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Compensatory Role of Human Immunodeficiency Virus Central Polypurine Tract Sequence in Kinetically Disrupted Reverse Transcription▽

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We tested whether the additional positive-strand DNA synthesis initiation of human immunodeficiency virus type 1 (HIV-1) from the central polypurine tract (cPPT) facilitates efficient completion of kinetically disturbed proviral DNA synthesis induced by dysfunctional reverse transcriptase (RT) mutants or limited cellular deoxynucleoside triphosphate (dNTP) pools. Indeed, the cPPT enabled the HIV-1 vectors harboring RT mutants with reduced dNTP binding affinity to transduce human lung fibroblasts (HLFs), without which these mutant vectors normally fail to transduce. The cPPT showed little effect on wild-type HIV-1 vector transduction in HLF, whereas it significantly enhanced vector transduction in HLFs engineered to contain reduced dNTP pools, suggesting a novel compensatory role for cPPT in viruses harboring kinetically impaired RT.

Lentiviruses such as human immunodeficiency virus type 1 (HIV-1) uniquely infect terminally differentiated/nondividing cells (8, 13, 14, 24). Previously, we reported intracellular deoxynucleoside triphosphate (dNTP) concentrations in human macrophages (20 to 50 nM) to be ~80- to 100-fold lower than in other virus target cell types, i.e., activated/dividing $CD4^+$ T cells (2 to 4 μM) (9), and this was recently confirmed by Perez-Bercoff et al. (18). Our follow-up studies revealed that reverse transcriptases (RTs) of lentiviruses efficiently polymerize DNA even at the low dNTP concentration found in macrophages due to their high binding affinity for the dNTP substrate (9, 16, 20). Alternatively, RTs of oncoretroviruses display efficient DNA synthesis only at the high dNTP concentrations found in dividing cells (9, 16, 20). The low dNTP binding affinity of oncoretroviral RTs must be sufficient to support viral replication because oncoretroviruses exclusively infect dividing cells containing high dNTP concentrations. A key implication of these findings is that the tight dNTP binding affinity of HIV-1 RT could be one of the mechanistic elements contributing to the unique infectivity of HIV-1 to nondividing macrophages.

The central polypurine track (cPPT) is a *cis* element of lentiviruses providing a second RNA primer for the initiation of positive [(+)]-strand DNA synthesis at the center of lentivirus genomes (3–6, 11, 21, 23), and the DNA flap structure was proposed to augment nuclear import of the viral preintegration complex in nondividing cells (2, 17, 26). Previous studies reported enhancement of vector transduction efficiency by the cPPT, and this cPPT effect was discussed mainly within the context of the nuclear targeting function of the cPPT and DNA flap (2, 7, 22). However, the nuclear import role of cPPT was also challenged by Dvorin et al. (10) and will likely remain controversial until further evidence is available.

In this report, we tested a hypothesis that an additional initiation of (+)-strand DNA synthesis from the cPPT RNA primer facilitates the efficient completion of proviral DNA synthesis because RTs need to synthesize only half of the genome during (+)-strand synthesis. Furthermore, we hypothesized that the role of the cPPT can be more evident under conditions that restrict proviral DNA synthesis kinetics, such as kinetically defective RT mutants or limited cellular dNTP pools. To test this hypothesis, we constructed an HIV-1 vector system transferring the enhanced green fluorescent protein (eGFP) gene with an insertion of a 15-mer HIV-1 cPPT sequence at the center of the HIV-1 transfer construct (pHR'CMV-GFP) (Fig. 1A) (27). In this construct, the 3′-end PPT primer and cPPT primer replicate almost equally sized (2.1- and 2.0-kb, respectively) (+)-strand proviral DNAs. We also introduced two HIV-1 RT mutations, Q151N and V148I, into the HIV-1 vector-packaging plasmid (pCMVΔR8.2) (Fig. 1B) (1, 15, 19), and these RT mutations are known to exhibit restricted polymerase activity specifically at low dNTP concentrations due to reduced dNTP binding affinity (9, 25).

