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Functional Central Polypurine Tract Provides Downstream Protection of the Human Immunodeficiency Virus Type 1 Genome from Editing by APOBEC3G and APOBEC3B

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Lentiviruses utilize two polypurine tracts for initiation of plus-strand viral DNA synthesis. We have examined to what extent human immunodeficiency virus type 1 plus-strand initiation at the central polypurine tract (cPPT) could protect the viral genome from DNA editing by APOBEC3G and APOBEC3B. The presence of a functional cPPT, but not of a mutated cPPT, extensively reduced editing by both APOBEC3G and APOBEC3B of sequences downstream, but not upstream, of the cPPT, with significant protection observed as far as 400 bp downstream. Thus, in addition to other potential functions, the cPPT could help protect lentiviruses from editing by cytidine deaminases of the APOBEC family.

Retroviral replication requires reverse transcription of particle-associated genomic viral RNA into double-stranded DNA. Reverse transcription follows sequential steps, during which discrete segments of DNA are synthesized by reverse transcriptase, with each of these segments being initiated at a precise site by a specific primer (30). Synthesis of the first (minus) strand of DNA uses genomic viral RNA as a template and is initiated by a tRNA primer that defines the 3′ boundary of the U5 region of the long terminal repeat (LTR). Synthesis of the second (plus) strand of DNA uses minus-strand DNA as a template and is initiated by a short RNA primer corresponding to a purine-rich sequence, the polypurine tract (PPT) (24, 27). While all retroviruses carry a PPT near the 3′ end of the retroviral genome, defining the 5′ boundary of the U3 region of the LTR, several retroviruses, including lentiviruses, carry a second PPT near the center of the genome. Central polypurine tracts (cPPT) have mainly been described for lentiviruses, including visna virus, feline immunodeficiency virus, bovine immunodeficiency virus, equine infectious anemia virus, simian immunodeficiency virus (SIV), human immunodeficiency virus type 1 (HIV-1), and HIV-2, but they have also been found in spumaretroviruses and in the yeast retrotransposons Ty1 and Ty3 (3, 12, 29, 32, 33). Due to the presence of the cPPT, the plus strands of these viruses are synthesized as two distinct segments separated by a unique discontinuity (6, 7, 10, 15, 16, 18, 31, 33). The downstream segment, initiated at the cPPT, covers the 3′ half of the genome, including the whole 3′ LTR. The upstream segment, initiated at the 3′ PPT, covers the 5′ LTR and the 5′ half of the genome. In HIV-1 and other viruses with a cPPT, this upstream segment terminates approximately 100 nucleotides beyond the cPPT itself, by virtue of the biophysical properties of a central termination sequence (CTS) (7). In these viruses, the central plus-strand discontinuity is therefore represented by an ~100-nucleotide overlap between the two segments, also known as the DNA "flap" (7, 10, 29, 33).

The nature of the selective advantage conferred by the existence of a cPPT, a CTS, and a DNA flap has been mainly evaluated for HIV-1. Mutants of HIV-1 carrying purine-to-pyrimidine substitutions in the cPPT or mutations in the CTS clearly replicate at lower levels than wild-type virus (6, 7, 14), but the mechanism through which the replication of these mutants is impaired is not fully elucidated. Strong evidence has been presented that these HIV-1 mutants are defective in viral DNA nuclear import (1, 26, 36), but some authors have contested these results (9, 20). It is possible that the cPPT system provides more than a single advantage for the replication of retroviruses. We hypothesize here that one such additional function could be that initiating plus-strand synthesis at two separate sites would reduce the amount of time during which minus-strand viral DNA remains single-stranded during the reverse transcription process, thereby reducing its vulnerability to cellular enzymes. Most notably, cytidine deaminases of the APOBEC family are known to edit newly synthesized single-stranded viral DNA following encapsidation of these enzymes into HIV virions (2, 11, 19, 22). APOBEC enzymes act as natural defense barriers against HIV infection through extensive DNA editing and lethal mutagenesis. Several human isoforms of APOBEC enzymes have been found to exert antiviral activity against HIV-1 (2, 34). The best characterized of these isoforms, APOBEC3G, expressed at high levels in T lymphocytes and monocytes/macrophages, is the target of the virally encoded Vif protein, which promotes its rapid degradation by the proteasome and prevents its encapsidation. Another human APOBEC, APOBEC3B, also edits HIV-1 DNA but is not neutralized by Vif (8, 34). In this study, we have examined the extent to which the presence of a functional central polypurine tract in the HIV genome can reduce editing of viral DNA by APOBEC3G and APOBEC3B downstream of the cPPT site, thereby protecting important HIV coding regions, notably in *pol* and in *vif* itself, from mutagenesis.

