The HIV-1 Central Polypurine Tract Functions as a Second Line of Defense against APOBEC3G/F[∇]

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HIV-1 and certain other retroviruses initiate plus-strand synthesis in the center of the genome as well as at the standard retroviral 3' polypurine tract. This peculiarity of reverse transcription results in a central DNA “flap” structure that has been of controversial functional significance. We mutated both HIV-1 flap-generating elements, the central polypurine tract (cPPT) and the central termination sequence (CTS). To avoid an ambiguity of previous studies, we did so without affecting integrase coding. DNA flap formation was disrupted but single-cycle infection was unaffected in all target cells tested, regardless of cell cycle status. Spreading HIV-1 infection was also normal in most T cell lines, and flap mutant viruses replicated equivalently to the wild type in nondividing cells, including macrophages. However, spreading infection of flap mutant HIV-1 was impaired in non-*vif*-permissive cells (HuT78, H9, and primary human peripheral blood mononuclear cells [PBMCs]), suggesting APOBEC3G (A3G) restriction. Single-cycle infections confirmed that *vif*-intact flap mutant HIV-1 is restricted by producer cell A3G/F. Combining the Δvif and cPPT-CTS mutations increased A3G restriction synergistically. Moreover, RNA interference knockdown of A3G in HuT78 cells released the block to flap mutant HIV-1 replication. Flap mutant HIV-1 also accrued markedly increased A3G-mediated G→A hypermutation compared to that of wild-type HIV-1 (a full log₁₀ in the 0.36 kb downstream of the mutant cPPT). We suggest that the triple-stranded DNA structure, the flap, is not the consequential outcome. The salient functional feature is central plus-strand initiation, which functions as a second line of defense against single-stranded DNA editing by A3 proteins that survive producer cell degradation by *Vif*.

Retroviral reverse transcription begins when a host cell tRNA is used to prime the synthesis of a short segment of minus-strand cDNA. Following the first strand transfer and elongation of the minus strand, the primer for subsequent plus-strand DNA synthesis is a short purine-rich remnant of the viral RNA, the polypurine tract (PPT), which is located immediately upstream (U+) of the U3 element (52, 61). Lentiviruses and some spumaviruses also initiate plus-strand synthesis at a second, central PPT (cPPT), which for lentiviruses is located nearly precisely in the center of the genome, in the integrase open reading frame (ORF) (8, 12, 13, 27, 62, 66, 71). A similar central initiation has been observed in the *Saccharomyces cerevisiae* retrotransposon Ty1 (29). When the U+ half of the lentiviral plus strand is synthesized following the second strand transfer, DNA synthesis proceeds past the cPPT, displacing the centrally initiated downstream (D+) plus strand for approximately 90 to 100 nucleotides (nt) until synthesis stops at the central termination sequence (CTS) (11, 13, 62, 71). The outcome is a triple-stranded “central DNA flap” in which the redundant 0.1 kb of the D+ strand is unpaired. The central DNA flap can be detected by Southern blotting as a plus-strand discontinuity in the preintegration viral cDNA, and the specific U+- and D+-strand termini have been determined (12, 13, 62, 71).

A number of functional roles have been attributed to the

cPPT-CTS/flap, although consensus has notably been lacking. It has been reported to enhance the HIV-1 infection of both dividing and nondividing cells and also to enhance HIV-1-based lentiviral vector transduction in the brain and other cell types (11, 17, 24, 33, 51, 60, 67, 76). Specific claims for a role in nuclear import of the HIV-1 preintegration complex have been made (2–4, 18, 24, 53, 60, 67, 76). However, these roles have been questioned by other studies (21, 40, 48, 73). Riviere et al. recently concluded that the DNA flap aids the nuclear import step in both dividing and nondividing cells (53), and Arhel et al. concluded on the basis of electron microscopic observations that the flap promotes efficient uncoating at the nuclear pore (4).

The restriction factor APOBEC3G (A3G) is a cytidine deaminase that is incorporated into HIV-1 particles during virion assembly and acts during subsequent reverse transcription to catalyze deamination of viral minus-strand cytidines to uracil (6, 28, 44, 45, 56, 77). Since A3G deaminates only single-stranded DNA (14, 74), the net result is an accumulation of plus-strand G→A mutations, which is lethal when sufficiently extensive. There is also substantial evidence for noncatalytic (nonediting) mechanisms that inhibit the formation of intact cDNA by interfering with reverse transcription (7, 31, 50). The viral *Vif* protein counters A3G by binding to it in producer cells and acting as an adaptor between it and an E3 ubiquitin ligase complex comprised of Cullin5, elongin B, elongin C, and an unidentified E2-conjugating enzyme, thereby inducing polyubiquitination and proteasomal degradation of the restriction factor (16, 47, 49, 57, 63, 75). Hence, the phenotype of *Vif*-minus HIV-1 depends on the presence or absence of A3G in virus-producing cells rather than in target cells (25).

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	cPPT										CTS									
WT	AAT	TTT	AAA	AGA	AAA	GGG	GGG	A	...	(68 nt)...	CAA	AAA	CAA	ATT	ACA	AAA	ATT	CAA	AAT	TTT
	N	F	K	R	K	G	G				Q	K	Q	I	T	K	I	Q	N	F
DM	AA C	TT C	AA C	CG C	AA C	GG C	GG T	A	...	(68 nt)...	CA C	AA C	CAG	ATT	AC T	AA C	ATT	CA C	AA C	TTT
	N	F	K	R	K	G	G				Q	K	Q	I	T	K	I	Q	N	F
cPPT-D	AA C	TT C	AA C	CG C	CG C	GG T	GG T	A												
	N	F	K	R	R	G	G													

FIG. 1. Wild-type and mutant cPPT-CTS sequences. Nucleotide and amino acid changes are indicated by white letters in a black background. The double mutant (DM) used here is compared to the cPPT-D mutant made by Zennou et al. (76) and subsequently used in other studies (1, 2, 18, 21, 40, 48, 60). The CTS, where the U+ strand terminates after approximately 90 nt of D+ strand displacement synthesis (13, 62, 71), consists of a string of CA_nT_m motifs with terminations clustering at *ter1* (CA₅T₂) and *ter2* (CA₄T₄), indicated here by overhead brackets. By comparison, one CA₅T₂ motif triggers a single termination stop in the feline immunodeficiency virus (FIV) CTS (71). RT termination is induced in part by the A tracts in these motifs, which foster minor groove compression in the DNA duplex (37, 62). In addition to introducing *ter1* and *ter2* mutations, we introduced three other mutations, again synonymously, in the A tracts of the CA₅ and CA₃T₂ motifs that lie directly upstream (i) because a minor fraction of U+ strand terminations can be detected in the CA₅ motif (13) and (ii) to forestall any potential unmasking of termination activity there by the downstream mutations.

