measure two-LTR DNA circles [41] in P4-CCR5 cells infected with wild-type, single and double mutant HIV-1 LAI vsvG viruses. At 24 h post-infection, quantification of 2-LTR junctions revealed a reduction in 2-LTR copy numbers of 3-6-fold for all single and double DNA Flap mutants compared to wild-type virus, indicating a defect in nuclear import (Figure 5A). As previously observed (P. Charneau, unpublished data), the CTS mutant has a nuclear import defect comparable to cPPT mutants. Mutations in the cPPT lead to linear DNA lacking the central DNA Flap while mutations in the CTS lead to linear DNA containing an aberrantly sized DNA Flap. Since both DNA Flap mutant types have impaired nuclear import, it is the integrity of the DNA Flap structure, rather than the presence or not of a (+) strand discontinuity, that accounts for nuclear import. Furthermore, DNA Flap mutant viruses maintained a defect in 2-LTR copy numbers over a 72 h time course (Figure 5B), indicating an overall defect in nuclear import rather than a delay. D vsv-G and DM-D vsv-G viruses remained strongly defective for nuclear import throughout the time course (Figure 5B). Similar to results obtained from infectivity experiments (Figure 2), the combination of cPPT and CTS mutants led to a further decrease in 2-LTR copy numbers compared with single mutants. Using this test, DM-225T and DM-D maintain residual levels of 2-LTR circles of 10-20% of wild-type virus, indicating that disruption of the central DNA Flap severely impairs but does not abrogate nuclear import.
Two-LTR circles represent only a minute fraction of HIV-1 DNA in infected cells (< 1% of nuclear viral DNA), and small variations in their levels will lead to important changes in inferred nuclear import levels. One-LTR circles are more abundant (ca. 35% of nuclear viral DNA) [9], but cannot be distinguished by quantitative PCR. We therefore used restriction enzyme analysis coupled to Southern blotting to detect total, linear non-integrated, and circularised forms of HIV-1 DNA in MT4 cells [9]. In three independent experiments, we found that at 48 h post-infection with wild-type HIV-1LAI, under 1% of viral DNA was in the form of 2-LTR circles, 35-45% as 1-LTR circles, and 10-15% as linear unintegrated DNA (Figure 6A), which is concordant with previous work [9]. Linear viral DNA molecules undergo rapid integration or circularisation soon after entry in the nucleus [42-45]; therefore, measured linear DNA corresponds predominantly to cytoplasmic viral DNA, and its accumulation is indicative of a nuclear import defect. The ratio of 1- and 2-LTR forms over linear cytoplasmic viral DNA at 48 h post-infection, which was of 4 on average for wild-type HIV-1, was reduced,
in the case of mutations in the cPPT, to 0.2-0.6 indicating a nuclear import defect (Figure 6A). Using this Southern blot assay, we did not notice an additional impairment of nuclear import for DM viruses. Furthermore, despite the reduction in nuclear import, nuclear forms of viral DNA (1-LTR) could still be detected for both single and double DNA Flap mutants, confirming the residual level of nuclear import obtained with 2-LTR quantitative PCR.

While these findings suggested that a residual proportion of viral pre-integration complexes might enter the nucleus in the absence of the central DNA Flap, we could not exclude that a small proportion of DNA circles form independently of nuclear import, through auto-integration events in the cytoplasm for instance [45]. To verify this, we quantified integrated viral DNA using Alu-PCR. If viral DNA circularisation occurs independently of HIV-1 nuclear import or at the expense of HIV-1 integration, which is essential for productive infection, integrated viral DNA would not be detected following infection with DNA Flap mutant viruses. We found that cPPT and CTS mutations led to significant reduction in detected integrated HIV-1 genome and that the combination of cPPT and CTS mutations in double mutants led to even greater defects in integration (Figure 6B). The integration defect for the 223 mutant was only 2-fold, which is concordant with the single cycle infectivity and quantitative PCR of 2-LTR circles data (Figure 2). Importantly, integrated signal could be detected for all viruses, including the most disrupted DM-D virus, equivalent to 5-10% of wild-type levels, consistent with the result that all mutant viruses have some residual nuclear import.