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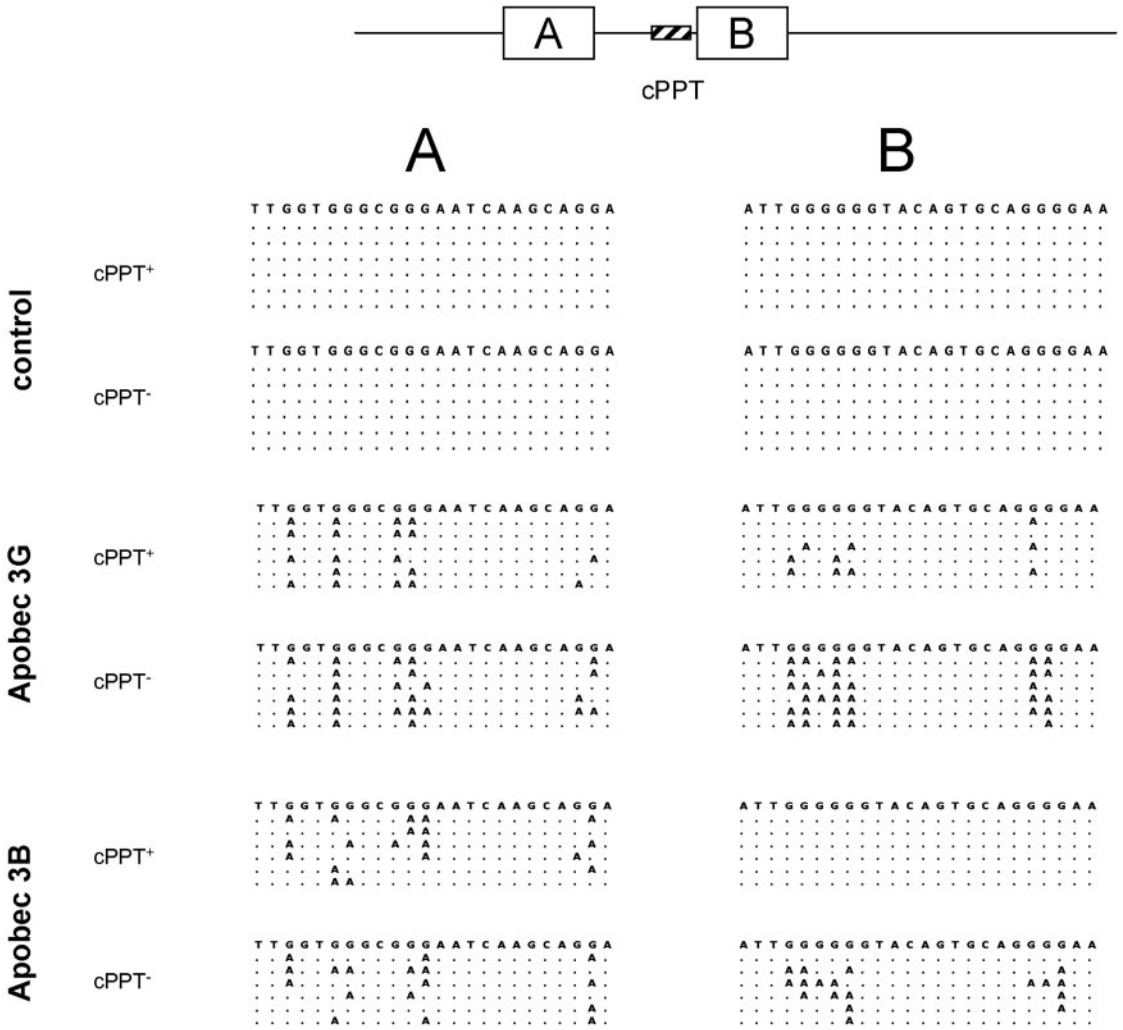

