

# HIV-1 Genome Nuclear Import Is Mediated by a Central DNA Flap

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## Summary

HIV-1 and other lentiviruses have the unique property among retroviruses to replicate in nondividing cells. This property relies on the use of a nuclear import pathway enabling the viral DNA to cross the nuclear membrane of the host cell. In HIV-1 reverse transcription, a central strand displacement event consecutive to central initiation and termination of plus strand synthesis creates a plus strand overlap: the central DNA flap. We show here that the central DNA flap acts as a *cis*-determinant of HIV-1 DNA nuclear import. Wild-type viral linear DNA is almost entirely imported into the nucleus where it integrates or circularizes. In contrast, mutant viral DNA, which lacks the DNA flap, accumulates in infected cells as unintegrated linear DNA, at the vicinity of the nuclear membrane. Consistently, HIV-1 vectors devoid of DNA flap exhibit a strong defect of nuclear import, which can be corrected to wild-type levels by reinsertion of the DNA flap sequence.

## Introduction

Lentiviruses have evolved a nuclear import strategy, which allows their DNA genome to cross the nuclear membrane of a host cell. This active nuclear import of lentiviruses accounts for their unique capacity, among retroviruses, to replicate efficiently in nondividing target cells such as tissue macrophages. The restriction of replication of oncoviruses like MoMLV to dividing cells appears to be due to the requirement for disruption of the nuclear membrane barrier during mitosis, allowing MLV preintegration complexes (PICs) to enter the nucleus (Roe et al., 1993). Mitosis-independent replication of lentiviruses, at the origin of their *in vivo* replication strategy and hence of their pathogenicity, has also enabled the generation of lentiviral gene transfer vectors with promising therapeutic applications (Poznansky et al., 1991; Naldini et al., 1996).

The mitosis-independent replication of lentiviruses was first demonstrated by the productive infection of nonmitotic chondroid cells by the VISNA lentivirus (Thormar, 1963). HIV-1 was also shown to replicate in

differentiated primary macrophages (Gartner et al., 1986). HIV DNA integrates in the chromatin of nonmitotic target cells (Weinberg et al., 1991), implying that HIV-1 PICs are able to cross the nuclear membrane of host cells (Bukrinsky et al., 1992). Thus, mitosis-independent nuclear import is a pivotal event responsible for the ability of HIV and other lentiviruses to replicate in nondividing cells.

The search for the viral determinants responsible for the active nuclear import of the HIV-1 DNA genome has constituted an active but controversial field of investigation. The presence of putative nuclear localization signals (NLS) within the matrix (MA) and Vpr viral proteins has led to the proposition that they could act in a redundant manner in HIV-1 DNA nuclear import (Bukrinsky et al., 1993; Emerman et al., 1994; von Schwedler et al., 1994). It has been proposed that phosphorylation of a small subset (1%) of MA molecules at a C-terminal tyrosine residue is essential to HIV-1 genome nuclear import (Gallay et al., 1995). More recently, putative NLS motifs have also been identified in the integrase protein (IN) (Gallay et al., 1997). However, the contribution of these proteins to the karyophilic properties of HIV-1 PICs is currently a matter of strong debate (Freed and Martin, 1994; Freed et al., 1995, 1997; Fouchier et al., 1997).

We showed previously that HIV-1 has evolved a complex reverse transcription strategy, in which plus strand DNA is synthesized as two discrete half-genomic segments. A central copy of the polypurine tract *cis*-active sequence (cPPT), present in all lentiviral genomes, initiates synthesis of a downstream plus strand. The upstream plus strand segment initiated at the 3' PPT will, after a strand transfer, proceed until the center of the genome and terminate after a discrete strand displacement event (Figure 1B). This last chronological event of HIV-1 reverse transcription is controlled by the central termination sequence (CTS), which ejects HIV-1 reverse transcriptase (RT) at this site in the specific context of strand displacement synthesis (Charneau et al., 1994). Thus, the final product of HIV-1 reverse transcription is a linear DNA molecule bearing in its center a stable 99 nucleotide-long plus strand overlap, here referred to as the central DNA flap. Mutations in the cPPT or CTS *cis*-active sequences severely impair HIV replication and DNA flap formation, suggesting a direct role of the central DNA flap in the retroviral life cycle (Charneau et al., 1992, 1994; Hungnes et al., 1992).

In this study, we establish that the central DNA flap of HIV-1 is involved in viral genome nuclear import. Thus, the reverse-transcribed HIV-1 genome bears a *cis*-acting determinant for its nuclear import. We also show that HIV-1 gene transfer vectors lacking the central DNA flap exhibit a strong nuclear import defect. The insertion of the DNA flap sequence into a previously described HIV-1 vector (Naldini et al., 1996) increases the transduction efficiency by complementing the nuclear import defect of the original vector DNA to wild-type levels. This finding provides additional and independent evidence for the role of the central DNA flap in HIV-1 nuclear

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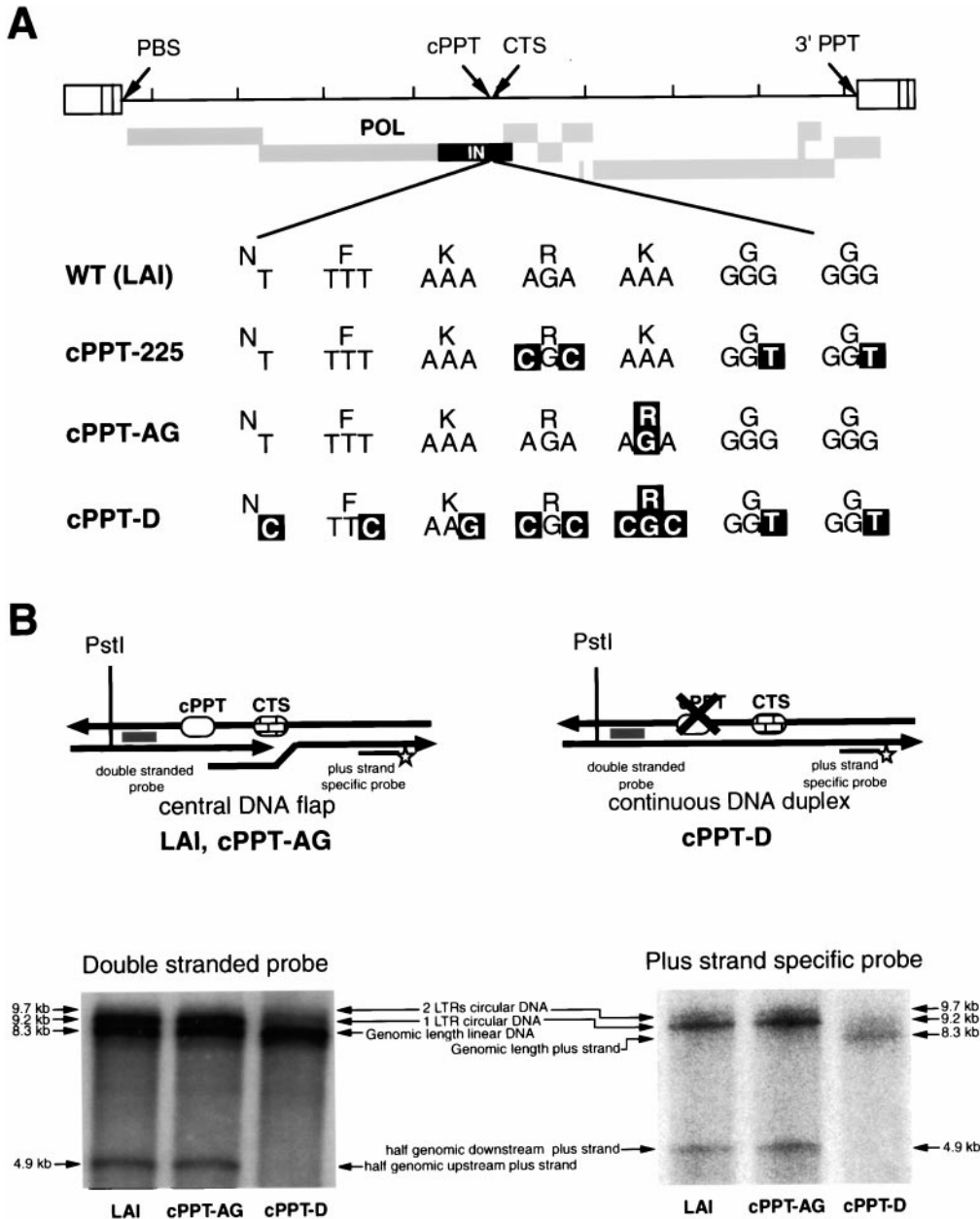


Figure 1. Reverse-Transcribed DNA from a cPPT Mutant Virus Is a Continuous Duplex Linear Molecule, Devoid of the Central DNA Flap

(A) Mutations introduced in the HIV-1 cPPT sequence. Conservative and semi-conservative cPPT mutant viruses were constructed. Conservative mutant cPPT-225 virus contains four mutations respecting the integrase overlapping coding sequence. Semiconservative mutant cPPT-D comprises 10 mutations in the 19-mer cPPT. Mutant cPPT-AG is its control virus in which a single purine-to-purine mutation introduces the same amino acid change in the overlapping integrase coding region. Mutations are shown in reversed type.