5'→3' hypermutation gradients have been identified in HIV-1 genomes (5, 36, 64, 68, 74). Yu et al. detected a general 5'→3' increase in Δ vif HIV-1 G→A substitutions and limited editing immediately downstream of the 3' long terminal repeat (LTR)-proximal PPT (74). Those investigators did not sequence the central region of the virus, but in several studies, some of which predate the discovery of A3G restriction, a "twin peaks" effect has been evident, with a relatively reduced G→A mutation frequency downstream of both the 3'-LTR-proximal PPT and the cPPT and with mutational maxima 5' to these elements (5, 9, 36, 64, 68). Wurtzer et al. also reported that mutation of the cPPT resulted in increased A3G-mediated G→A mutations in the 360-nt segment downstream of the cPPT, albeit with a Δ vif virus (72). These mutation pattern biases have been hypothesized to reflect two factors: the 3'→5' processivity of A3G, which results in preferential deamination toward the 5' ends of single-stranded DNAs (14), and a longer duration of the unduplexed state for viral minus-strand segments that lie further downstream from plus-strand initiation (44, 64, 74).

However, whether A3G affects the functional replication properties of central DNA flap-plus and -minus viruses is not known. In each study that identified suggestive G→A mutation gradients, the data were confined to proviral DNA sequence analyses (5, 36, 64, 68, 72). Differential impacts of cPPT mutation on viral replication in Vif-permissive versus nonpermissive cells have not been reported, nor have effects on viral replication been compared for vif-plus and vif-minus HIV-1. In the present study, we systematically reevaluated the role of the cPPT-CTS in HIV-1 replication, while specifically analyzing A3G/F restriction. We used single-cycle reporter viruses and full-length infectious HIV-1 molecular clones that have the cPPT and CTS inactivated but preserve wild-type (WT) IN coding. Our data do not support a role for the central DNA flap in determining nondividing cell infection, and they suggest that the "flap" DNA structure itself is likely an outcome of reverse transcription that requires final repair but is of limited functional consequence. We show that cPPT-CTS mutant viruses display specific phenotypes, even when Vif is intact, and these depend on the presence or absence of A3G restriction. We propose that the major role of the central plus-strand initiation is not to generate a triple-stranded DNA structure or

to mediate viral nuclear import but rather to limit the exposure of the unpaired HIV-1 minus-strand cDNA to cellular antiviral effectors that target it, such as A3 proteins that escape Vif-mediated destruction.

MATERIALS AND METHODS

Molecular clones and restriction analyses. A combined cPPT-CTS mutant (Fig. 1), termed a double mutant (DM), was constructed by overlap extension PCR using HIV-1 NL4-3 (pNL4-3) as a template. Primers used were (OE S1/-5326) 5'-CAGAAGAAGCAGAGCTAGAACTGG-3', (OE AS2/-5327) 5'-CCTCTGTCGAGTAACGCCTATTC-3', (OE AS1/-5329) 5'-GTATGTCTGTGCTATATGTCTACTATTTCCCTGCCTGACTGTACCCCAATACC GCCCTTGGCGCTTGAAGTTGTGGATGAATACTGCCATTG-3', and (OE S2/-5328) 5'-GTAGACATAATAGCAACAGACATACAACTAAAGAATTACAGAGCAGATTACTAAGATTGAGAACTTTCGGGTTTATTACAGG GACAGC-3'. The amplicon, which spans HIV-1 NL4-3 nt 3436 to 5819 (numbering follows that for GenBank accession no. M19921.1), was cleaved with AgeI and SalI and inserted between these sites in the reporter virus HIV-1_{luc}, an HIV-1 NL4-3 derivative in which *nef* is replaced by the firefly luciferase cDNA (*luc*); it also has a 426-nt deletion in *env* and a frameshift in *vpr* (41). The presence of desired nucleotide changes and the absence of other changes were confirmed by DNA sequencing. To generate an infectious NL4-3 DM proviral clone, the AgeI-SalI fragment of pNL4-3 was exchanged for the corresponding fragment of the DM version of HIV-1_{luc}, yielding pNL4-3DM. An R5-tropic virus was also constructed from this clone by exchange of the XbaI-BamHI fragment of HIV-1 ADA. We introduced the Δ vif mutation into viruses by deleting between NdeI and PstI (34). Plasmids pcDNA-A3G, pNL4-1, and pNL4-1Vif were kindly provided by K. Strebel (NIAID). For restriction experiments, viruses and reporters were produced in 293T cells in the presence of increasing amounts of A3G. Each HIV-1_{luc} proviral DNA (1.0 μ g) was cotransfected with pcDNA-A3G (0.0125, 0.025, 0.05, or 0.1 μ g), and 0.3 μ g of pMD.G and total DNA (1.4 μ g) was kept at a constant level with an empty pcDNA vector plasmid. Each transfection was performed in 4.5×10^5 293T cells in a six-well plate well. Viruses were harvested at 24 h and filtered, and the p24 antigen level was determined. SupT1 cells (45,000 cells) in 24-well plates were then challenged with p24-normalized inputs, and cellular luciferase activity was determined 3 days later. A3F was expressed from pcDNA3.1-A3F, obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, courtesy of B. M. Peterlin, Y. Zheng, and B. R. Cullen.