Taken together, our data indicate that all DNA Flap mutants analysed have a strongly impaired nuclear import, but maintain a residual level of nuclear import which amounts to 5-15% of wild-type HIV-1, depending on the assay used. The mechanism for this DNA-Flap-independent nuclear import is unknown. The proportion of circularised and integrated HIV-1 following nuclear import is similar to that observed for wild-type virus. However, levels of integrated virus are insufficient to establish spreading infection.

Discussion

The central DNA Flap and its importance for HIV-1 replication have been controversial for many years. Its role as a cis-acting determinant of nuclear import has proven all-important for gene transfer based on lentiviral vectors and in the context of replicative viruses [5,9-13,17-21,23,24,26-31,33,34,39]. This defect is independent of envelope tropism (R5, X4, VSV-G) or cell type used (cell lines, primary lymphocytes, APOBEC3G/ F status). Still, a certain element of contention remains regarding the extent of the DNA Flap’s importance, with some reports contesting its role in nuclear import and replication [16,35-38].

We believe this controversy may be resolved by considering two points. Firstly, the benefit to infection/transduction brought by the central DNA Flap will be overseen in saturating experimental conditions with assays that cannot distinguish single from multiple infectious events per cell. Concordantly, high MOIs in the context of lentiviral transductions attenuate defects measured in the absence of the central DNA Flap [11]. Similarly, this paper shows that the infectivity defect of DNA Flap mutants is more apparent for wild-type envelope HIV-1 than for VSV-G pseudotyped HIV-1, which is 10-fold more infectious; and more apparent in PBMCs than MT4 cells, which are highly permissive. In transduction experiments using HIV-1 derived vectors coding for eGFP, the benefit of the DNA Flap in terms
of the percentage of transduced cells will also decrease with increasing MOI, with transduction efficiencies that can reach 100% for both Flap+ and Flap- vectors in permissive cells (Figure 7). The same loss of a differential phenotype at high and low MOI was also observed following infection of GHOST cells with the viruses from the Hu et al., 2010 study (Figure 2F). In contrast, the difference in mean fluorescent intensity (MFI), which reflects cumulative nuclear entry events, will increase (Figure 7). Therefore, in the case of multiple infectious events per cell, the true impact of the central DNA Flap may only be reliably assessed by combining both percentage of infected cells and MFI [31].

Secondly, DNA Flap mutant viruses display an equal nuclear import defect in both dividing and non-dividing cells [9,10,39,46]. Therefore, assays that compare cycling cells with aphidicolin-arrested cells will invariably see no difference in terms of nuclear import and infectivity of DNA Flap mutant viruses. These experiments are based on the hypothesis that HIV-1 passes through the nuclear pore in non-dividing cells, but uses an alternative route for nuclear entry during mitosis. However, several arguments suggest that this might not be the case. (1) A mitosis-independent nuclear import in cycling cells has been reported [47]. (2) A genome-wide RNA interference-based screen comparing HIV-1 and MLV infections identified unique nuclear import factors for HIV-1 even though the study was carried out in cycling cells [48]. (3) The assumption that HIV-1 might passively gain access to the chromatin upon mitosis, if based on the belief that cytoplasmic and nuclear contents mix homogeneously throughout mitosis, is not valid. Indeed, evidence suggests that mitotic cells maintain spatial information through gradients, such as the RanGTP gradient that surrounds chromatin [49,50]. (4) HIV-1 mutants with a nuclear import defect in cell cycle-arrested cells often maintain this defect in cycling cells [9,10,39,46,51]. (5) The replication of certain lentiviruses (such as EIAV, CAEV and VISNA) is entirely limited to macrophages, which do not divide.