FIG. 1. APOBEC3G- and APOBEC3B-mediated G-to-A mutagenesis of newly synthesized HIV-1 DNA upstream and downstream of the cPPT in HIV-1 mutants carrying a functional cPPT ($cPPT^+$) or a mutated, nonfunctional cPPT ($cPPT^-$). G-to-A mutations were quantified in two segments of the genome located immediately upstream (segment A) and downstream (segment B) of the cPPT. Six clones from each APOBEC-cPPT allele are shown, corresponding to those with the most G-to-A mutations across the whole 829-bp HIV DNA insert.

We compared the editing effects of two APOBEC enzymes, APOBEC3G and APOBEC3B, on regions upstream and downstream of the cPPT of HIV-1 with viruses carrying a functional or mutant cPPT. The viruses studied in these experiments were the previously described cPPTAG and cPPTD mutants (36). The cPPTD mutant of the HIV-1 LAI isolate was created by the introduction of a total of eight purine-to-pyrimidine changes in the cPPT, resulting in a dramatic decrease in plus-strand priming at this site and in a 5- to 10-fold replicative defect compared with wild-type LAI (36; data not shown). Because the integrase coding region of the cPPTD mutant was also modified, bearing a K-to-R amino acid substitution at position 188 of IN, the control virus for the effect of the substitution of pyrimidines for purines in the cPPT was the cPPTAG mutant, in which the same K188R substitution was created through an AAA-to-AGA change, which does not introduce pyrimidines in the cPPT (36). The infectivity of the cPPTAG mutant is not significantly different from that of wild-type LAI (36; data not shown). To measure APOBEC editing in the context of a wild-type or mutant cPPT, 1×10^6 HeLa cells were cotransfected with 7 μg of cPPTAG or cPPTD proviral plasmid (henceforth referred to as $cPPT^+$ and $cPPT^-$ viruses, respectively) and 3 μg of a plasmid expressing either APOBEC3G (11) or APOBEC3B (unpublished data) as a fusion protein in which the C-terminal tail contains a V5 epitope. The levels of expression of the two APOBEC isoforms were approximately equivalent, as evaluated by Western blotting using an anti-V5 antibody (data not shown). Cells were washed 24 h after transfection, and viral supernatants were collected 24 h later. Notably, in all APOBEC3G experiments, the cotransfected virus always carried a deletion of the *vif* gene, making it possible for APOBEC3G to be encapsidated in viral particles and to subsequently exert its editing function. After 48 h, supernatants were harvested, and viral particles were quantified by an HIV-1 p24-specific enzyme-linked immunosorbent assay. After treatment with DNase I, viral particles