(B) Structural analysis of HIV-1 DNA. The structure of reverse transcription products of wild-type, cPPT-AG, and cPPT-D mutant viruses in infected cells was analyzed by alkaline denaturation and hybridization. Probes used were either a double-stranded DNA probe complementary of both minus and upstream plus strands (left panel), or a downstream plus strand-specific oligonucleotide probe (right panel). To distinguish between DNA strands originating from the linear DNA or from one- and two-LTR DNA circles, the viral DNA was digested by PstI, prior to denaturation and electrophoresis. In these conditions, DNA strands originating from one- and two-LTR DNA circles appear as 9.1 kb and 9.7 kb bands, respectively. Genomic or half-genomic length DNA strands from linear DNA appears as 8.3 kb or 4.9 kb bands, respectively.

Plus strand from wild-type and cPPT-AG viruses is entirely synthesized as two half-genomic discrete segments (right panel). In contrast, all detectable minus and plus strands from cPPT-D linear DNA are of genomic length (left and right panels). The final reverse transcription product of wild-type and cPPT-AG viruses is a linear DNA containing a plus strand discontinuity at the center of the genome characteristic of the central DNA flap (Charneau et al., 1994). Reverse transcription product of the cPPT-D virus is a continuous duplex DNA molecule devoid of the central DNA flap.

import. Hence, the distinctive features of lentiviral reverse transcription account for the unique capacity of lentiviruses, among retroviruses, to replicate in nondividing cells.

## Results

### The Final Reverse Transcription Product of a cPPT Mutant Virus Is a Continuous Duplex Linear Molecule, Devoid of the Central DNA Flap

In our previous work, we showed that conservative mutations in the cPPT and CTS sequences severely impaired virus replication and altered the structure of the central DNA flap (Charneau et al., 1992, 1994). In order to disrupt more profoundly the sequence of the cPPT, semiconservative mutations were introduced in the overlapping integrase coding region. In the mutant virus cPPT-D, the lysine-to-arginine change at position 188 allowed the introduction of a total of 10 mutations into the 19 nucleotide sequence of the PPT primer (Figure 1A). In order to check the effect of this amino acid change on the integrase function, a control cPPT-AG mutant virus was constructed in which a single mutation from purine to purine, respecting the polypurine nature of cPPT, induced the same amino acid change.

We analyzed the structure of the reverse-transcribed DNA isolated from MT4 cells infected with wild-type LAI, cPPT-AG, or cPPT-D viruses (Figure 1B). After alkaline denaturation of samples, Southern blot hybridizations were performed using either a double-stranded DNA probe localized in the 5' part of the genome (left panel) or a downstream plus strand-specific oligonucleotide probe (right panel). When hybridization was performed with the double stranded DNA probe, an upstream half-genomic length plus strand was detected in the case of the wild-type and cPPT-AG viruses. In contrast, only genomic length DNA strands were detected in the case of cPPT-D mutant virus. Hybridization with the oligonucleotide revealed that plus strand from both wild-type and cPPT-AG viruses is entirely synthesized as two half-genomic segments. Thus, the single mutation introduced in the cPPT of the cPPT-AG virus does not affect the central initiation of plus strand synthesis. In the case of the cPPT-D mutant virus, only a genomic length plus strand was detected. In this central initiation mutant, synthesis of the downstream plus strand segment initiated at the cPPT is abolished; no strand displacement occurs at the center of the genome. The elongation of the transferred plus strand strong stop initiated at the 3' PPT proceeds all along the genome. The reverse transcription product of a cPPT mutant virus is therefore a continuous duplex linear DNA, lacking the central DNA flap.

### Central Initiation of Reverse Transcription Is an Essential Step of the HIV-1 Replicative Cycle

Infectivity of wild-type LAI, cPPT-AG, and cPPT-D viruses was first evaluated in classical kinetic replication experiments in cell cultures. PHA-stimulated peripheral blood lymphocytes (PBLs) and MT4 cells were infected with equal numbers of viral particles, and reverse transcriptase activity was followed over time in the culture

supernatants (Figure 2A). Growth curves of the wild-type HIV-1 LAI and cPPT-AG control viruses were similar in both cell systems. In contrast, when PBLs were infected with the cPPT-D mutant virus, no replication was detected during the 15 days of culture. The same was true for MT4 cells, despite their high susceptibility to HIV infection.

Virus infectivity was then quantitatively analyzed by titrations based on a single round of replication (Figure 2B) using P4 indicator cells (HeLa CD4 LTR-LacZ) (Charneau et al., 1994). These one-cycle titrations confirmed the almost complete loss of infectivity of the cPPT-D mutant virus. In P4 cells, infectivity of the cPPT-AG control was identical to that of the wild-type virus, whereas infectivity of the cPPT-D mutant was strongly reduced, to levels close to background (Figure 2B, left panel). The same results were obtained in aphidicolin-treated, nondividing P4 cells (Figure 2B, right panel). These results establish that the central initiation of reverse transcription is necessary for HIV replication in nondividing as well as in proliferating cells.

### Mutations in the cPPT Do Not Affect Virus Production, Reverse Transcription Efficiency, nor the Ability of Viral DNA to Integrate In Vitro

We first checked whether the different mutations introduced into the cPPT-AG and cPPT-D plasmid proviruses affect the late steps of the replicative cycle. Virus production was quantified, according to the p24 content of the supernatants, after transient transfections of HeLa cells by proviral plasmids. The production of the cPPT mutant viruses was found to be no significantly different from that of the wild-type LAI virus (Figure 3A). Therefore, as the late stages are normal, the defective step involved in the phenotype of the cPPT-D mutant virus must belong to the early phase of the HIV replicative cycle.

The effect of mutations in cPPT on viral DNA synthesis was then evaluated by quantitating the DNA synthesized in a single round of reverse transcription (Figure 3B). An internal MscI restriction fragment from the viral DNA of infected cells was detected by Southern blot and quantified using the corresponding MscI DNA fragment as a probe. Since the internal MscI fragment is common to the integrated proviral DNA and the unintegrated linear and circular molecules, its quantitation reflects the total amount of viral DNA, irrespective of its integrated or unintegrated state. To limit the analysis to the first cycle of reverse transcription, DNA from infected P4 cells was harvested 12 hr after infection, before initiation of a second round of infection. The total amount of DNA reverse transcribed in a single cycle of reverse transcription was the same after infection with the cPPT-D mutant, the cPPT-AG control, or the wild-type virus. These experiments showed that whereas mutations in cPPT abolish virus replication, they do not affect the rate of DNA synthesis. The replicative defect of cPPT mutant viruses implicates a step subsequent to viral DNA synthesis.

The in vitro integration ability of PICs from wild-type HIV-1 and central initiation mutants was compared (Figure 3C) using a quantitative in vitro integration assay described by Farnet (Farnet and Haseltine, 1990), with minor modifications. Since HIV-1 replication complexes

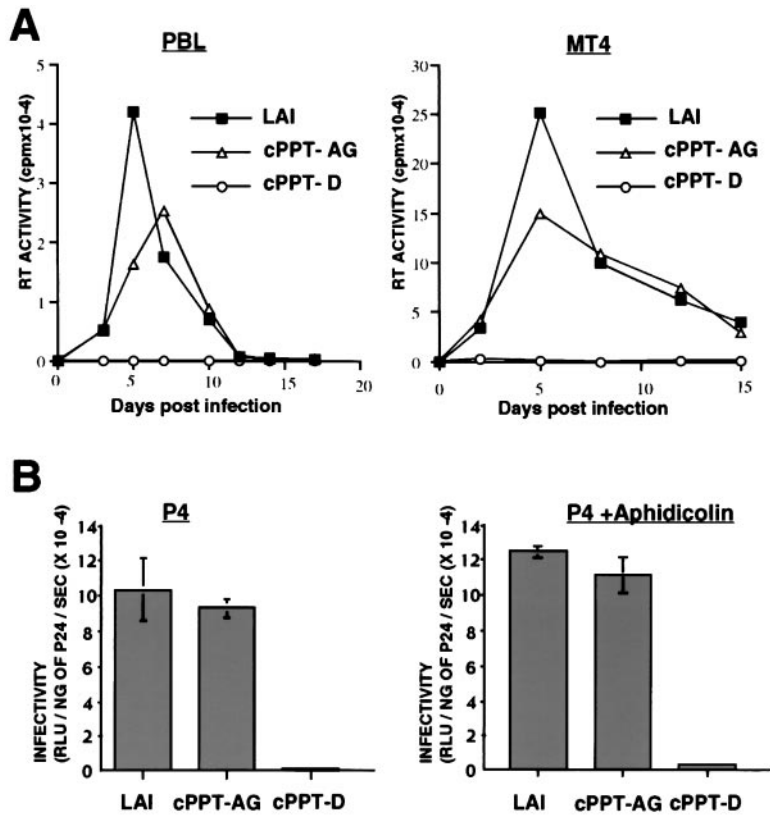


Figure 2. Central Initiation of Reverse Transcription Is an Essential Step of HIV-1 Replicative Cycle

(A) Impact of the mutations in the cPPT on HIV-1 infectivity. Virus replication kinetics on PHA-stimulated PBLs (left panel) and MT4 cells (right panel). Cells were infected with equivalent amounts of viral particles according to the capsid antigen (p24) contents of viral supernatants. Virus production was followed over time by RT activity in cell supernatants.