Mechanism of DNA-Flap-dependent nuclear import
We have previously shown that the central DNA Flap mediates nuclear import by promoting viral capsid uncoating at the nuclear pore [17]. However, the molecular mechanisms underlying DNA-Flap-dependent maturation of HIV-1 capsids remain unknown. Since DNA-Flap-dependent uncoating can be reconstituted in vitro in purified vector particles undergoing endogenous reverse transcription, it is not likely that any cellular component(s) is implicated in the uncoating process unless it is already present in the particle. One possible hypothesis is that formation of the central DNA Flap at the end of reverse transcription triggers major morphological rearrangements that lead to the release of the HIV-1 pre-integration complex from the capsid core. Intriguingly, CTS mutations, which lead to DNA Flap structures with aberrant length spread over a distance of 500 nt downstream of the cPPT [6], severely impair nuclear import and infectivity. These data suggest that a central DNA Flap of aberrant length is as detrimental to viral replication as is the absence of the central DNA Flap.

Mechanism of DNA-Flap-independent nuclear import
The mechanism for the DNA-Flap-independent nuclear import uncovered by this study is unknown, but evidence suggests that it might involve at least some of the same host factors as DNA-Flap dependent nuclear import since Flap-negative viruses are as sensitive to the depletion of TNPO3 or Nup153 as wild-type virus [52,53]. DNA Flap-independent nuclear import cannot be due to viral complexes entering the nucleus via an alternative route during mitosis, since the DNA-Flap-
independent nuclear import reported here may be observed in both cycling and non-cycling cells [9,10,39,46]. We have noted that smaller lentiviral genomes, such as minimal HIV-1 derived vectors, have a higher proportion of DNA-Flap-independent nuclear import (P. Charneau, unpublished data), suggesting that the smaller the viral genome, the less dependent it is on the central DNA Flap for passage through the nuclear pore.

Taken together, our data establish that the integrity of the central DNA Flap is required to support a spreading infection and confirm that all cPPT and CTS mutants have a nuclear import defect. Although this defect may vary in its amplitude depending on the nuclear import assay and the MOI used, it is consistently observed. Of note, central (+) strand initiation may also carry further benefits for HIV-1 replication besides assisting viral nuclear import, such as protection from APOBEC3/F editing as previously shown [14,16]. Here, we show that even the most disrupted DNA Flap mutants still maintain residual nuclear import, but that this does not support spreading infection in human lymphocytic cells. It will be interesting to determine the mechanisms for this DNA Flap-independent nuclear import.

Methods

Cells and viruses

The P4-CCR5 reporter cells are HeLa CD4+ CXCR4+ CCR5+ carrying the Lac Z gene under the control of the HIV-1 LTR promoter [6]. MT4 cells are HTLV-1 transformed human CD4+ T cells that allow acute cytopathic HIV-1 infection [54]. The 293T cells are human embryonic kidney cells. Citrate human blood was obtained from healthy donors (Établissement Français du Sang) and PBMCs were isolated following Ficoll gradient. GHOST3 cells are HOS CD4+ CXCR4+ CCR5+ carrying the green fluorescent protein (GFP) gene under the control of the HIV-2 LTR promoter.

The viral molecular clone used in our study was HIV-1BRU also called LAV (Lymphadenopathy-associated virus), based on the 1983 isolate from a homosexual patient with lymphadenopathy [55]. This molecular clone is HIV-1LAI for all intents and purposes and is referred to as such in the manuscript. In the case of pseudotyping with VSV-G, we used a Δenv molecular clone that was generated by deletion of the 1.3 kb KpnI-BglII fragment in HIV-1 env [56]. Molecular clones from the Hu et al., 2010 study are NL4-3 Vpr+, WT, mcPPT (cPPT mutant) or DM (cPPT and CTS double mutant).