First, we tested whether the additional initiation of (+)-strand DNA synthesis from the cPPT RNA primer at the center of the HIV-1 genome can compensate for delayed proviral DNA synthesis induced by enzymatically defective RT mutants such as the Q151N and V148I mutants, which exhibit reduced dNTP binding affinity. For this test, we transduced primary human lung fibroblasts (HLFs) cultured with 10% serum, which were previously found to contain ~150 to ~300 nM dNTP concentrations (12), with equal pg p24 levels of negative (−) and (+) cPPT wild-type (WT) or mutant RT HIV-1 vectors, and transduction efficiency was determined by fluorescence-activated cell sorting (FACS) for eGFP expression 48 h posttransduction. As shown in Fig. 2A, the WT HIV-1 vector efficiently transduces HLFs even without cPPT, and the cPPT insertion enhanced transduction efficiency only slightly in HLFs (Fig. 2B). This minimal effect of the cPPT on transduction efficiency (~20%) implies that HLF dNTP pools

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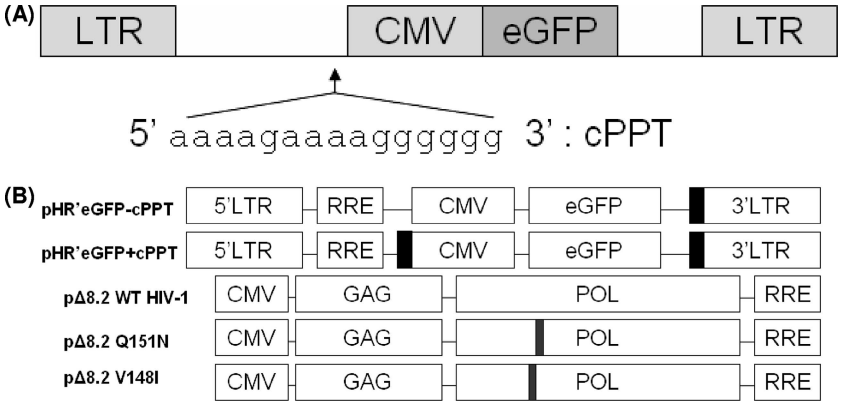

FIG. 1. HIV-1 vector constructs with cPPT and RT mutations. (A) The transfer pHR'CMV-GFP transfer vector (27) expressing eGFP from the cytomegalovirus (CMV) promoter was modified to contain a 15-bp cPPT sequence (5′-AAAAGAAAAGGGGGG-3′) at the ClaI site at the beginning of the CMV promoter sequence. In this construct, 3′ PPT and cPPT primers synthesize 2,180- and 2,030-nucleotide-long (+)-strand DNAs. (B) The Q151N and V148I mutations were created in the pCMVΔR8.2 HIV-1 packaging plasmid by site-directed mutagenesis. These vectors were pseudotyped by use of the vesicular stomatitis virus G envelope.

are sufficient and that WT HIV-1 RT is active enough to efficiently complete proviral DNA synthesis without the cPPT.

In contrast, as shown in Fig. 2, both (−) cPPT Q151N and V148I mutant vectors failed to efficiently transduce HLFs, which is consistent with the previous biochemical finding that these two RT mutants display restricted polymerase activity at the dNTP concentration found in HLFs (12). However, as shown in Fig. 2, the cPPT sequence enabled these RT mutant vectors to transduce HLFs and produce GFP at least 20 h earlier (data not shown), supporting our hypothesis.

Next, we tested whether the enhanced transduction of the RT mutant vectors by cPPT is due to improved proviral DNA synthesis kinetics by use of quantitative real-time PCR for two-long-terminal-repeat (2LTR) circles. As shown in Fig. 3A, while the (−) cPPT Q151N and V148I mutant vectors produced very low copy numbers of 2LTR circles in HLFs 48 h