Cell culture and virus production. 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SupT1, MT-4, HuT78, and H9 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Buffy coats of normal human donors were obtained from the Mayo Clinic Blood Bank, and peripheral blood mononuclear cells (PBMCs) were derived by Ficoll centrifugation. PBMCs were cultured in RPMI 1640 with 10% heat-inactivated filtered human AB serum (Irvine Scientific) and recombinant human interleukin-2. CD14⁺ human monocyte-derived macrophages were prepared from Ficoll-separated PBMCs by allowing the macrophages to adhere to plastic and culturing them in RPMI 1640

medium with 10% filtered, heat-inactivated human AB serum for 3 to 7 days; they were replated with 50% trypsin plus 50% Versene at 2×10^5 cells/well in 24-well plates and fed with 50% fresh medium every 3 days. HIV-1 stocks were prepared by 293T cell transfection as described previously (41). An exogenous ^{32}P -based reverse transcriptase (RT) assay or an HIV-1 p24 antigen capture assay was used to assess viral production and normalize particle inputs (54). Viral titers were measured on GHOST cells as described previously (41) by transduction with serial dilutions followed by fluorescence-activated cell sorting (FACS) analysis for green fluorescent protein (GFP).

Viral infections. PBMCs, macrophages, SupT1, H9, HuT78, and MT-4 cells were infected with RT- or p24-normalized wild-type and mutant viruses for spreading infection assays. For single-round infectivity assays with HIV-1_{luc}, cells were infected with input-normalized vesicular stomatitis virus G (VSV-G)-pseudotyped virus. Three to 5 days later, adherent cells were washed in phosphate-buffered saline (PBS) to which cold PBS-1% Tween 20 (100 to 200 μl per well of a 6-well plate) had been added, placed on ice for 10 to 30 min, and centrifuged at 14,000 rpm for 1 min. Twenty microliters was pipetted in duplicate or triplicate into black 96-well plates (OptiPlate; PerkinElmer Life Science), 100 μl of SteadyGlo or BrightGlo (Promega) was added, followed by incubation in the dark for 5 min at room temperature, and luciferase activity was scored on a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer). Suspension cells were centrifuged at 2,500 rpm for 7 min first, followed by application of the procedure for adherent cells. For growth-arrested HeLa cell experiments, 10 $\mu\text{g}/\text{ml}$ aphidicolin was used and growth arrest was verified as described previously (54). For A3G coexpression experiments, 293T cells were cotransfected with wild-type or cPPT-CTS mutant proviral plasmids and corresponding APOBEC3 plasmids using Eugene 6 (Roche) and washed the next morning; 48 h later, the supernatant was harvested and p24-normalized inputs were used to infect 4.5×10^4 SupT1 cells.

Southern blotting for flap DNA detection. S1 nuclease and Southern methods for detecting the plus-strand gap are similar to those used previously by us (71) and Charneau et al. (11). Twenty million MT-4 cells were infected with WT or DM HIV-1 LAI virus inputs that had been normalized to a multiplicity of infection (MOI) of 0.1 (by prior GHOST cell titration) and treated with Turbo DNase (Ambion). The cells were washed after overnight incubation with the viruses. Low-molecular-weight DNA was isolated at day 3 by Hirt extraction (30) and digested with BamHI. The samples were then treated with S1 nuclease (Worthington Biochemical Corp.) to cleave single-stranded DNA or left untreated as indicated below. These samples were separated on a 1.2% agarose gel and transferred to a SuperCharge nitrocellulose membrane, which was probed with a ^{32}P -labeled 2.66-kb KpnI fragment of pNL4-3 and washed as described previously (71).

Immunoblotting. Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP-40, 150 mM Tris-HCl, pH 8.0) with added protease inhibitors (Complete mini; Boehringer) and clarified, and protein concentrations were determined using the Bradford assay. Fractions and lysates were boiled in Laemmli buffer with β -mercaptoethanol for 10 min, electrophoresed in 10% Tris-HCl gels (Bio-Rad), and transferred overnight to Immobilon P membranes (Millipore). Blocked membranes were incubated overnight with the primary antibodies (Ab) anti-APOBEC3G (NIH AIDS Research and Reference Reagent Program) at 1:1,000, anti-myc (monoclonal Ab [Mab] 9E10; Covance) at 1:1,000, and anti-Vif (NIH AIDS Research and Reference Reagent Program) at 1:1,000 and washed with Tris-buffered saline-Tween 20 (TBST) three times for 10 min each. Secondary Ab were either goat anti-rabbit-horseradish peroxidase (HRP) (Calbiochem) at 1:5,000 or goat anti-mouse-HRP (KPL) at 1:5,000, with incubation for 1 h at room temperature and washing with TBST 3 times for 10 min each. Membranes were incubated in SuperSignal West Pico chemiluminescent substrate (Pierce) for 1 to 2 min and exposed to film.

Stable knockdown of APOBEC3G in HuT78 cells. A3G mRNA was targeted at three 19-nt sequences (5'-GTACCACCCAGAGATGAGA-3', 5'-GATCATGAATTATGACGAA-3', and 5'-ACACGTGAGCCTGTGCATC-3') with short hairpin RNAs (shRNAs) expressed from pSilencer2.1U6Hygro as described previously (42). The three shRNA plasmids were linearized in the prokaryotic backbone and electroporated together into HuT78 cells (315 V, 10 ms, 1 pulse, 4-mm cuvette). Stable cell lines were selected and maintained in 300 $\mu\text{g}/\text{ml}$ hygromycin.