The generation of 223 [7], 225T [17], cPPT-D [9] and CTS [6] mutants by site directed mutagenesis has been described previously. Double mutants were generated by splice overlap extension PCR using the following primers to amplify the cPPT region, DM1 5’-ACATA-CAGACAATGGCGCAG-3’ and DM2 5’-TGCTATTATGTCTACTATTC-3’ and the following primers to amplify the CTS region, DM3 5’-ATAGTAGACATAATAGCAAC-3’ and DM4 5’-TATGTGACACCAATTC-3’.

Virus production

Viruses were produced by transient transfection of 293T cells using calcium phosphate co-precipitation with the wild-type or mutant proviral plasmids based on pLAI or pNL43 Vpr-. In the case of pseudotyping with VSV-G, pLAIΔenv was co-transfected with the VSV-G envelope expression plasmid pHMV-G [57]. Virus concentration in supernatants was measured by p24 ELISA according to the manufacturer’s instructions (Perkin Elmer).

Quantitative PCR

P4-CCR5 cells were infected with LAI-vsvG viruses (500 ng p24 antigen per 2 × 10⁶ cells in 2 ml) for 2 h at 37°C and thereafter maintained at a concentration of 0.33 × 10⁶ cells/ml. For each viral strain, a control of infected cells cultured in the presence of 5 μM nevirapine, a nonnucleosidic RT inhibitor, was included. Total cellular DNA was extracted at given time points post-infection according to manufacturer’s instruction (Qiagen) 2-LTR junctions were quantified by real-time PCR using a Realplex instrument (Eppendorf) with 5’AACTAGG-GAACCcACTGCTTAAG3’ forward primer, 5’TCCA-CAG ATCAAGGATATCTTGTC3’ reverse primer and 5’FAM ACACTACTTGAAGCACTCAAGGC AAGCT TT TAMRA3’ probe [58]. Pol gene copy number was determined with 5’TITATAGTTGA ATAGATAAGGGCCA3’ forward primer, 5°CACGCTGCTAAC-TATTCTTTTGTCA3’ reverse primer and 5’FAM AATCACCTAGCAATGTCTCCTCAATATTAC TAMRA3’. Amplification was carried out for each reaction in 20 μl with 300 nM of each primer, 200 nM of probe, 5 μl of total-cell DNA and 10 μl of 2X of FastStart Universal Probe Master (Roche). Assessment of integration by Alu-PCR was performed as previously described [59] at 24 h post-infection.

Restriction enzyme analysis of intracellular viral DNA forms by Southern blotting

MT4 cells were infected with HIV-1LAI vsvG viruses (500 ng p24 antigen per 10⁶ cells in 1 ml) for 2 h at 37°C and thereafter maintained at a concentration of 0.5 × 10⁶ cells/ml. At 48 h post-infection, total DNA was isolated by lysis in 10 mM Tris, 10 mM EDTA and 0.6% SDS. Samples were treated by RNase A (100 μg/ml) for 1 h at 37°C, and protease K (100 μg/ml) for 2 h at 55°C. DNA was extracted by phenol chloroform and digested with DpnI to remove contaminating bacterial plasmid DNA. Viral DNA was then digested with MscI.
and XhoI, which create well-defined fragments on the basis of which the different intracellular forms of viral DNA can be distinguished [9]. Digested samples were then analysed by Southern blotting, using a probe that overlaps one of the two Mscl sites in the HIV-1 genome [9]. Southern blots were quantified by phosphorimager (Molecular Dynamics) and the ImageQuant software.

Single-cycle titration in P4-CCR5 and GHOST3 cells
P4-CCR5 infections were carried out in triplicate in 96-well plates (10,000 cells per well) with increasing doses of LAI or LAI-vsvG. β-Galactosidase activity was measured at 48 h postinfection by using a chemiluminescent β-galactosidase reporter gene assay according to manufacturer’s instructions (Roche). GHOST3 infections were carried out in 6-well plates (400,000 cells per well) with increasing doses of LAI or NL43 Vpr-. The percentage of GFP+ cells was assessed 48 h.p.i using flow cytometry (BD Calibur).