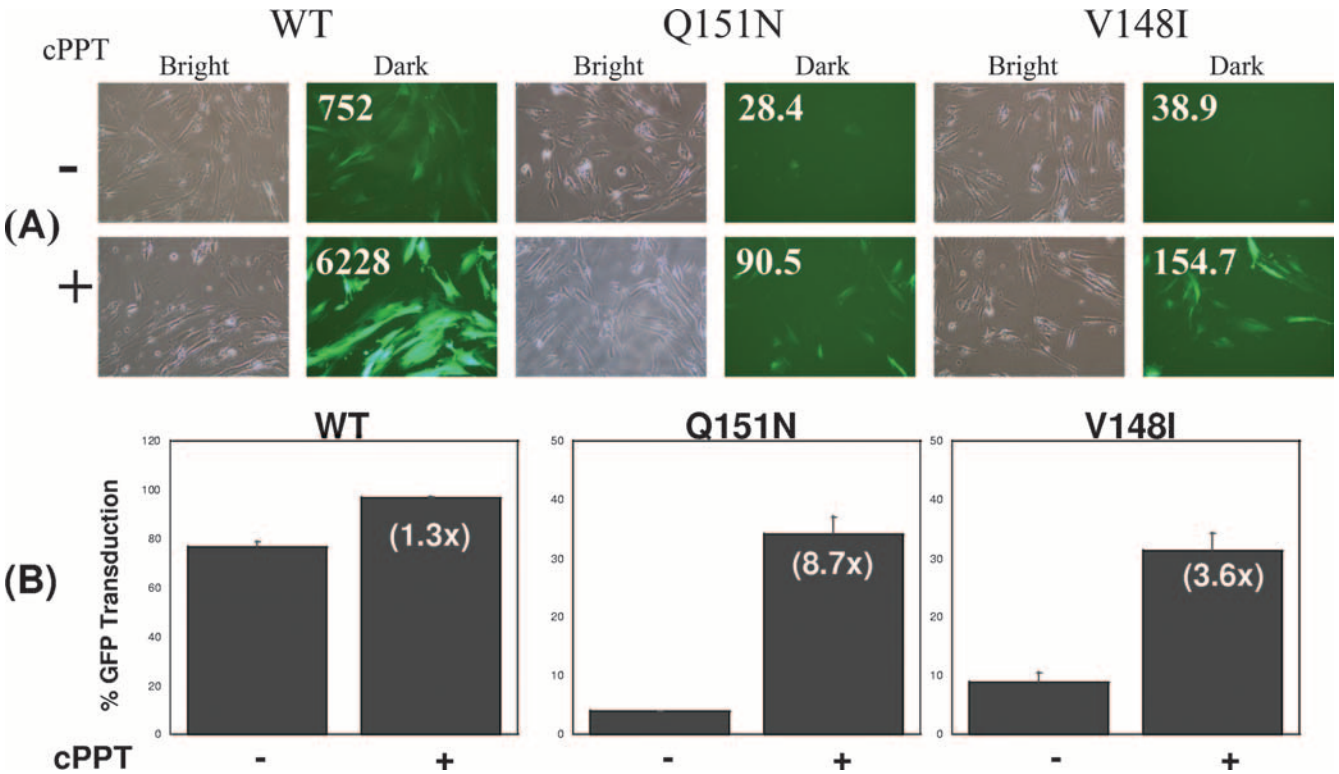

FIG. 2. Transduction of WT and RT mutant HIV-1 vectors in primary HLFs. HLFs cultured with 10% serum (3×10^5) were transduced with 2.8×10^5-pg p24 of WT or Q151N or V148I RT mutant vector with the inset mean fluorescence intensity values (A), and the percentages of the transduced cells were determined by FACS for eGFP expression at 48 h posttransduction (B). The experiments were performed in triplicate and values are presented with standard deviation of the mean.