(B) Single cycle virus titrations in dividing or nondividing aphidicolin-treated P4 cells (HeLa CD4-LTR LacZ).  $\beta$ -galactosidase activity was measured using a chemiluminescent assay. Results are expressed as relative light units (RLU)/s/ng p24 of the inoculum, mean  $\pm$  SD of four independent experiments.

reside only transiently in the cytoplasm of freshly infected cells (Barbosa et al., 1994; this report), the preparation of sufficient amounts of HIV PICs requires massive infection and cellular fractionation within 4 to 6 hr. This was achieved by coculture of H9 cells chronically infected by either wild-type or cPPT-225 virus and uninfected HUT 78 target cells. The cPPT-225 mutant virus (Figure 1A) was chosen for these experiments instead of the noninfectious cPPT-D, which is unable to establish a chronic infection. The residual infectivity of cPPT-225 mutant virus is low but sufficient to allow it to slowly propagate in cell cultures (Charneau et al., 1992). HIV PICs were isolated from the cytoplasm of infected cells and incubated in the presence of a linearized Bluescript plasmid target DNA. Integration was revealed by the presence of a 12.7 kb fragment, reactive to the HIV-1 probe, corresponding to the expected size of the 9.7 kb linear HIV genome integrated into the 3 kb target DNA. The amount of linear DNA integrated into the plasmid DNA did not differ between the wild-type and cPPT 225 mutant virus (Figure 3C). Hence HIV-1 PICs from the cPPT mutant retained their full ability to integrate *in vitro*.

In summary, the defective replication step of central DNA flap mutant viruses must lie after reverse transcription but before integration of the viral genome into the host cell chromatin.

#### Impaired Nuclear Import of Central DNA Flap Mutant Viruses

The foregoing experiments suggested that the replicative defect of central DNA flap mutant viruses was related to the access of HIV PICs to the chromatin of the

target cell. Hence we tested the hypothesis of a nuclear import defect of DNA from cPPT mutant viruses. Studies on the nuclear import of HIV-1 PICs are hampered by lack of a quantitative and reproducible assay for nuclear import at the level of the viral DNA. Once reverse transcribed in the cytoplasm, the retroviral linear DNA is imported into the nucleus where it either integrates or circularizes. Unintegrated retroviral DNA circles, containing one or two long terminal repeats (LTRs), are found exclusively within the nucleus, and thus represent convenient markers of viral DNA nuclear import. To assess HIV DNA nuclear import, previous studies used PCR amplification of unintegrated two-LTR DNA circles. However, as two-LTR circles represent a minute fraction of the HIV DNA in infected cells (Barbosa et al., 1994; this report), their detection is very sensitive to minor alterations of cell physiology or virus infectivity. Therefore, we designed a novel assay, which permits a quantitative follow-up by Southern blot of the synthesis, circularization, and integration of HIV DNA. To restrict the analysis to a single cycle of replication, infections were performed in presence of Saquinavir, an HIV-1 protease inhibitor.

Briefly, DNA from P4-infected cells is prepared at various time points and digested with MscI, a restriction enzyme that cuts the HIV-1 genome twice. Using a PCR-generated DNA probe exactly overlapping the 5' MscI site, several specific bands are revealed (Figure 4A). The internal 1.9 kb MscI fragment is common to all viral DNA species irrespective of their integrated or unintegrated state, and quantitation of this band indicates the total amount of viral DNA in infected cells. A 2.6 kb band

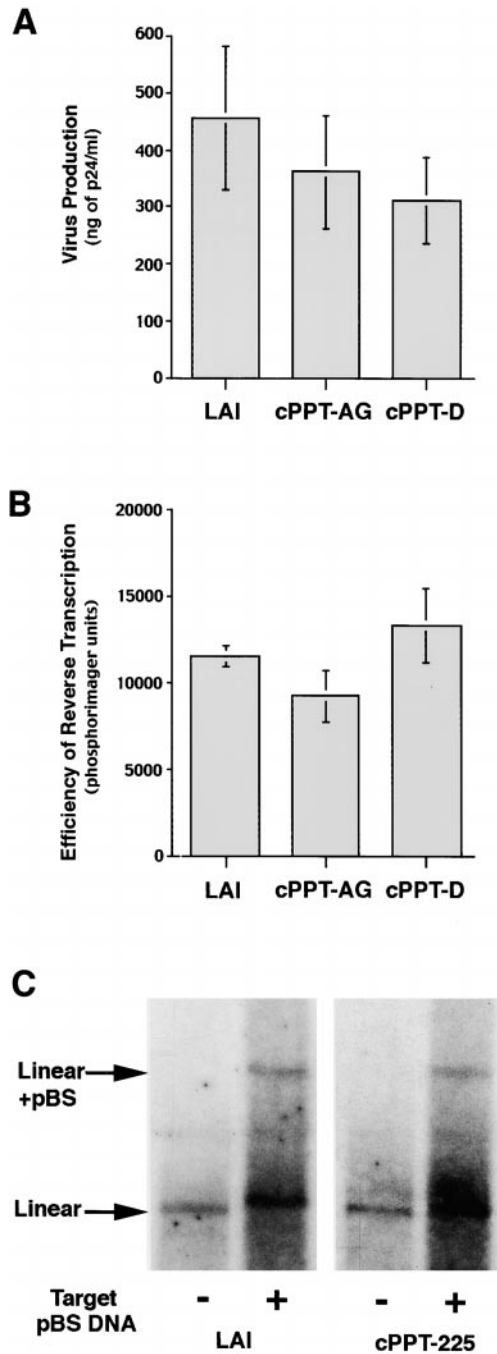


Figure 3. Mutations in the cPPT Do Not Affect Virus Production, Viral DNA Synthesis, nor the Ability of Viral DNA to Integrate In Vitro (A) Effect of mutations in the cPPT on virus production. HeLa cells were transiently transfected with pLAI, pcPPT-AG, or pcPPT-D plasmids. Virus production was measured by quantitation of p24 viral antigen in cell supernatants, 48 hr posttransfection. (B) Effect of mutations in the cPPT on reverse transcription efficiency. P4 cells were infected with the same amounts of viral particles (300 ng of p24 antigen per  $10^6$  cells) and DNA was extracted 12 hr later. Total amounts of reverse-transcribed viral DNA, represented by an internal MscI HIV-1 fragment, were detected by Southern blot using the same DNA fragment as a probe and quantitated using a phosphorimager. (C) Effect of mutations in the cPPT on in vitro integration. MT4 cells were cocultivated with H9-LAI or H9-cPPT-225 chronically infected

corresponds to the distal 5' MscI fragment of unintegrated linear HIV DNA. To minimize transfer bias due to the large size of specific fragments from the DNA circles, the DNA is further cut with XhoI. One- and two-LTR circular DNAs then appears as 2.8 and 3.4 kb bands, respectively. Since the DNA probe exactly overlaps the 5' MscI site, the intensity of each band is directly proportional to the quantity of the corresponding viral DNA species. The amount of integrated proviral DNA is calculated by subtracting from the total amount of viral DNA the signals of unintegrated linear and circular viral DNAs. A parallel quantitation of the same infected cell population was performed after a Hirt fractionation to separate low-molecular weight unintegrated viral DNA from high-molecular weight integrated proviral DNA. This gave rise to identical results, thus validating the one-step subtractive calculation (not shown).

As indicated by the kinetics of accumulation of total viral DNA (1.9 kb internal fragment), the synthesis of viral DNA proceeded for 24 to 48 hr after infection, reflecting an asynchronous infection process. The amounts of total viral DNA from cPPT-AG and cPPT-D mutant viruses were similar to those of wild-type HIV-1 LAI. As previously shown (Figure 3B), the mutations in cPPT did not influence the rate of DNA synthesis. Detectable amounts of full-length unintegrated linear DNA were present in cells as early as 6 hr after infection (Figure 4B). Integrated proviruses and DNA circles were first detected 12 hr after infection. Integration and circularization proceeded to completion over a further 36 hr.

On completion of one cycle of infection, in the case of the wild-type virus, about 55% of the viral DNA had integrated into the host cell DNA, about 35% had circularized into one-LTR circles, and a small fraction of less than 10% remained in the form of stable unintegrated linear DNA (Figure 4C). DNA from the cPPT-AG control virus was processed in a very similar manner to DNA from the wild-type virus. Notably, two-LTR circular DNA, although detectable at 48 hr after infection, was present only in trace amounts.