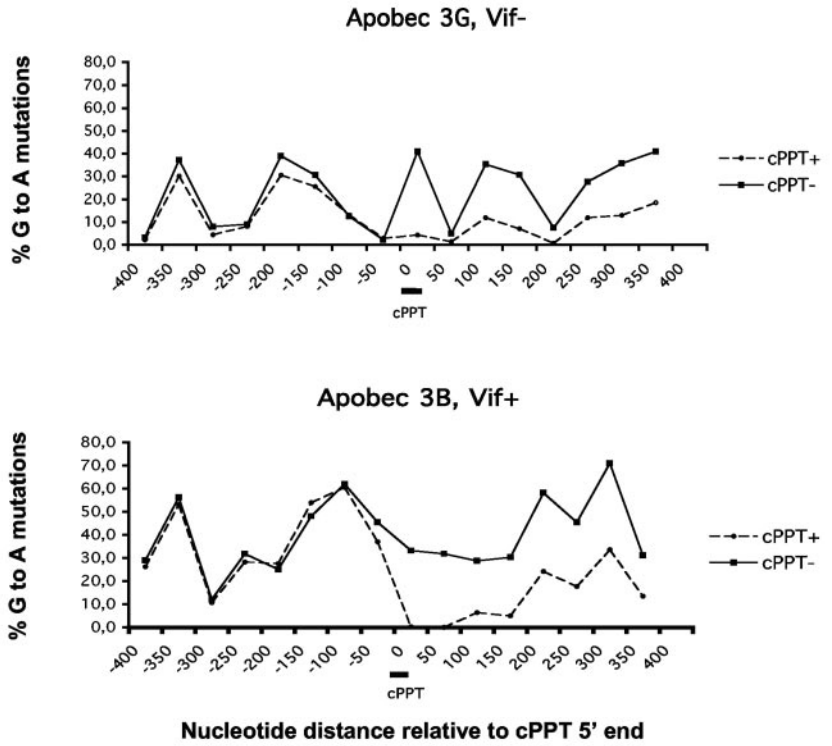

FIG. 2. APOBEC3G and APOBEC3B editing as a function of the distance from the cPPT in cPPT$^+$ and cPPT$^-$ viruses. Editing was calculated as percentages of potential target sites for both APOBEC3G and APOBEC3B (cumulated GG and GA sequences) in windows of 50 bp. Sequence numbering is relative to the 5′ end of the cPPT. Dashed lines, functional cPPT; continuous lines, mutated, nonfunctional cPPT.

corresponding to 5 to 20 nanograms of HIV-1 p24, according to the experiment, were used to infect HeLa-CD4-LTR-LacZ cells (P4 cells) using a spinoculation method (4). After an incubation period of 12 h, allowing for synthesis of viral DNA in the course of a single cycle of replication, total cell-associated DNA was isolated and PCR amplified with primers 5′-GACAAGTAGACTGTAGTCCAGGAATATGG and 5′-GGGATGTGTACTTCTGAACTTATTCTTGG, generating a segment spanning the *pol* coding region of HIV-1 LAI from nucleotides 3966 to 4795 and centered on the cPPT. These amplification products were then cloned into a TOPO plasmid and sequenced.

In a first series of analyses, we examined the presence of characteristic G-to-A substitutions in the plus strand of newly synthesized viral DNA, according to the presence of a functional cPPT in the HIV-1 genome and the expression of APOBEC enzymes in virus-producing cells. Figure 1 shows the plus-strand sequences of two segments of the HIV-1 LAI genome, located upstream and downstream of the cPPT site, for six clones from each APOBEC-cPPT allele pair. These selected clones were those carrying the most G-to-A mutations across the whole 829-bp insert among the 14 to 22 total clones sequenced for each pair in a single infection experiment. The A segment covers nucleotides 4201 to 4224 of the HIV-1 LAI genome, while the B segment covers nucleotides 4382 to 4405, immediately downstream of the cPPT, which extends from nucleotides 4367 to 4381. The two segments were selected for analysis in view of their rich content in potential target sequences for both APOBEC3G and APOBEC3B, which favor G-to-A substitutions at GG and GA sites, respectively (2, 8, 11, 22). No traces of editing in segment A or B were seen in the absence of APOBEC, regardless of the cPPT allele (Fig. 1, control). In the presence of either APOBEC3G or APOBEC3B, however, a strong effect of the presence of an intact cPPT on the editing of segment B, but not that of segment A, was observed. With APOBEC3G, the mean number of G-to-A changes/clone in the A segment was 3.17 for the cPPT$^+$ virus and 4.17 for the cPPT$^-$ virus (not significant), while the mean number of G-to-A changes in the B segment was significantly lower for the cPPT$^+$ virus (1.7 changes/clone) than for the cPPT$^-$ virus (5.5 changes/clone) ($P < 0.05$; Mann-Whitney test). This effect was also observed with APOBEC3B. In the presence of this enzyme, the mean number of G-to-A changes/clone in the A segment was again not significantly different according to the cPPT allele (3.17 for cPPT$^+$ virus and 2.83 for cPPT$^-$ virus [not significant]). In the B segment, however, the mean number of G-to-A changes was significantly lower for the cPPT$^+$ allele (0 changes/clone) than for the cPPT$^-$ allele (3.0 changes/clone) ($P < 0.05$).