Hypermutation analysis. Reporter viruses produced by 293T cell transfection using the calcium phosphate method and a 1.0:0.4:0.3 ratio of proviral to A3G to VSV-G DNA were treated with Ambion Turbo DNase (1 $\mu\text{l}/\text{ml}$ at 37 degrees for 30 min) to remove plasmid DNA. p24-normalized infections of 293T target cells were carried out for 12 h (4.5×10^5 cells per well plated the day before infection). Cells were washed and suspended in PBS, and DNA was harvested

with the DNeasy kit (Qiagen). DpnI digestion was then used to eradicate any remaining plasmid DNA carryover. An 830-bp fragment centered on the cPPT was amplified using the primers of Wurtzer et al. (72), 5'-GACAAGTAGACTGTAGCCAGGAATATGG-3' and 5'-GGGATGTGTACTTCTGAACCTATTTTGG-3', using Phusion hot start DNA polymerase, with initial denaturation at 98°C for 30 s and then 35 cycles of 98°C for 10 s, annealing at 55°C for 0 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. Blunt-end cloning of PCR products was performed with the StrataClone Ultra Blunt PCR cloning kit (Stratagene), and nucleotide sequences of 720 nt that were centered on the cPPT were determined for each virus by Sanger sequencing for at least 5 independent randomly selected clones.

RESULTS

Single-cycle infection analyses of cPPT-CTS mutant viruses.

Previous functional studies of the HIV-1 central DNA flap have utilized the cPPT-D mutant constructed by Zennou et al. (76), which, in addition to disrupting the cPPT nucleotide sequence, results in a lysine-to-arginine change at position 188 in the integrase protein (1, 2, 18, 21, 40, 48, 60, 76). K₁₈₈ is part of the highly conserved K₁₈₆R₁₈₇K₁₈₈ motif that participates in the integrase dimer interface (70). Although conservative from the standpoint of amino acid properties, K₁₈₈R is unlikely to be entirely neutral, as it is found only rarely in subtype B sequence databases and never in infectious molecular clones of HIV-1. In addition, mutants with single-amino-acid integrase mutations often produce complex effects throughout the life cycle, including during viral assembly, reverse transcription, and integration (23). Therefore, to inactivate the cPPT-CTS definitively without this potentially confounding change in integrase coding, we introduced eight synonymous nucleotide changes into the HIV-1 NL4-3 cPPT and seven into the CTS, as shown in Fig. 1. The legend provides the rationale for the choice of CTS substitutions.

Mutant cPPT and CTS (mcPPT and mCTS) elements were initially studied alone and in combination (the double mutant, or DM). The mutations were introduced into full-length HIV-1 clones (NL4-3 and LAI) and also into the NL4-3-based luciferase reporter virus HIV-1_{luc} (41). Replication-competent variants containing the CCR5-tropic HIV-1 ADA envelope, designated NL4-3(ADA), were constructed as well.

Late life cycle events, i.e., Gag/Pol precursor expression and processing and supernatant p24 production, were unaffected by the mutations (data not shown). In single-cycle infection assays using 293T cell-produced viruses to infect a variety of target cells, no flap-specific phenotype was discernible (Fig. 2). CD4-positive T cell lines, including SupT1, MT-4, H9, and HuT78, and primary PBMCs all displayed similar luciferase activities after infection by particle-normalized wild-type and flap mutant reporter viruses at a range of inputs.

To confirm these results, infectivities of WT and DM viruses were reassessed by a different method, endpoint dilution titration on GHOST indicator cells. *env*-deleted reporter viruses entering via VSV-G (Fig. 3A) and replication-competent HIV-1 entering via CD4 and CXCR4 (Fig. 3B) showed similar infectivities in the respective flap-intact and mutant-flap versions. To establish whether we had disrupted central plus-strand formation as predicted, low-molecular-weight DNA was isolated from MT-4 cells that had been infected 3 days earlier at the same MOI (0.1, with pretitration on GHOST cells) with 293T cell-produced WT and DM viruses. After S1 nuclease treatment to cleave at points of single-stranded DNA, South-

FIG. 2. Single-cycle infection in diverse cell lines and primary cells. (A) MT-4 cells; (B) SupT1 cells; (C) H9 cells; (D) HuT78 cells; (E) PBMCs. The reporter virus HIV-1_{luc} is HIV-1 NL4-3 modified to encode firefly luciferase in the *nef* ORF; it also has a 426-nt deletion in *env* and a frameshift in *vpr*. Genetically homogenous stocks of VSV-G-pseudotyped HIV-1_{luc} variants (WT and DM) were produced by 293T cell transfection and used to challenge 4.5×10^4 cells. Inputs were normalized by p24 antigen or RT activity. Intracellular luciferase activity was assayed 5 days after infection.

ern blotting was performed. Flap formation, clearly detected in the WT virus, was abrogated in the DM virus (Fig. 3C).

Levels of infection of nondividing and dividing target cells are equivalent. Lentiviruses infect dividing and nondividing cells with equivalent efficiencies (39). This property underlies their ability to propagate in terminally differentiated macrophages, which in the case of some ungulate lentiviruses represents the principal cell type infected *in vivo*, and it is a main rationale for lentiviral vector applications in human gene therapy (43). The mechanistic basis has been persistently enigmatic, having been variously reported to depend upon nuclear import determinants in HIV-1 MA, CA, IN, and Vpr (reviewed in reference 65). Enhancing the infection of nondividing cells has also been functionally attributed to the cPPT-CTS (1–4, 18, 24, 60, 67, 76). Here, we found that HIV-1 reporter virus infection of macrophages was not significantly impaired by the mutations of the mcPPT, mCTS, and DM viruses (Fig. 4A). We further analyzed this issue in growth-arrested HeLa cells. Again, no flap-specific phenotype was observed (Fig. 4B).