In the case of the cPPT-D mutant virus, a marked alteration in the pattern of intracellular viral DNA was evident, with a clear and persistent accumulation of unintegrated linear molecules. At 48 hr after infection, only very small amounts of one-LTR circular DNA and integrated proviral DNA had been generated and more than 90% of the cPPT-D mutant DNA remained blocked in the unintegrated linear form (Figure 4C). The same experiment performed with an integration-defective virus gave an inverse intracellular DNA profile: the intranuclear accumulation of integration-defective linear molecules produced four to five times more circular DNA than in the case of the wild-type virus (data not shown). In fact, the only possible intranuclear processing of integration-defective linear viral DNA is its circularization by the homologous recombination and ligation pathways of the host cell.

cells. In vitro integration of viral preintegration complexes, isolated from the cytoplasm of infected cells, was performed as previously described (Farnet and Haseltine, 1990). Each lane is loaded with cytoplasmic DNA from  $2 \times 10^8$  infected cells.

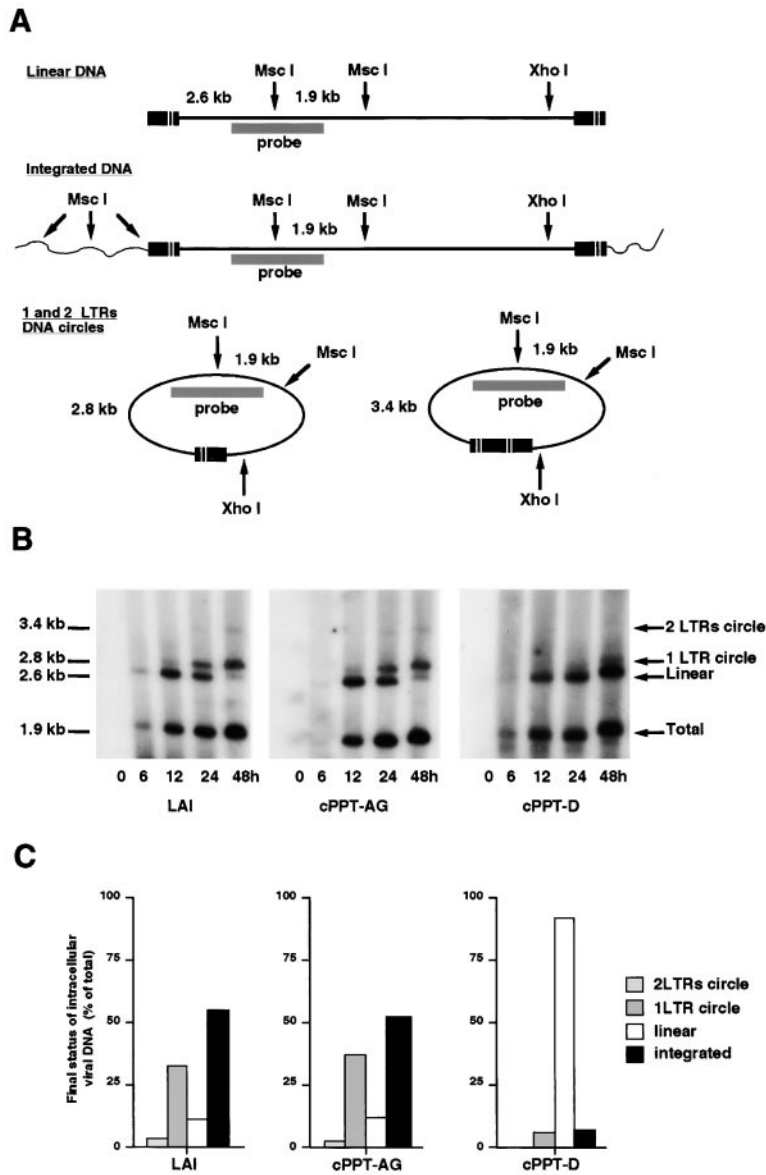


Figure 4. Central DNA Flap Mutant Viruses Are Defective in Nuclear Import of Viral DNA

(A) Strategy for the quantitative follow up of the synthesis, circularization and integration of HIV-1 DNA. DNA from infected cells was extracted at various times postinfection, digested with MscI and XhoI, and analyzed by Southern blot using a probe overlapping the 5' MscI site. The internal 1.9 kb DNA fragment, common to all viral DNA species irrespective of their integrated or unintegrated state, indicates the total amount of viral DNA in infected cells. The 2.6 kb, 2.8 kb, and 3.4 kb signals correspond respectively to unintegrated linear DNA, one-, and two-LTR circular DNAs. Since the PCR-generated probe exactly overlaps the MscI site, the intensity of each band is directly proportional to the amount of the corresponding viral DNA species. Thus, the amount of integrated proviral DNA can be calculated by subtracting from the total amount of viral DNA the signals of unintegrated linear and circular viral DNA.

(B) Southern blot analysis of viral DNA processing in infected cells. P4 cells were infected with equivalent amounts of each virus normalized on the p24 contents of the supernatants. Infected cells were lysed at different times postinfection, DNA was extracted, and used for the quantitative analysis described above.

(C) Intracellular viral DNA profiles, on completion of one cycle of infection (48 hr postinfection). Results are expressed as percentages of total viral DNA. Similar intracellular viral DNA profiles were obtained using MT4 cells (data not shown).

A nuclear import defect is expected to decrease the proportion of nuclear viral DNA species (integrated proviruses and one- and two-LTR circles) and to concomitantly increase the proportion of nontranslocated linear DNA molecules. Thus, the intracellular DNA profile of cPPT-D mutant virus strongly suggests a defect of viral DNA nuclear import.

**Linear DNA from Central DNA Flap Mutant Viruses Accumulates at the Vicinity of the Nuclear Membrane**  
To further characterize the nuclear import defect of central DNA flap mutant viruses, we addressed the question of whether the mutated linear DNA molecules accumulate in a particular subcellular compartment. The nuclear import process can be divided into two main phases, docking of the nuclear component to the nuclear membrane and its translocation through the nuclear pore complex (NPC). We first conducted classical nuclei/cytoplasm fractionation of infected cells, followed by Southern blot detection of viral DNA.

The totality of viral DNA of all viruses was associated with the nuclei of infected P4 cells, 24 hr after infection (Figure 5A). The same analysis was performed at an early time point, using a massive infection provided by the cocultivation between chronically infected H9 cells by the cPPT-225 or by the wild-type virus and uninfected MT4 cells. As early as 6 hr after infection, the vast majority of the detectable linear DNA molecules from both viruses fractionates with the nuclei (Figure 5B). Only a minor fraction of these linear DNA molecules are found in the cytoplasm. This suggested that mutations in the cPPT do not affect the docking process of HIV DNA to the nuclear membrane.

To confirm that central flap-deficient DNA molecules do accumulate at the nuclear membrane, we used fluorescence in situ hybridization (FISH) to directly visualize the intracellular location of HIV genomes. P4 cells were infected at a high multiplicity in one-cycle conditions, hybridized with a full-length HIV-1 genome probe, and observed by deconvolution microscopy. Specific dots

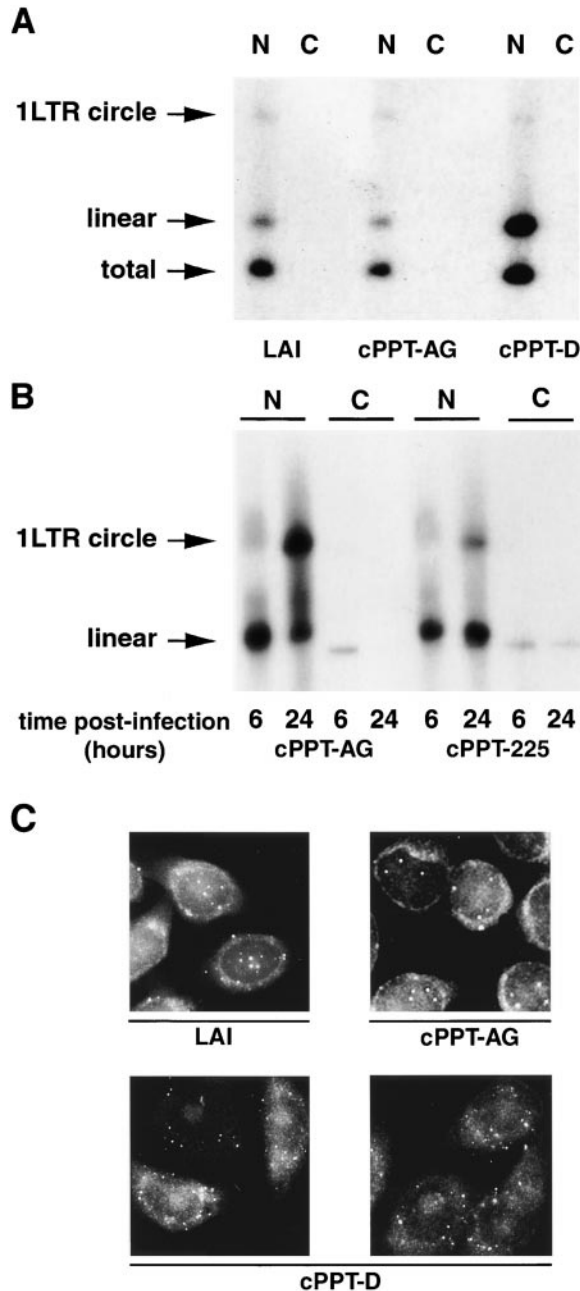


Figure 5. Linear DNA from Central DNA Flap Mutant Viruses Accumulates at the Vicinity of the Nuclear Membrane