We next wished to quantify the effect of the cPPT on DNA editing as a function of the distance of the edited site from the cPPT. This analysis was conducted by sequencing an 800-nucleotide segment of HIV *pol* centered on the cPPT in a total of 75 clones (38 cPPT$^+$ and 37 cPPT$^-$ clones) for APOBEC3B cotransfections and 66 clones (32 cPPT$^+$ and 34 cPPT$^-$ clones) for APOBEC3G cotransfections in two independent series of cotransfection and infection experiments. Editing was quantified by performing simple counts of the G-to-A changes in

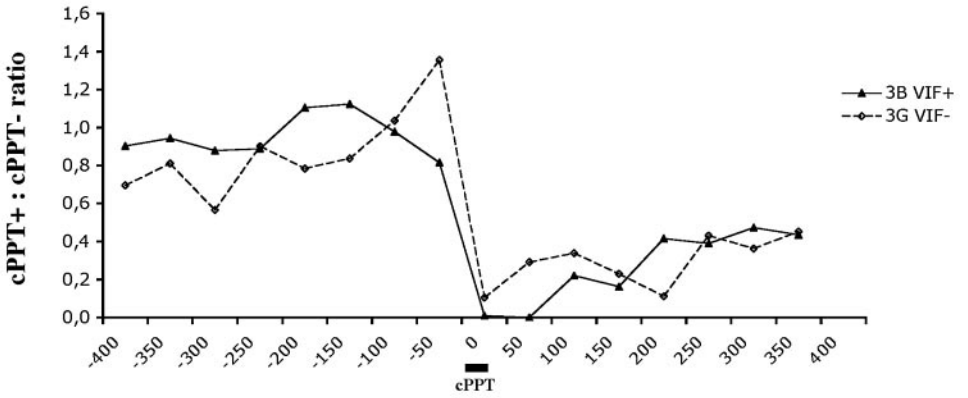

FIG. 3. Protective effect of the cPPT from APOBEC3G and APOBEC3B editing. The data presented are ratios of the editing values (presented in Fig. 2) for the $cPPT^+$ virus to those for the $cPPT^-$ virus. Dashed line, APOBEC3G; continuous line, APOBEC3B.

windows of 50 nucleotides and dividing these values by the number of combined potential target sites for APOBEC3G and APOBEC3B (GG plus GA sites) within each window. We chose to combine both GG and GA sites for analysis of editing by either enzyme in view of the reported significant overlap in their substrate specificities (2, 8). As shown in Fig. 2, the extents of editing of viral DNA upstream of the cPPT by both APOBEC3G and APOBEC3B were essentially identical for viruses carrying either of the two cPPT alleles. In contrast, downstream of the cPPT, the editing curves markedly diverged. The profiles of these curves were clearly different between APOBEC3G and APOBEC3B, reflecting the differences in frequency and distribution of preferred target sequences for these two enzymes, but both curves appeared equally affected by the presence or the absence of a functional cPPT. To further evaluate this distance effect, the results were expressed as ratios between the rates of editing in $cPPT^+$ virus clones and those in $cPPT^-$ virus clones with both APOBEC3G and APOBEC3B (Fig. 3). This analysis, indicating the relative susceptibilities to editing of the two viruses, which reflect the extent of protection conferred by the cPPT, revealed very similar profiles for APOBEC3B and APOBEC3G. As expected, the degree of protection was almost absolute immediately downstream of the cPPT and decreased gradually further downstream. The protection, however, was still about 50% 400 bp downstream of the cPPT, a position that corresponds to the Vif coding region.