Spreading-infection analyses. X4-tropic HIV-1 NL4-3 or R5-tropic HIV-1 NL4-3(ADA) viruses with and without the

double mutations were used to infect human CD4⁺ T cell lines (SupT1, MT-4, H9, and HuT78), primary human PBMCs, and primary human monocyte-derived macrophages (Fig. 5). Replication kinetics were similar for flap-intact and mutant-flap viruses in MT-4 and SupT1 cells (Fig. 5A and B). In contrast, spreading replication of the DM virus was blocked in H9 cells and HuT78 cells and was variably impaired in PBMCs (Fig. 5C to E). Interestingly, macrophages were able to support the replication of both the DM and wild-type viruses (Fig. 5F). These results, which contrasted with the complete lack of CPPT-CTS-specific phenotypes when such cells were infected with single-cycle reporter viruses produced in 293T cells (Fig. 2), implicated a producer cell-dependent mechanism. Moreover, since H9 cells, HuT78 cells, and PBMCs express A3G and are accordingly not Vif mutant permissive, while SupT1 and MT-4 cells are Vif mutant permissive, we suspected that the phenotypes of the DM virus, though wild type for Vif, might primarily reflect restriction by A3G/F. Western blotting for Vif excluded the possibilities that unapparent *vif* disruption had been introduced during cloning of the various cPPT-CTS mutant viruses or that cPPT-CTS mutation somehow inter-

FIG. 3. Infectivity by endpoint dilution titration and verification of central DNA flap prevention. Intact and mutant HIV-1s entering via VSV-G (A) or via gp120 (B) were tested. Serial dilutions of different viruses produced in 293T cells were used to infect GHOST cells, with inputs normalized for RT activity. Forty-eight hours after infection, GFP-positive cells were scored by FACS and infectivities were calculated as numbers of infectious units per 1,000 cpm of RT activity. Values are means $\pm$ standard deviations (SD) of results of three independent experiments. (C) Southern blot of low-molecular-weight DNA from infected cells. Twenty million MT-4 cells were infected at an MOI of 0.1 with GHOST cell titer-normalized WT or DM LAI virus that had been pretreated with DNase I. Low-molecular-weight DNA was isolated on day 3 by Hirt extraction (30), digested with BamHI, and treated with S1 or left untreated. Note the equal intensities of the 8.5- and 1.2-kb bands for both viruses, indicating equal inputs, while the 3.7-kb band cleaved from the 8.5-kb band is not detected for the DM virus. This experiment was repeated with the HIV-1 NL4-3 version, which produced identical results (data not shown); the mcPPT virus also failed to form a detectable flap (data not shown).

feres with Vif expression specifically (Fig. 5G). In PBMCs, relative A3G levels determined at the time of viral inoculation correlated roughly with degree of impairment (Fig. 5E). An important additional feature that emerged from repeated challenge experiments is that the impairment to replication in non-*vif*⁻ permissive CD4⁺ T cell lines is substantial but not absolute. In some experiments in which we monitored the cultures to long time points, eventual outgrowth of the DM virus was observed in HuT78 cells but always with a major delay compared to the time of outgrowth for wild-type HIV-1 NL4-3 (Fig. 5H). We also observed that viruses having a mutation in either the cPPT or CTS element alone were similarly impaired. To minimize the revertant potential and consolidate experiments, we utilized the DM in further work.

APOBEC3G restriction of flap mutant HIV-1. To analyze further the hypothesis that the cPPT-CTS enhances viral replication in the presence of A3G, we constructed Δvif versions of the WT and DM NL4-3 reporter viruses using the deletion

strategy previously validated by Kao et al. (34) and produced particle-normalized viral stocks by 293T cell transfection. In the absence of A3G, no difference in infectivity was seen between the four viruses in multiple experiments (data not shown). However, production in the presence of A3G revealed that the flap mutation produced a specific and dose-dependent vulnerability to A3G restriction.

Figure 6A shows the results with low A3G inputs, i.e., in experiments in which the A3G/proviral DNA ratios were 1:80 to 1:10. As expected, A3G had no effect on WT virus. In contrast, each mutant virus was impaired. The effects were consistent and could be quantified at a range of A3G inputs. For example, at the 0.05- μ g A3G DNA input (ratio, 1:20), the WT, DM, Δvif , and DM plus Δvif (DM+ Δvif) viruses displayed 95%, 26.4%, 2.79%, and 0.42% infectivity, respectively. At the 0.1- μ g-A3G DNA input (ratio, 1:10), the percentages of infectivity were 92%, 23.1%, 1.2%, and 0.07%, respectively. cPPT-CTS inactivation acted synergistically with *vif* deletion (Fig.

FIG. 4. Nondividing cell infection. (A) Macrophages. Normal human donor monocyte-derived macrophages were infected with VSV-G-pseudotyped HIV-1_{luc} or its cPPT-CTS mutants. Luciferase activities were determined 3 days later. Values are means $\pm$ SD of duplicate measurements. (B) Infection of growth-arrested HeLa cells. Cells were pretreated (white bars) or not treated (black bars) with aphidicolin, 10 μ g/ml, for 16 h (54), which induced full G₁ arrest (data not shown). HIV-1_{luc} or the cPPT-CTS mutants (each VSV-G pseudotyped) were inoculated while growth arrest was maintained, and intracellular luciferase activities were determined 3 days later. Values are means $\pm$ SD of triplicate measurements.

6A). Note that the addition of the cPPT-CTS mutation to Δ vif HIV-1 added approximately a full log of further inhibition. The mean value for the DM+ Δ vif virus across the 0.0125- μ g- to 0.1- μ g A3G DNA range was 10.5% of the Δ vif virus value; at the 0.1- μ g-A3G input, the DM+ Δ vif virus value was only 5.6% of the Δ vif virus value and the single-round infectivity of the DM+ Δ vif virus was more than 3 logs impaired versus the infectivity of the WT virus. Note also that at the lowest pcDNA-A3G input (ratio, 1:80), the DM+ Δ vif virus had only 5% of the infectivity of the no-A3G control. These data suggest that Vif and central plus-strand initiation defend HIV-1 cooperatively against A3G restriction.

At much higher producer cell A3G expression plasmid inputs, the Δ vif mutation became dominant, although the effect of the DM mutation was still observed (Fig. 6B). Inhibitory effects on WT virus were also incurred with A3G overexpression, as has been reported previously (45, 46, 56, 58, 77). Even so, A3G produced a substantially greater deficit in single-round infectivity for the flap mutant (41-fold versus 8.6-fold for the WT). At this level of expression, A3G was effectively lethal to both the Δ vif and Δ vif+DM viruses, producing 3.7- and 4.7-log inhibition of single-cycle infectivity, respectively (Fig. 6B). In addition, this result was consistent over broad ranges of HIV-1 particle inputs in the target cells (data not shown). Finally, we saw similar outcomes with APOBEC3F, including

the synergy with vif deletion, although A3F was considerably less inhibitory over the entire range (Fig. 6C).