(A) Nucleus/cytoplasm fractionation of infected P4 cells. Southern blot analysis of viral DNA from nuclear (N) and cytoplasmic (C) fractions, 24 hr postinfection. Fractionation was based on Triton lysis. DNA was restricted with *MscI* and hybridized using the *MscI* site overlapping probe. (B) Nucleus/cytoplasm fractionation of MT4 cells, cocultivated with HIV-1 LAI or cPPT-225 chronically H9-infected cells, at 6 and 24 hr postinfection. DNA was restricted with *EcoRI* and hybridized using the *MscI* site overlapping probe. (C) Detection of individual HIV-1 genomes by fluorescence in situ hybridization (FISH). P4 cells were infected at high multiplicity ( $2 \mu\text{g}$  of p24 antigen per  $10^6$  cells), and hybridized using a full-length HIV-1 genome probe. Fluorescent signals were amplified by tyramid precipitation. Optical sections through cells were analyzed by deconvolution microscopy.

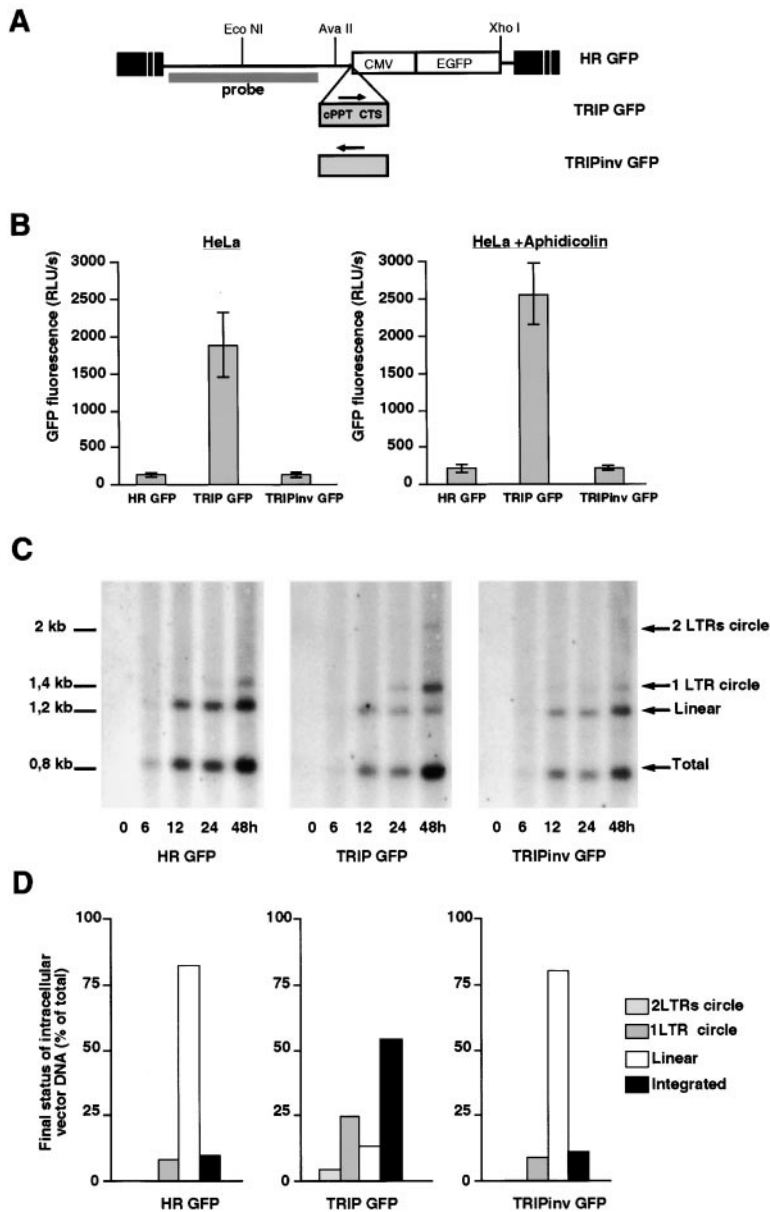
were found predominantly within the nucleus in the case of the wild-type and cPPT-AG control viruses (Figure 5C). Since FISH cannot distinguish between the different HIV DNA species, these intranuclear viral DNA molecules could have been integrated proviruses or unintegrated DNA circles. Some rare genomes were associated with the nuclear membrane and probably represented the residual linear DNA detected by Southern blotting at the same time after infection. In contrast, HIV genomes were predominantly localized at the nuclear membrane and almost completely absent from the nucleus in the case of the cPPT-D mutant virus. As the Southern blot DNA profile indicated that practically all cPPT-D DNA was blocked in the linear form, we can assume that these HIV genomes associated with the nuclear membrane were unintegrated linear DNA molecules. This direct visualization of viral DNA molecules in infected cells confirmed the association of the viral DNA of central flap mutant viruses with the nuclear membrane.

Altogether, we may conclude from these results that the central DNA flap of HIV-1, created by central initiation and termination steps during reverse transcription, is necessary for HIV-1 PICs to enter the host cell nucleus. In the absence of DNA flap, viral DNA nuclear import is severely impaired at a stage immediately preceding or during the translocation of HIV-1 DNA through the nuclear pore.

#### Impact of the Central DNA Flap on Gene Transduction by an HIV-1-Based Vector

Having identified the central DNA flap as a key determinant for the nuclear import of HIV-1 genome, we tested the effect of inserting the central *cis*-active sequences of HIV-1 into the previously described HR HIV-1 vector (Naldini et al., 1996) (Figure 6A). To monitor gene transduction, a gene encoding the green fluorescent protein (GFP) was further inserted. The vector containing the triple-stranded DNA flap was called TRIP GFP. Controls included similar constructs with mutated cPPT or CTS and a wild-type central region inserted in reverse, non-functional orientation (TRIPinv GFP). The presence of a DNA flap in reverse-transcribed TRIP GFP vector DNA, and its absence from HR GFP and TRIPinv GFP DNA was confirmed by alkaline denaturation and Southern blot hybridization (data not shown).

The number of vector particles produced by transient transfection was normalized prior to transduction according to the levels of capsid protein (p24), reverse transcriptase activity and the quantity of genomic vector RNA in transfected cell supernatants. Similar productions of the various vectors were obtained, with a linear correlation between the three normalization criteria (data not shown). Hence insertion of the central region of the HIV-1 genome into the HR vector did not influence the rate of genomic RNA encapsidation. Dividing or non-dividing aphidicolin-treated HeLa cells were transduced with HR-GFP or TRIP-GFP vector particles. GFP expression was monitored 48 hr later by fluorescence quantitation. Pseudo-transduction of GFP activity was calculated from the transduction of cells treated with an HIV-1 RT inhibitor ( $1 \mu\text{M}$  Nevirapine, Boehringer Ingelheim) and this background subtracted from the fluorescence



**Figure 6.** The Insertion of the Central DNA Flap in an HIV-1-Based Vector Enhances GFP Transduction and Nuclear Import of the Vector DNA Genome

(A) Schematic representation of vector genomes. cPPT and CTS central *cis*-active sequences of the HIV-1 genome, responsible for the formation of the DNA flap during reverse transcription, were inserted in a central position in the previously described HR GFP vector (Naldini et al., 1996). TRIPinv-GFP includes the central DNA flap sequence in the reverse, nonfunctional orientation.

(B) Comparative efficiency of GFP transduction using HIV vectors including or not a central DNA flap. Dividing or nondividing (aphidicolin-treated) HeLa cells were used as targets. GFP fluorescence was quantitated 48 hr posttransduction using a microplate fluorimeter. Results are expressed as the mean  $\pm$  SD of a representative experiment performed in triplicate. Pseudo-transduction of GFP activity was subtracted from the signal.