Replication kinetics
MT4 cells were infected with HIV-1LAI and cPPT/CTS mutants in 96-well plates (1 ng p24 antigen per 5 × 10⁶ cells in 200 μl). Every 2 days for 24 days, 100 μl of supernatant were collected and replaced by fresh medium. Viral concentration in supernatants was assessed by RT-assay. PBMCs, stimulated for 3 days with Concanavalin A (5 μg/ml), were infected with HIV-1LAI and cPPT/CTS mutants in 96-well plates (1 ng p24 antigen per 1 × 10⁵ cells in 200 μl). Every 3-4 days for 40 days, 100 μl of supernatant were collected and replaced with fresh medium containing IL2 (final concentration 10 ng/ml). Viral concentration in supernatants was assessed by p24 ELISA (Perkin Elmer).

Additional material

Additional file 1: Figure S1: cPPT and CTS mutations do not affect viral production. The p24 concentrations (ng/ml) are shown for 3 to 6 independent preparations of each virus, both wild-type and VSV-G pseudotyped envelopes. The graph shows mean values +/- SEM. One-way Anova analysis indicated that there is no statistically significant difference between any of the 16 mean values.

Additional file 2: Figure S2: GHOST cells were infected in parallel with viruses. GHOST cells were infected in parallel with viruses from the Hu et al., 2010 study (WT, mcPPT, DM) and our wild-type and mutant viruses (LAI D, DM D). The percentage of GFP-positive cells was assessed at 48 h.p.i using flow cytometry. Results are representative of three independent experiments.

Acknowledgements and funding
This work was funded by CONACyT (Consejo Nacional de Ciencia y Tecnologia), CNRS (Centre National de Recherche Scientifique), ANRS (Agence Nationale de Recherche sur le SIDA), FRM (Fondation pour la Recherche Médicale) and the Pasteur Institute.

Author details
1Molecular Virology and Vaccinology Unit, Department of Virology, Institut Pasteur, Paris, France. 2Institut Pasteur de Mexico, Mexico City. 3Centro de Investigacion en Enfermedades Infecciosas, Mexico, Mexico. 4Molecular Virology Laboratory, Inserm Unité de Génétique Humaine, Montpellier, France. 5Immunity and Cancer Unit, Institut Pasteur, Paris, France.

Authors’ contributions
CI generated the mutants and carried out Southern blotting experiments. MR and JP performed quantitative PCR. CR, MR, FDN, JF, RG, PS and NJA performed all other experiments. PC conceived the study. N/A participated in the design and coordination of the study, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 3 May 2011 Accepted: 10 November 2011
Published: 10 November 2011