These results show that at high A3G levels, Vif contributes more to the preservation of single-round infectivity than the cPPT-CTS, which was expected since the absence of any producer cell degradation of A3G in this circumstance is predicted to result in an overwhelmingly lethal mutagenesis and/or have other (nonediting) effects. Thus, the results are consistent with a model in which plus-strand initiation defends HIV-1 against those A3G molecules that survive Vif-mediated destruction to become incorporated in particles.

Replication of viruses with and without A3G knockdown.

We then constructed Δ vif versions of replication-competent DM and wild-type HIV-1 NL4-3. HuT78 and SupT1 cells were infected with p24-normalized inputs. The Δ vif, DM, and combined DM+ Δ vif viruses were unable to replicate in HuT78 cells but encountered no block in SupT1 cells, again confirming the cell type-specific behavior of flap mutant HIV-1 (Fig. 7A). In contrast, the restriction to the DM virus was partially relieved when A3G was partially knocked down in HuT78 cells (Fig. 7B and C). The residual A3G in these cells was, however, adequate to restrict the Δ vif and DM+ Δ vif viruses (Fig. 7C). We were unable to obtain a deeper knockdown to test whether that would be adequate to relieve the restriction of Δ vif viruses in this cell line. We were also unable to confer a fully WT phenotype to the DM virus, which suggests again that in these relatively A3G-depleted cells, some A3G is still able to survive Vif degradation. When Δ vif versions of the WT and DM viruses were compared to their vif-intact versions in PBMCs, the effect of the double mutation alone was discernible but notably milder than the vif gene deletion, suggesting that Vif was able to counter most but not all A3G in these cells (Fig. 7D). As in the T cell line experiments with replicating virus, we did not detect an additive effect of the double mutation to the profoundly impairing vif deletion in PBMCs (7D).

DM virus exhibits markedly increased susceptibility to A3G-mediated hypermutation. To analyze the effects of cPPT-CTS mutation on minus-strand editing, we produced vif-intact WT and DM reporter viruses in the presence and absence of A3G. 293T cells were infected with DNase preparations, and 12 h later, cellular DNA was isolated and subjected to DpnI restriction to further safeguard against plasmid DNA carryover. Target cell viral cDNA sequences upstream and downstream of the cPPT-CTS were then PCR amplified, cloned, and sequenced. The results, shown in Table 1, were unequivocal and are fully consistent with the model that emerges from the data of Fig. 2 through 7. In the presence of A3G, wild-type (Vif-intact) HIV-1 incurred some G $\rightarrow$ A hypermutation, but this was increased dramatically when the cPPT-CTS was mutant. Moreover, the segment downstream of the cPPT was considerably more vulnerable to hypermutation than the upstream segment of the cPPT (10.2-fold versus 2.2-fold) (Table 1). In the absence of A3G, neither WT nor DM virus incurred a single G $\rightarrow$ A mutation, whereas in the presence of A3G and the cPPT-CTS mutation, 3.78% of all nucleotides were mutant in the cPPT-distal 360 nt and 15% of G nucleotides were mutant. To determine whether this vulnerability could also be detected for vif-intact HIV-1 during spreading infection in the presence of endogenously expressed A3G, we also sequenced genomes of WT and DM viruses from the HuT78 cell replica-

FIG. 5. (A to F) Spreading infections. Replication of WT and DM viruses in diverse cells is shown. (A) MT-4 cells; (B) SupT1 cells; (C) H9 cells; (D) HuT78 cells; (E) PBMCs (numbers at the left of the blot at the bottom are molecular weights in thousands) (F) macrophages. Virus inputs were p24 antigen or RT activity normalized for the two viruses in each case. The initial cell number was 1×10^6 for CD4⁺ T cell lines and PBMCs. Macrophages were plated in 6-well plates at 2×10^5 per well. (G) Western blotting for Vif. Proviral DNAs were transfected into 293T cells, and Vif expression was analyzed at 48 h. Lanes: 1, Vif expression plasmid pNL4-1vif; 2, pNL4-3; 3, mock-transfected cells; 4, pNL4-3 DM. For lanes 2 and 4, p24 levels in the respective supernatants were also equivalent (data not shown). (H) Replication of WT, mcPPT, mCTS, and DM viruses in HuT78 cells, followed for 32 days. Cells were infected with p24-matched inocula on day 0, followed by five washings on day 1.

FIG. 6. APOBEC3 protein restriction analysis. (A) Single-round HIV-1 infectivity at lower A3G inputs. The four reporter viruses were produced in 293T cells in the presence of increasing amounts of A3G, and p24-normalized inputs were used to infect SupT1 cells. Each HIV-1_{luc} reporter virus proviral DNA (1.0 μ g) was cotransfected with pcDNA-A3G (0, 12.5, 25, 50, or 100 ng). The VSV-G expression plasmid pMD.G, 0.3 μ g, was also included, and the amount of total DNA (1.4 μ g) was kept constant with an empty pcDNA vector. (B) Single-round infectivity at maximum A3G input. Here, 1.0 μ g of pcDNA-A3G was cotransfected with 1.0 μ g of each HIV-1_{luc} reporter virus DNA and 0.3 μ g of pMD.G. Inputs to SupT1 target cells were RT activity normalized (5.6×10^5 cpm per virus). (C) Same experiment as described for panel A, but with the indicated amounts of A3F rather than the A3G expression vector. Error bars represent SD of triplicate luciferase activity measurements.

tion experiment whose results are shown in Fig. 5H. Virion RNA was harvested at the peak of p24 production, reverse transcribed, and then analyzed as described in Table 1. The results (Table 2) showed that the DM virus but not WT virus had accrued G→A mutations downstream of the cPPT (4.3 G→A changes per 10 kb).