(C) Southern blot analysis of vector DNA processing in transduced cells. Transduced HeLa cells were lysed at different times postinfection, DNA was extracted, restricted, and Southern blotted using a similar strategy as for the viruses. MscI digestion is replaced by EcoNI and AvaII, and the probe is a PCR DNA fragment overlapping exactly the EcoNI site. (D) Quantitative analysis of vector DNA intracellular status, 48 hr posttransduction. Results are presented as percentages of total vector DNA.

signal. Under these conditions, the presence of the DNA flap sequence in the HIV vector increased GFP transduction in HeLa cells by more than 10-fold (Figure 6B). A similar enhancement of gene transduction was observed in other target cell lines such as MT4 or 293T (data not shown). This effect was lost if the DNA flap sequence was inserted in the reverse orientation (Figure 6B) or mutated in the cPPT (not shown).

#### The Presence of a DNA Flap in HIV-1 Vectors Increases the Rate of Vector Genome Nuclear Import to Wild-Type Levels

It was then of interest to determine whether the increase in GFP fluorescence induced by insertion of a DNA flap sequence in the HIV vector was due to its effect on the nuclear import of vector DNA. To address this question, we adapted our quantitative Southern blot assay for

intracellular viral DNA to the vector system. DNA from vector-transduced cells was digested with EcoNI and AvaII to produce an internal 0.8 kb fragment, and with XhoI. Using a PCR-generated DNA probe exactly overlapping the EcoNI site, signals specific for the unintegrated linear vector genome, and for one- and two-LTR DNA circles were expected at 1.2 kb, 1.4 kb, and 2 kb, respectively. The processing of vector DNA was analyzed at various time points after transduction of HeLa cells.

The total quantity of vector DNA synthesized in transduced cells was comparable for vectors containing the DNA flap or not (Figure 6C). Once again, insertion of the cPPT and CTS sequences, in either orientation, into the HR vector did not influence the rate of reverse transcription of its genome. Interestingly, we found the intracellular fate of DNA from the HR-GFP and TRIPinv-GFP



vectors (Figure 6D) to closely resemble that of DNA from the central flap-defective virus (cPPT-D). The fate of vector DNA from TRIP-GFP followed that of DNA from the wild-type HIV-1 LAI virus (Figure 4C).

A defect of DNA nuclear import was evident in the case of the HR-GFP and TRIPinv-GFP vectors. The intracellular fate of DNA from these vectors was characterized by a strong accumulation of unintegrated linear molecules, together with small amounts of integrated provirus and one- and two-LTR circles. On completion of the processing of vector DNA in transduced cells, 70% to 80% of the DNA from HR-GFP and TRIPinv-GFP constructs remained in the form of unintegrated linear molecules. Only 10% to 15% was present as unintegrated one-LTR circles and 5% to 10% as integrated proviruses. This low but detectable amount of integrated vector DNA would account for the gene transduction obtained using HR-GFP or TRIPinv-GFP vectors.

This quantitative assay also showed that insertion of the DNA flap sequence into the HR vector in the correct orientation complemented its nuclear import deficiency to wild-type levels. The final state of TRIP-GFP DNA in transduced cells was similar to that observed with wild-type HIV-1 virus: 50% or more of the vector DNA integrated the chromatin of the target cell, an important fraction circularized and a few molecules remained as unintegrated linear DNA (compare Figures 6D and 4C). Of note, insertion of the DNA flap sequence into the HR vector did not influence GFP expression at the transcriptional level. This was checked by transfection of HeLa cells with pHR-GFP, pTRIP-GFP, and pTRIPinv-GFP plasmids and fluorescence quantitation (data not shown).

It may be inferred from these results that the increase in GFP transduction obtained with the TRIP-GFP vector is entirely imputable to strong stimulation of its nuclear import by the presence of the central DNA flap. The insertion of the same HIV DNA fragment encompassing the cPPT and CTS but present as a double-stranded DNA in TRIPinv-GFP vector genome did not influence its nuclear import. This finding again emphasizes the crucial role of the HIV DNA flap in the nuclear import of viral and vector DNA.

## Discussion

This work evidences an original mechanism of HIV-1 nuclear import with a crucial role of a three-stranded DNA structure, the central DNA flap, in this process. HIV-1 has evolved a complex reverse transcription strategy, whereby a central strand displacement event, consecutive to the central initiation and termination of reverse transcription, creates a DNA flap at the center of unintegrated linear HIV-1 DNA molecules. This DNA flap acts in turn as a *cis*-active determinant of the nuclear import of the HIV-1 genome. Thus, the central initiation and termination, two distinctive steps of HIV-1 reverse transcription, account for the capacity of HIV-1 to infect nondividing target cells.

We show here that lack of the DNA flap leads to a virus that is almost noninfectious in dividing or nondividing cells. Although mutations in cPPT did not affect the rate of synthesis of viral DNA or its ability to integrate *in vitro*, most of the reverse-transcribed DNA molecules

from the cPPT mutant virus accumulate over time as unintegrated linear DNA (Figure 7A). In contrast, linear DNA from the wild-type virus was almost fully processed into integrated proviruses and DNA circles. The intracellular DNA profile of cPPT mutant viruses pointed to a defect of nuclear import, the viral DNA accumulating as linear molecules as a consequence of its lack of access to the nuclear compartment where it could integrate or circularize. A late defect of viral DNA import, most probably affecting translocation through the NPC, was demonstrated by fractionation of infected cells and direct visualization (FISH) of intracellular viral DNA. The flap-defective linear DNA molecules were found to associate with the nuclear membrane.

In this report, we focused our analysis on cPPT mutant viruses, which are characterized by the absence of a central DNA flap. Most of the experiments presented here were also conducted with our previously described CTS mutant virus (Charneau et al., 1994), with the same results (data not shown). In the CTS mutant virus, reverse transcription produces linear DNA molecules containing larger, randomly distributed plus strand overlaps, as compared to the discrete central DNA flap of the wild-type virus. Thus, not only the presence of the DNA flap but also its structural integrity is necessary for the nuclear import of HIV DNA.

The DNA flap is also operative in the context of an HIV-1-based vector system. Its insertion into a vector devoid of DNA flap reverts a strong defect of nuclear import of the vector DNA to wild-type levels of nuclear import.

### The Central DNA Flap Is a Common Nuclear Import Determinant of Lentiviruses

The location of the central DNA flap has been precisely defined in the case of HIV-1. Central strand displacement starts at the first nucleotide following the cPPT sequence (Charneau and Clavel, 1991) and stops in general 99 nucleotides downstream, at the *ter2* site of the CTS sequence (Charneau et al., 1994). The three-dimensional configuration of the three DNA strands of the flap is as yet unknown. Nevertheless, the presence of a DNA flap at the center of the genome can probably be generalized to all lentiviruses. A central copy of PPT is a common feature of all lentiviral genomes and a putative CTS terminator element, revealed by the presence of (A)<sub>n</sub> and (T)<sub>n</sub> tracts, also exists approximately 100 nucleotides downstream (Charneau et al., 1994). The central DNA flap of the ungulate lentivirus EIAV has been characterized recently (Stetor et al., 1999). A central strand discontinuity in VISNA virus DNA, referred to as a gap but most probably a nick resulting from the central strand displacement, was revealed by S1 nuclease cleavage (Harris et al., 1981).

Since mitosis-independent replication has been described for most lentiviruses, we assume that the role of the DNA flap in nuclear import described here for HIV-1 will be generalized to all lentiviruses.

### Mechanistic Hypothesis for the Role of the Central DNA Flap in HIV-1 Nuclear Import

A three-stranded DNA structure acting as a *cis*-determinant of nuclear import is a novel biological phenomenon