References
1. Chameau P, Clavel F. A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polypurine tract. J Virol 1991, 65:2415-2421.
2. Harris JD, Scott JV, Traynor B, Brahic M, Stewring L, Ventura P, Haase AT, Peluso R: Viva virus DNA: discovery of a novel gapped structure. Virology 1981, 113:573-583.
3. Hungnes O, Tjøtta E, Grinde B: The plus strand is discontinuous in a subpopulation of unintegrated HIV-1 DNA. Arch Virol 1991, 116:133-141.
4. Stetar SR, Rausch JW, Guo MJ, Burnham JP, Boone LR, Waring MJ, Le Grice SF: Characterization of (+) strand initiation and termination sequences located at the center of the equine infectious anemia virus genome. Biochemistry 1999, 38:3656-3667.
5. Whitwam T, Peretz M, Poechila E: Identification of a central DNA flap in feline immunodeficiency virus. J Virol 2001, 75:9407-9414.
6. Chameau P, Mirambeau G, Roux P, Paulous S, Buc H, Clavel F: HIV-1 reverse transcription A termination step at the center of the genome. J Mol Biol 1994, 241:651-662.
7. Chameau P, Alizon M, Clavel F: A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. J Virol 1992, 66:2814-2820.
8. Hungnes O, Tjøtta E, Grinde B: Mutations in the central polypurine tract of HIV-1 result in delayed replication. Virology 1992, 190:440-442.
9. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Chameau P: HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 2000, 101:173-185.
10. Arhel N, Munier S, Souque P, Mollier K, Chameau P: Nuclear import defect of human immunodeficiency virus type 1 DNA flap mutants is not dependent on the viral strain or target cell type. J Virol 2006, 80:10262-10269.
11. De Rijck J, Debyser Z: The central DNA flap of the human immunodeficiency virus type 1 is important for viral replication. Biochem Biophys Res Commun 2006, 349:1100-1110.
12. Arhel N, Souquere-Besse S, Chameau P: Wild-type and central DNA flap defective HIV-1 lentiviral vector genomes: intracellular visualization at ultrastructural resolution levels. Retrovirology 2006, 3:39.
13. Hollenbeck AI, Ailles LE, Bakovic S, Geuna M, Naldini L: Mutations in the central polypurine tract of HIV-1 result in delayed replication. Virology 1992, 190:440-442.
14. Wurtzer S, Goubard A, Mammano F, Saragosti S, Lecossier D, Hance AJ, Arhel N, Munier S, Souque P, Mollier K, Charneau P: Mutations in the central polypurine tract of HIV-1 result in delayed replication. Virology 1992, 190:440-442.
15. Yu Q, König R, Pillai S, Chiles K, Kearney M, Palmer S, Richman D, Coffin JM, Landau NR: HIV-1 reverse transcription A termination step at the center of the genome. J Mol Biol 1994, 241:651-662.
16. Hu C, Saenz DT, Fadel HJ, Walker W, Peretz M, Poechila E: The HIV-1 central polypurine tract functions as a second line of defense against APOBEC3G/F. J Virol 2010, 84:11981-11993.
17. Arhel NJ, Souquere-Besse S, Munier S, Souque P, Guadagnini S, Rutherford S, Prévost MC, Allen TD, Charneau P: HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore. *EMBO J* 2007, 26:3025-3037.

18. Baekelandt V, Claes A, Eggermont K, Lauwers E, De Strooper B, Nuttin B, Debayer Z: Characterization of lentiviral vector-mediated gene transfer in adult mouse brain. *Hum Gene Ther* 2002, 13:841-853.

19. Barry SC, Harder B, Brezinski M, Flint LY, Seppen J, Osborne WR: Lentivirus vectors encoding both central polypurine tract and posttranscriptional regulatory element provide enhanced transduction and transgene expression. *Hum Gene Ther* 2001, 12:1103-1108.

20. Breckpot K, Dullaers M, Bonehill A, van Meirvenne S, Heirman C, de Greef C, De Derudder M, Van Maele B, De Clercq E, Debyser Z: Impact of the central DNA flap in HIV-1-derived lentiviral vectors. *Mol Ther* 2003, 6:841-853.

21. Dardalhon V, Herpers B, Noraz N, Pflumio F, Guetard D, Leveau C, Dubart-Kupperschmitt A, Charneau P, Taylor N: Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap. *Gene Ther* 2001, 8:190-198.

22. De Ryck J, Van Maele B, Debayer Z: Positional effects of the central DNA flap in HIV-1-derived lentiviral vectors. *Biochim Biophys Acta* 2005, 1728:979-994.

23. Demason C, Parsley K, Brouns G, Scherr M, Battmer K, Kinnon C, Grez M, Thrasher A: A central DNA flap in lentiviral vectors enhances transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentivector containing an internal spleen focus forming virus promoter. *Hum Gene Ther* 2002, 13:803-813.

24. Giannini C, Morosan S, Thalha JS, Battaglia S, Mollier K, Thaller A: Analysis of the viral elements required in the generation of HIV-1 Vif- mutant particles from restrictive infected cells. *Virology* 2009, 38:114-122.