Finally, because prior investigations have implicated both integrase and the central DNA flap in viral nuclear import, we investigated a possible connection to the HIV-1 integrase interactor LEDGF/p75 (41). p24-normalized DM and wild-type HIV-1

NL4-3 were used to infect SupT1 cells that have a stringent LEDGF/p75 knockdown (41). WT and DM viruses were equivalently impaired in these cells compared to in control shRNA-expressing cells; i.e., no significant flap-specific effect was seen (data not shown).

DISCUSSION

The full set of mechanisms by which A3G exerts its antiviral activity is a matter of ongoing debate, but there is a consensus

FIG. 7. Differential replication properties in Vif-permissive and nonpermissive cells. (A) Wild-type, DM, ΔVif, and DM+ΔVif virus replication in SupT1 versus HuT78 cells. For each virus, 4 ng of p24 was used to infect 2×10^6 cells. (B) A3G knockdown (immunoblotting). (C) Virus replication in HuT78 and HuT78 A3G knockdown cells. Cells were inoculated with equal inputs and followed in parallel for 3 weeks. (D) Wild-type, DM, ΔVif, and DM+ΔVif virus replication in human PBMCs.

that Vif defends HIV-1 against this restriction factor primarily by serving as an adaptor protein that directs A3G into a proteasomal degradation cycle in the virus-producing cell. As this is a defense system that functions by reducing A3G supply to the assembling virion rather than directly abrogating the func-

tion of the protein, any leakage that allows some A3G to be incorporated might threaten viral genome integrity. This threat exists whether or not A3G acts exclusively by uracylating minus-strand DNA or by additional editing-independent mechanisms, but the catalytic nature of the former provides a means for just a few molecules to generate significant genome damage. Indeed, Browne et al. have recently shown that only a few (and possibly one) A3G molecules per virion can inhibit

TABLE 1. Hypermutation analysis^a

Virus with or without A3G	Total no. of nt sequenced	No. of G→A mutations (%) ^b		No. of changes in cPPT
		360 nt upstream of cPPT	360 nt downstream of cPPT	
WT	4,320	0 (0)	0 (0)	0
DM	4,320	0 (0)	0 (0)	0
WT + A3G	4,320	19 (0.88)	8 (0.37)	2
DM + A3G	3,600	35 (1.94)	68 (3.78)	1

^a G→A mutations in proviral DNA were tallied 0.36 kb upstream and downstream of the cPPT sequence in the WT and DM viruses, both of which are wild type for *vif*. Calculated as a percentage of G nucleotides, in the presence of A3G, 15.1% of G residues were mutated in the DM virus downstream of the cPPT versus 1.48% for the WT virus. A total of three G→A changes were observed within the cPPT sequence itself (right-most column).

^b Values in parentheses are percentages of the total number of nucleotides (A+G+C+T) sequenced.

TABLE 2. G→A hypermutation after viral replication in HuT78 cells^a

Virus	Total no. of nt sequenced	No. of G→A mutations (per 10 ⁴ nt)		No. of changes in cPPT
		360 nt upstream of cPPT	360 nt downstream of cPPT	
WT	21,580	3 (2.8)	0 (0.0)	0
DM	23,240	3 (2.6)	5 (4.3)	0

^a G→A mutations were tallied 0.36 kb upstream and downstream of the cPPT sequence in the WT and DM viruses from Fig. 5H at the time of peak p24 production. The methods are as described in Table 1 except that, here, cDNA was prepared from virion RNA to ascertain genome mutational accumulation in actively propagating viruses.

HIV-1 infectivity (10). Our data provide experimental support for the concept that plus-strand synthesis from dual locations reduces exposure of unpaired minus-strand segments to antiviral restriction to the extent that clear effects on viral replication occur. In the absence of central plus-strand priming, all minus-strand DNA upstream of the 3' U3 element remains exposed as single-stranded DNA until after the second strand transfer and subsequent plus-strand elongation. The kinetic mechanism for cPPT-CTS function that our data support is also consistent with the precisely central location of the cPPT primer. Central plus-strand initiation is thus a second line of viral defense against those A3G molecules that evade Vif-directed destruction and become virion incorporated. From this line of reasoning, the cPPT-CTS could also function to defend lentiviruses against other antiviral activities for which the relevant target is single-stranded viral cDNA.

We show that cPPT-CTS mutant HIV-1 incurs a substantial block to replication mediated by APOBEC3 proteins and that these effects are evident in non-*vif*-permissive cells for *vif*-intact HIV-1. Another informative feature is that the restriction to the DM virus could be relieved partially by reducing the level of A3G in such cells (Fig. 7C). It has been noted that there is likely to be a balance between Vif and A3 proteins, such that A3 proteins that escape Vif suppression may be either beneficial or detrimental (44). Achieving an equilibrium between Vif and A3G that allows limited, sublethal editing may be of adaptive value to a lentivirus by driving sequence diversification *in vivo* (35, 59). It has thus been considered that the relatively elaborate strategy of encoding a specific viral counterprotein that regulates A3G levels in cells, rather than one that categorically blocks A3G incorporation or action as in other retroviruses (19, 20), has evolved for this reason (44). Our data support a model in which the cPPT-CTS contributes to controlling this balance and provides a second line of defense against "spillover" A3G, i.e., one that functions when A3G is abundant enough that some intracellular A3G molecules survive destruction during the viral production phase and become virion incorporated.

The viral replication data for the cPPT-CTS mutant are strongly supported by the genome-sequencing experiments (Table 1), which show that the presence of a functional cPPT-CTS significantly reduced an unambiguous, mechanistically informative endpoint: A3G-mediated cytidine deamination downstream of the cPPT in *vif*-intact HIV-1. The results extend the genome-editing data of Wurtzer et al., who carried out a similar experiment for A3G with a Δvif virus (72). We were also able to identify a genetic impact during viral replication in the presence of endogenous A3G (Table 2). These data are also consistent with previous observations of limited G→A hypermutation downstream of PPTs in the sequences of natural HIV-1 genomes (5, 9, 36, 64, 68). Sequencing of more genome segments will be needed to determine whether regions farther downstream of the cPPT are progressively vulnerable to hypermutation in the DM virus. An alternative possibility is that a delay in conversion to double-strandedness resulting from cPPT mutation is most consequential close to the D+ strand initiation and might be mitigated in other U+ and D+ segments by additional mechanisms. The common selection pressure would be the need to reduce APOBEC3 protein access to the unpaired minus strand.