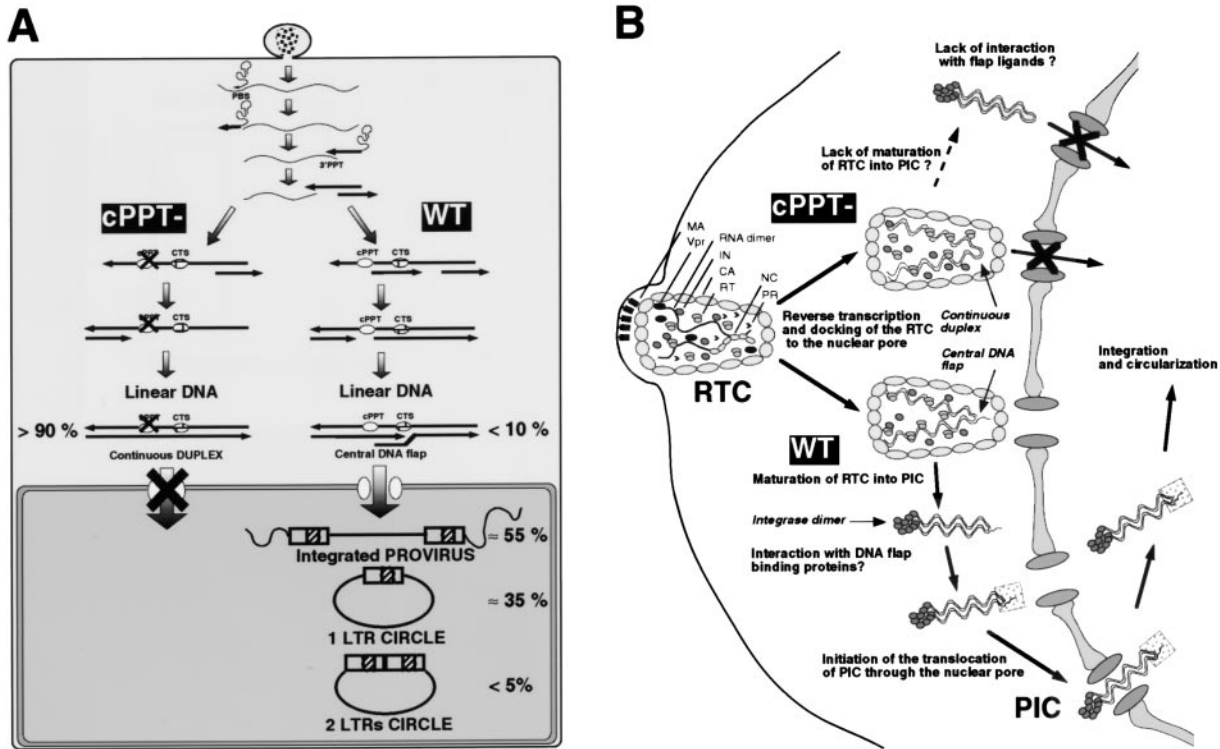


Figure 7. A Model for DNA Flap-Dependent HIV-1 Genome Nuclear Import

(A) Overview of the observed phenotype of central DNA flap mutant viruses. Central initiation and termination steps of HIV-1 reverse transcription create a long DNA flap structure. HIV-1 plus strand is synthesized as two discrete half-genomic segments. A downstream segment is initiated at a central copy of the polypurine tract sequence (cPPT). The upstream segment terminates downstream of the cPPT, after a 99 nucleotide-long strand displacement event, blocked by the central termination sequence (CTS). At completion of a single cycle of infection, viral DNA from wild-type virus is almost fully processed into integrated provirus (=55%), 1-LTR (=35%), and 2-LTR circular DNA (<5%), while a small fraction remains as linear DNA (<10%). The reverse transcription product of a central initiation mutant virus is a continuous double-stranded linear DNA lacking the central DNA flap. Viral DNA from cPPT-D mutant virus accumulates in infected cells as linear nontranslocated DNA molecules and localizes at the vicinity of the nuclear membrane.

(B) Maturation of the reverse transcription complex (RTC) into a preintegration complex (PIC) and linear DNA translocation through the nuclear pore as two speculative mechanisms for flap-dependent HIV-1 genome nuclear import (see Discussion).

with no known cellular or viral counterparts. Any hypothesis of a molecular mechanism describing the role of the central DNA flap in HIV nuclear import is therefore speculative. The central DNA flap could act as a viral determinant for initiation of the crawling of the HIV DNA filament through the nuclear pore. This could be achieved through direct interaction of the DNA flap with components of the pore, or alternatively through interaction with cellular or viral proteins that shuttle between the cytoplasm and the nucleus of the host cell and could drag the HIV genome into the nucleus. Translocation of the 9.7 kb HIV genome through a nuclear pore of maximum diameter of 26 nm must occur in a specific orientation, after recognition of one extremity of the HIV-1 DNA filament to initiate the translocation. A similar situation arises in the nuclear export of messenger RNA, where export of the RNA filament through the pore is guided by the 5' cap structure (Hamm and Mattaj, 1990).

Although the conformation of HIV-1 PICs is not well known, it has been established that the extremities of the linear DNA are bridged together, probably after dimerization of the integrase proteins bound at the tips of the LTRs (Miller et al., 1997). Interestingly, the cPPT and CTS *cis*-active sequences are found at a central

position in all lentiviral genomes. One logical structure for lentiviral PICs would be a double DNA filament, symmetrically folded on either side of the central flap by the integrase dimerization (Figure 7B). The central DNA flap would then constitute one apex of a filamentous HIV-1 PIC and the integrase dimer the opposite apex. In cPPT mutant PICs, the absence of a DNA flap would lead to their lack of recognition by the nuclear pore machinery or the shuttling proteins.

Another hypothesis to explain the properties of flap mutant viruses would involve a defect in the maturation of HIV capsids into PICs, prior the translocation of viral DNA into the nucleus. According to this model, flap-defective viral DNA would remain trapped in integral viral capsids, unable to translocate. Retroviral reverse transcription does not take place at high dilution of the viral components in the cytoplasm of infected cells, but requires the structural environment of a reverse transcription complex where these components are confined in a capsid protein assembly. The HIV capsid size exceeds the maximum exclusion diameter of a nuclear pore. Therefore, before viral DNA can enter the nucleus, the HIV reverse transcription complexes must undergo maturation into PICs of size compatible with transloca-

tion through the nuclear pores (Karageorgos et al., 1993). The maturation of viral capsids prior to nuclear translocation is well established in several other viral systems in which the replicative cycle involves translocation of the DNA genome through the nuclear membrane of the host cell (Whittaker and Helenius, 1998). The HIV reverse transcription complex contains numerous copies of RT polymerase (about 30 to 50 per capsid). Owing to the important distributivity of HIV-1 reverse transcriptase, a high stoichiometry of enzyme to viral RNA template is necessary to overcome a number of limiting steps of reverse transcription such as strand transfers or polymerization pauses during plus and minus strand synthesis, and accurate formation of the central DNA flap (Klarman et al., 1993; Charneau et al., 1994). This strongly suggests that central termination, the last event of lentiviral reverse transcription, occurs within an integral capsid structure. Central termination, which marks the end of viral DNA synthesis, could be a required signal for viral DNA decapsidation and its subsequent translocation into the nucleus (Figure 7B).

These two putative molecular mechanisms for the DNA flap-mediated nuclear import of HIV-1 are not mutually exclusive. The formation of a central DNA flap could trigger the maturation of viral capsids into PICs, thus making the DNA flap accessible to shuttling proteins. In any case, the fact that the integrity of the central DNA flap is required for entry of the HIV-1 genome into the host cell nucleus implies that the entire process of DNA synthesis, including the last central strand displacement event, is completed prior to translocation of the HIV PIC through the nuclear pore.

#### Cell Mitosis Does Not Provide an Alternative Pathway for the Entry of Flap-Defective Viral DNA into the Host Cell Nucleus

The present work shows that central flap mutant viruses are strongly hampered in their replication capacity not only in nondividing but also in dividing target cells. Conversely, insertion of a DNA flap sequence in an HIV-1-based vector stimulates gene transduction in both dividing and nondividing cells. This differs from the published phenotype of MA/Vpr mutant viruses, where a replication defect has been described exclusively in nondividing cells (Bukrinsky et al., 1993; Heinzinger et al., 1994; von Schwedler et al., 1994). Of note, there is as yet no direct experimental evidence for a mitosis-dependent nuclear import pathway of lentiviral DNA genomes. As the published phenotype of MA/Vpr mutant viruses must be viewed with some caution, the same caution must be applied to the starting hypothesis that a nuclear import deficiency in lentiviruses should lead to a replication defect exclusively in nondividing cells. Whether lentiviruses can adopt a mitosis-dependent nuclear import strategy, or whether the active nuclear import of lentiviral genomes occurs in both dividing and nondividing cells, remains an open question.

#### Implications for the Design of Lentiviral Vectors

Since the infection of nondividing target cells by lentiviruses relies on their use of an active nuclear import pathway, it is essential to maintain the lentiviral nuclear

import determinants in derived vector constructs. Classical retroviral vector constructs are replacement vectors in which the entire viral coding sequences between the LTRs are deleted and replaced by the sequences of interest. In the case of lentiviral vectors, this classical strategy leads to the deletion of the cPPT and CTS central *cis*-active sequences. The crucial role of the central DNA flap in HIV nuclear import implies that such replacement vector constructs are not optimal. Consistently, insertion of the HIV DNA flap into the HR GFP replacement vector (Naldini et al., 1996) enhanced its gene transduction efficiency by complementing a nuclear import defect of the HR vector genome to a rate of DNA import close to that of wild-type HIV-1.

It is noteworthy that while HIV vectors lacking a DNA flap were still capable of gene transduction (Figure 6B), the residual replication of viruses mutated in cPPT is absent or extremely low (Figures 2B and 2C). One possible explanation is that flap-independent nuclear import could occur in the case of a small vector genome (about 4 kb for HR-GFP), but the presence of a DNA flap would be required for the import of the 9.7 kb native HIV-1 genome. In fact, the active if relatively inefficient nuclear import of DNA molecules as large as 3 to 4 kb has been reported (Hagstrom et al., 1997).

The *cis*-active sequences responsible for formation of the DNA flap are found at the center of all lentiviral genomes. This central position could have evolved on account of its structural implications for the conformation of PICs, in so far as symmetrical folding of the left and right arms of the linear DNA molecule around the flap might be necessary for its efficient translocation through the NPCs (Figure 7B). If this is true for the virus, then a central position of the DNA flap might also be required for the efficient nuclear import of vector genomes.