Taken together, our results show that the additional plus-strand priming site provided by the cPPT in HIV-1 significantly reduces the susceptibility to APOBEC editing of a fairly large region of the viral genome located downstream of this priming site. This observation concurs with and complements that of Yu et al., who found that editing of the HIV genome by APOBEC3G in the absence of Vif followed a gradient whereby regions closest to the site of initiation of the plus strand at the 3′ PPT, such as *gag*, were less prone to editing than *env*, which is located just upstream of this priming site (35). We extend these observations to the central PPT and propose that this additional priming site for HIV reverse transcription may help reduce the impact of APOBEC enzymes on HIV sequence integrity during replication. We hypothesize that this protection may be of particular importance in the defense against isoforms of APOBEC that are not neutralized by Vif, such as APOBEC3B. As seen in our experiments, the protection conferred by the cPPT against APOBEC editing is equally efficient against APOBEC3G and APOBEC3B.

Whether the protective effect of the cPPT on HIV DNA editing explains, at least in part, the selective advantage conferred by this structure on HIV replication and participates in the described loss of infectivity of cPPT mutants has not been evaluated here. We hypothesize that protection from APOBEC editing may come in addition to other beneficial functions of the cPPT and the DNA flap of HIV-1, including their described role in mediating HIV DNA nuclear import. It is possible, but unlikely, that the positioning of the cPPT at the center of the genome reflects a need for selective protection of particular coding regions located immediately downstream, such as the end of the integrase gene or the *vif* gene itself. Rather, we believe that the central positioning of the cPPT is statistically the best option for providing balanced protection from editing on either side of the genome, at the cost of a single additional plus-strand priming site. Indeed, even better protection of HIV against editing would have been conferred by multiple plus-strand specific initiation sites. The evolutionary cost of multiple *cis*-acting sequences, however, might be too great for a virus with a small genome size, as it would create important protein coding and diversity constraints. It is also noteworthy that avian retroviruses synthesize their plus-strand DNAs as multiple segments which are subjected to extensive strand displacement by upstream DNA polymerization (13, 17). Such a system would certainly provide strong protection against minus-strand editing enzymes, but we feel that it might also create an important burden for the overall efficiency and kinetics of viral DNA synthesis.

Whether the protection conferred by the cPPT becomes most valuable in situations where high APOBEC3G expression is likely to saturate neutralization by Vif or when HIV infects cells expressing dangerous levels of APOBEC isoforms such as

APOBEC3B, which is not neutralized by Vif, remains to be determined. In this respect, it is noteworthy that most viruses bearing a cPPT also carry genes that inhibit the activities of some APOBEC isoforms, although equine infectious anemia virus and the Ty1 and Ty3 retrotransposons of yeast may be exceptions to this rule (5, 21, 23, 25, 28, 32, 33). Most lentiviruses carry a Vif gene, and in spumaretroviruses, which carry a cPPT, the BET protein has recently been found to inhibit DNA editing by APOBEC enzymes (25). This coincidence may reflect the observation that in cells targeted by these viruses, the presence of potentially deleterious levels of cytidine deaminases prone to encapsidation into retroviral particles could create a need to both directly counteract the editing activity of these enzymes and to promote rapid completion of a double-stranded DNA viral genome through dual initiation of the plus strand.

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