FIG. 8. Kinetic model of cPPT defense against minus-strand attack. The diagram summarizes the following reassessment of HIV-1 cPPT function. Although we cannot exclude the possibility that the penultimate triple-stranded DNA flap structure has a function in the life cycle, we have so far obtained no evidence for this. We doubt that this DNA structure is a specific mediator of nuclear import, since abrogation of its formation in single-cycle studies had no discernible effect in our hands on the infection of dividing, nondividing, primary, or immortalized cells. We favor instead a kinetic model that returns emphasis to the cPPT's main role as a reverse transcription primer, which is congruent with its location at the center of the genome. The model predicts that earlier conversion to double-strandedness in the downstream half of the genome is protective against host defenses that target single-stranded DNA, including but perhaps not limited to A3 proteins. Finally, a role in A3G defense does not exclude the possibility of other kinetic advantages to central plus-strand initiation if faster completion of double-strandedness acts to stabilize the viral complex or license its progress to the integrated state in other ways.

The ability of the DM virus to undergo spreading replication in macrophages in our hands and the ability of the cPPT-D mutant made by Zennou et al. (76) to do so in the experiments of Dvorin et al. (21) bear emphasis in view of prior proposals of a cPPT-CTS requirement for the propagation of HIV-1 in nondividing cells (76). Recent work has shown that A3G and A3F levels are low in macrophages in the absence of immune effector cytokines, such as alpha interferon, which induce their expression (38). Type III interferon also upregulates A3G expression in macrophages (32), and Sarkis et al. reported that A3G was upregulated by alpha interferon in both hepatocytes and macrophages but not in T cells or 293T cells; upregulation in macrophages was also strongly donor dependent (55). Therefore, Vif alone may be adequate to prevent virion A3G incorporation in macrophages that are not specifically induced. Our results are consistent as well with the observations of Limon et al. (40) and Dvorin et al. (21). These groups did not examine A3G-positive and -negative cell lines specifically, but Dvorin et al. also found no difference in HIV-1 replication in MT-4 cells and CEM-SS/CCR5 cells but saw modest impairment of cPPT-D mutant NL4-3 replication in PBMCs and to a lesser extent in macrophages, with the PBMC replication differences being donor variable in magnitude, as in our study. Limon et al. similarly found that the cPPT-D mutant was unimpaired in Jurkat and MT-4 cells but found a variable impairment in PBMCs (40).

We propose a threshold spillover model (summarized in Fig. 8) in which central plus-strand synthesis protects the unpaired minus strand against A3G molecules that survive the Vif-mediated destruction cycle and gain entry into assembling virions, and it may therefore provide a way to reconcile at least some

of the conflicting prior reports in the field with primary macrophages and PBMCs. The discrepancies could in the main reflect donor- and cell type-dependent A3G expression variability in combination with the particular culture conditions and reagents present in a given experiment, e.g., the adventitious presence of inducers of A3G expression. In fact, even Δvif HIV-1 has been reported to undergo spreading replication in macrophages at an attenuated level compared to that in *vif*-intact HIV-1 (15). (In PBMCs, on the other hand, we [Fig. 7D] and others [26, 69] observed that Δvif viruses produced only low levels of p24, which are not clearly indicative of spreading after initial provirus establishment.) It is possible, as well, that even in immortalized CD4⁺ T cell lines, A3G may exert low levels of selection pressure for *Vif* and cPPT-CTS maintenance, since small amounts of A3G protein (a log or two lower than levels in PBMCs, H9 cells, or HuT78 cells) can be detected by optimized Western blotting in our hands even in *vif*-permissive lines (SupT1, MT-4, Jurkat, A3.01, CEM-SS) generally regarded as negative for A3G expression (data not shown). Cell lines in which we never detect trace A3G are 293T and Molt4.

We suggest that future analyses of the cPPT-CTS would benefit from using either the mcPPT or DM virus. The DM mutation set disrupts both elements of the cPPT-CTS, and both the mcPPT and DM viruses are phenotypically neutral in mutant-*Vif*-permissive cells and are coding neutral. As noted above, while the cPPT-D virus K₁₈₈R mutation (76) is conservative in terms of amino acid properties, single amino acid changes in integrase are well known to induce pleiotropic class II effects on various aspects of Gag/Pol function (22). In addition, proper experimental use of the cPPT-D mutant requires matched K₁₈₈R control viruses.

Finally, our results do not support a specific role for the central DNA flap in HIV-1 preintegration complex nuclear import. We suggest that the arrested progress of the reverse transcription complex due to APOBEC3 restriction mechanisms (editing or nonediting) may be the underlying feature that has led to past interpretations that conjectured nucleopore traversing as the specifically blocked step, in particular with the 2-LTR circle assay, which is indirect. We also suggest that the term “central DNA flap” is not ideal to describe or conceptualize the functional viral entity. The “flap,” i.e., the transient and penultimate DNA structure at the center of the reverse-transcribed genome, is less likely to be a potent biological mediator in the viral life cycle than a relatively (perhaps completely) inconsequential outcome of central plus-strand initiation, albeit one that requires repair to complete the final proviral state. The salient functional role of the cPPT rather appears to be an important kinetic one: minimizing the presence of vulnerable unpaired minus-strand DNA in the lentiviral reverse transcription complex. This is relevant—in fact it is critical (Table 1)—well before the central strand displacement that yields the flapped DNA structure can occur, i.e., as soon as the D+ strand initiates and begins to shield the unpaired minus strand from restrictions that recognize and attack single-stranded DNA. Future research is needed to determine whether temporal minimization of single-stranded DNA exposure serves to shield HIV-1 from cellular antiviral defenses other than APOBEC3 proteins.

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