25. Gropp M, Itskovson J, Singer O, Ben-Hur T, Reinhart E, Galun E, Reubroff BE: Stable genetic modification of human embryonic stem cells by lentiviral vectors. *Mol Ther* 2003, 7:281-287.

26. Hanaway A, Arosio D, Albanese A, Christ F, De Rijck J, Gijsbers R, Debyser Z: Nucleoporin NUP153-dependent nuclear import of HIV-1 DNA in vitro. *J Virol* 2000, 74:1534-1541.

27. Hanaway A, Debauche J, Arosio D, Albanese A, Christ F, De Rijck J, Gijsbers R, Debyser Z: Stable, and rapid approach for ex vivo human liver gene therapy via a lentiviral vector. *Hum Gene Ther* 2003, 14:291-300.

28. Hare S, Dintzis S, Sondag K, Wang J, Engelman A, Cherepanov P: Evidence for differential gene expression. *J Virol* 2001, 75:3025-3037.

29. Heirman C, De Deyn G, De Clercq E, Debyser Z: Structural basis for functional tetramerization of lentiviral integrase. *PLoS Pathog* 2009, 5:e1000515.

30. Hosier J, Brouns G, Scherr M, Battmer K, Grez M, Thrasher A: Evidence for differential gene expression. *J Virol* 2001, 75:3025-3037.

31. Iglesias G: Highly efficient lentiviral vector-mediated transduction of nondividing, fully cycling cells. *Mol Biol* 2009, 485:73-85.

32. Iglesias G: Virus-specific DNA in the cytoplasm of avian sarcoma-virus-infected cells is a precursor to covalently closed circular viral DNA in the nucleus. *J Virol* 1978, 25:104-114.

33. Iglesias G: Immunodominant CTL epitopes in human immunodeficiency virus type 1 Vif- mutant particles from restrictive infected cells. *Virology* 2009, 38:114-122.

34. Iglesias G: Current status of lentivirus vectors. *Curr Opin Biotechnol* 2001, 12:114-122.

35. Iglesias G: HIV-1 DNA nuclear import and gene transduction of human hematopoietic stem cells by lentiviral vectors. *Hum Gene Ther* 2002, 13:1793-1807.

36. Iglesias G: Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

37. Iglesias G: Treatment of the central DNA flap in HIV-1-derived lentiviral vectors. *Hum Gene Ther* 2001, 8:190-198.

38. Iglesias G: Modification of HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo. *Mol Ther* 2001, 4:164-173.

39. Iglesias G: Analysis of the viral elements required in the nuclear import of HIV-1 DNA. *J Virol* 2010, 84:729-739.

40. Iglesias G: Disruption of the central DNA flap is pathogenic in vivo. *J Virol* 2003, 77:1912-1920.

41. Iglesias G: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome. *PLoS Negl Trop Dis* 2011, 5:e1002724.

42. Iglesias G: Implications of lentiviral vector-mediated gene transfer for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.

43. Iglesias G: Lentiviral vectors for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.

44. Iglesias G: Improved lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

45. Iglesias G: Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

46. Iglesias G: Implications of lentiviral vector-mediated gene transfer for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.

47. Iglesias G: Lentiviral vectors for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.

48. Iglesias G: Improved lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

49. Iglesias G: Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

50. Iglesias G: Lentiviral vectors for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.

51. Iglesias G: Improved lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

52. Iglesias G: Lentiviral vectors for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.

53. Iglesias G: Improved lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol Ther* 2002, 6:199-209.

54. Iglesias G: Lentiviral vectors for human gene therapy. *Curr Opin Biotechnol* 2002, 13:1793-1807.
Brussel A, Sango P: Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J Virol 2003, 77:10119-10124.

doi:10.1186/1742-4690-8-92

Cite this article as: Iglesias et al: Residual HIV-1 DNA Flap-independent nuclear import of cPPT/CTS double mutant viruses does not support spreading infection. Retrovirology 2011 8:92.