#### Experimental Procedures

##### Cells

MT4 cells are HTLV-1 transformed human CD4<sup>+</sup> T cells that allow acute cytopathic HIV-1 infection. H9 cells are less permissive to HIV but allow chronic production after infection. MT4 H9 and HUT78 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Peripheral blood lymphocytes (PBLs) were obtained from healthy donors, stimulated with 1  $\mu$ g/ml of phytohemagglutinin (Wellcome), and maintained in the presence of Interleukin-2 (10% lymphocult; Biotest Diagnostics). 293T cells were grown in DMEM medium supplemented with 10% FCS. P4 indicator cells are HeLa CD4<sup>+</sup> cells carrying the *lacZ* gene under the control of the HIV-1 LTR (Charneau et al., 1994). P4 cells are grown in DMEM medium supplemented with 10% FCS and 500  $\mu$ g/ml G418.

##### DNA Constructs

Proviral plasmids: site directed mutagenesis was performed in M13mp18 carrying an EcoRI 1.1 kb insert (4684 to 5779) from the infectious molecular clone pLAI3. Mutagenic primers were used as follows: cPPT-AG, 5' pCAATTTTAAAAGAAGAGGGGGGATT 3'; cPPT-D, 5' pATTCATCCACAACCTCAAGCGCCGCGGTGGTATTGGGGGTAC 3'; pcPPT-AG, pcPPT-D, pcPPT-225, and pCTS were constructed by cloning back the mutated EcoRI fragment into pLAI3.

Vector plasmids: they derived from HR' CMVLacZ (Naldini et al., 1996). The EGFP gene (Clontech) replaced LacZ reporter gene. EGFP gene was amplified by PCR using Pfu polymerase (Stratagene) from pEGFP-N1 plasmid, adding BamHI and XhoI restriction sites in 5' and 3', respectively. PCR primers were as follows: Bam GFP,

5' CC GGATCC CCA CCG GTC GCC ACC 3'; Xho GFP, 5' CC CTCGAG CTA GAG TCG CGG CCG 3'.

HR GFP vector was constructed by cloning back this PCR fragment into BamHI and XhoI sites of pHR'CMVLacZ, replacing the LacZ ORF by EGFP.

A 178 bp fragment of pLAI3 (4793 to 4971), encompassing cPPT and CTS, was amplified by PCR. NarI restriction sites were added in 5' of the primers in the aim to insert this fragment into the unique ClaI site of HR GFP: Nar TRIP<sup>+</sup>, 5' GTC GTC GGCGCC GAATTC ACA AAT GGC AGT ATT CAT CC 3'; Nar TRIP<sup>-</sup>, 5' GTC GTC GGCGCC CCA AAG TGG ATC TCT GCT GTC C 3'.

Insertion of this flap sequence in the correct orientation gave rise to the TRIP GFP plasmid vector, and TRIPinv GFP in the reverse orientation. Alternatively, the same flap fragment was amplified from pcPPT-AG, pcPPT-D, pcPPT-225, and pCTS plasmids to generate vectors including the same mutations in the cPPT or in the CTS as the corresponding viruses.

### Virus and Vector Production

Viruses were produced by transient transfection of HeLa cells by the calcium phosphate coprecipitation technique. Vector particles were produced by transient cotransfection of 293T by the vector plasmid, an encapsidation plasmid (p8.2) and a VSV envelope expression plasmid (pHCMV-G; Yee et al., 1994), as previously described (Naldini et al., 1996). Nonspecific fluorescence due to secondary transfection caused by calcium/phosphate DNA coprecipitate in the vector supernatants was eliminated by treating the vector stocks with DNaseI (1 µg/ml in the presence of 1 µM MgCl<sub>2</sub>) for 15 min at 37°C, prior to ultracentrifugation.

### Structural Analysis of HIV-1 DNA Reverse

#### Transcription Products

MT4 cells were infected at high multiplicity (600 ng of p24 antigen per 10<sup>6</sup> cells). Low molecular weight DNA was extracted from infected cells 20 hr postinfection by Hirt fractionation. DNA was digested with PstI, precipitated, and resuspended in water. Samples were alkaline denatured in the presence of 0.4 N NaOH. DNA was then subject to electrophoresis in a 0.7% agarose gel, and analyzed by Southern blot. Probes used were either a double-stranded HIV-1 DNA fragment (from the position 1818 to 3498 of LAI genome, see below) hybridizing both minus and the upstream plus strands, or an oligonucleotide complementary to the downstream plus strand of the following sequence: D-probe, CATTGGTAGCTGCTGATTG CTACTTGTGATTGCTCC.

### Virus and Vector Titrations

One-cycle titration of viruses was performed in triplicate by infection of P4 cells plated in 96-well plates, with equivalent amounts of particles (1 ng of p24 viral antigen per well), in the presence of 20 µg/ml of DEAE-dextran. The protease inhibitor Saquinavir (Roche) was added at 1 µM throughout the experiment, to restrict the analysis to a single cycle of infection. Cell mitosis was inhibited by aphidicolin treatment (8 µM), from the day prior to infection. The β-galactosidase activity was measured 48 hr after infection using a chemiluminescent β-gal reporter gene assay (Boehringer).

HeLa cells were infected in triplicate with equivalent amount of vector particles (5 ng p24 per well). At 48 hr posttransduction, medium was replaced by 200 µl of TNB (Tris 50 mM, pH 7.5, NaCl 150 mM) and fluorescence of living cells quantitated using a microplate fluorimeter (Victor<sup>2</sup>, Wallac).

### Viral and Vector Intracellular DNA Profile Analysis

P4 or MT4 cells were infected at high multiplicity by viruses (150 ng of p24 per 10<sup>6</sup> cells, in the presence of 1 µM of Saquinavir) or transduced by vectors (25 ng of p24 per 10<sup>6</sup> cells), in the presence of 20 µg/ml of DEAE-dextran in the case of P4 cells. DNA from infected or transduced cells was extracted at various times, restricted, and analyzed by Southern blot. In all cases, contaminating bacterial plasmid DNA was removed from the analysis by DpnI digestion. DNA from infected cells was digested by MscI and XhoI and DNA from transduced cells by EcoNI, Avall, and XhoI. After electrophoresis and transfer of 10 µg of digested DNA, membranes

were hybridized with random primed <sup>32</sup>P-labeled DNA probes (Redi-prime II, Amersham).

Virus-specific DNA probe was amplified by PCR from pLAI3 plasmid template using the following primers: 5Msc, 5' AGA AGA AAT GAT GAC AGC ATG 3'; 3Msc, 5' TGC CAG TTC TAG CTC TG 3'.

The resulting 1680 bp DNA fragment (from positions 1818 to 3498 of pLAI3) overlaps the MscI restriction site at position 2655 of viral genomes.

Vector probe was synthesized by PCR on pTRIP GFP using the following primers: 5EcoNI, 5'CAG GGA CTT GAA AGC GAA AG 3'; 3EcoNI, 5'GCT TGT GTA ATT GTT AAT TTC TCT GTC 3'.

The vector probe is a 1027 bp fragment (from positions 649 to 1676 of pTRIP GFP) and overlaps the EcoNI site at position 1156 of vector genomes.

To assay the amount of reverse-transcribed DNA from wild-type and cPPT-AG and cPPT-D viruses, we followed a similar protocol except that the DNA extracted at 12 hr postinfection was restricted by MscI and DpnI. The probe used for hybridization was the MscI 1.9 kb internal fragment from pLAI3. Hybridization signals were quantified using a phosphorimager (Molecular Dynamics) and the ImageQuant software.

### In Situ Hybridization

P4 cells were infected at a high multiplicity (2 µg of p24 antigen of each virus per 10<sup>6</sup> cells), in the presence of 20 µg/ml DEAE dextran and Saquinavir was added at 1 µM throughout the experiment. At 24 hr postinfection cells were trypsinized, extensively washed (in order to remove viral particles associated to the plasma membrane), and replated on glass cover slides in 24-well plates. Cells were grown for a further 48 hr and fixed in 4% PFA/PBS for 20 min at room temperature. Cells were washed in PBS and permeabilized by 0.5% Triton/0.5% Saponin in PBS, for 5 min at room temperature. Dehydrated samples were treated by RNase A (200 µg/ml in 2× SSC), 1 hr at 37°C and by proteinase K (6 µg/ml in PBS), about 5 min. Samples were denatured by incubation in 70% deionized formamide/2× SSC 2 min at 70°C followed by 30% deionized formamide/2× SSC 2 min at 70°C. Hybridizations were performed overnight at 37°C using a nick-translated biotinylated pLAI3 plasmid (50% deionized formamide, 10% dextran sulfate, 10 µg/ml salmon sperm DNA, 0.1% Tween 20 in 2× SSC). Samples were extensively washed (serial washing in 2× SSC/50% formamide at room temperature and then at 50°C). Detection of hybridized probes was performed using the Tyramid-Streptavidin TSA-Direct kit (NEN) according to the manufacturer's instructions.

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