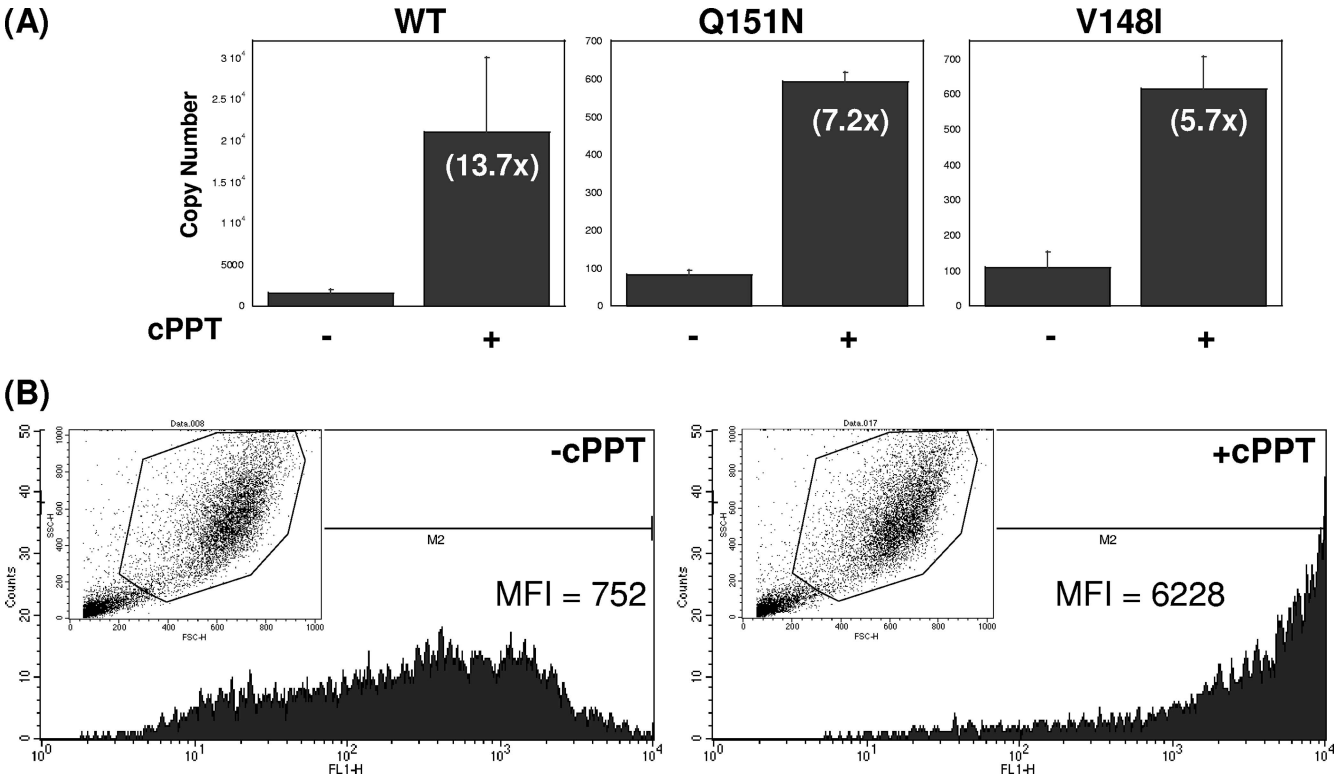

FIG. 3. Quantitative real-time PCR for 2LTR circles and GFP expression level of the transduced HLFs. (A) HLFs transduced with 2.8×10^5-pg p24 of (−) and (+) cPPT WT or Q151N or V148I RT mutant vector were harvested at 48 h posttransduction and their genomic DNAs were isolated. One microgram of genomic DNAs was used for the 2LTR circle quantitative real-time PCR as previously described (12), with standard deviation of the mean presented. (B) The intensities of green florescence in HLFs transduced by the (−) and (+) cPPT WT vectors at 48 h posttransduction were compared using the data obtained from the FACS data described for Fig. 2. MFI, mean fluorescence intensity; FSC, forward scatter; SSC; side scatter; -H, height.

posttransduction, the (+) cPPT mutant vectors were able to produce a significant level of 2LTR circle copies, indicative of efficient proviral DNA synthesis in HLFs. In contrast, even in the absence of the cPPT sequence, WT vector produced significantly high copy numbers of 2LTR circles without the cPPT, which is even greater than those observed for the RT mutant vector with cPPT (>1,500 copies per μg of genomic DNA). Clearly, these elevated copy numbers of the (+) cPPT RT mutant vectors as well as the (−) cPPT WT vectors were sufficient to express GFP in HLFs to a level detectable by FACS analysis, as discussed for Fig. 2.

Interestingly, however, as shown in Fig. 3A, the cPPT sequence also enhanced the 2LTR circle copy number of WT vector, unlike the FACS data, where the cPPT effect was minimal in the WT vector (Fig, 2B). We reasoned that the elevated 2LTR circle copy number by cPPT may affect the GFP expression kinetics in transduced cells by achieving early completion of viral replication and consequently higher levels of GFP per transduced cell. To test this, we analyzed the GFP intensity of cells transduced by (−) and (+) cPPT WT vectors by use of FACS 48 h posttransduction. As shown in Fig. 3B, the (+) cPPT WT vector exhibited a GFP signal 8.3 times as intense as that seen for cells transduced by the (−) cPPT vector, with a minimal difference in transduction efficiency (Fig. 2B). In fact, brighter GFP signals could be seen for HLFs transduced with the (+) cPPT WT vector than for cells transduced with the (−) cPPT WT vector (Fig. 2A). Therefore, the elevated 2LTR circle copy number in the (+) cPPT WT vector, which is indicative of accelerated completion of reverse transcription, appears to result in higher GFP accumulation in HLFs, although other factors, such as increased entry of viable virions, a higher percentage of virions completing reverse transcription, and earlier completion of reverse transcription, may contribute as well. Overall, the data shown in Fig. 2 and 3 support that the cPPT sequence enables HIV-1 variants harboring enzymatically dysfunctional RT mutants to infect cells by improving proviral DNA synthesis kinetics.

Next, we tested whether the cPPT can improve the transduction efficiency of the WT vector in serum-starved HLFs which contain reduced dNTP pools (50 to 60 nM [unpublished data]) compared to HLFs cultured with 10% serum (150 to 300 nM [12]). We also employed treatment with dNs (2.5 mM), which elevate intracellular dNTP concentrations (12). As shown in Fig. 4, the (−) cPPT WT vector displayed transduction efficiency ~20 times lower (1.8%) in serum-starved HLFs than in normally cultured HLFs (37%). However, this reduced transduction capability of the (−) cPPT vector in serum-starved HLFs could be partially rescued by dN treatment (4×) and cPPT insertion (10×). Similarly, dN treatment improved transduction of the (+) cPPT vector by 2.8-fold in serum-starved HLFs, and cPPT improved vector transduction in the serum-starved and dN-treated HLFs by 7-fold. The combina-

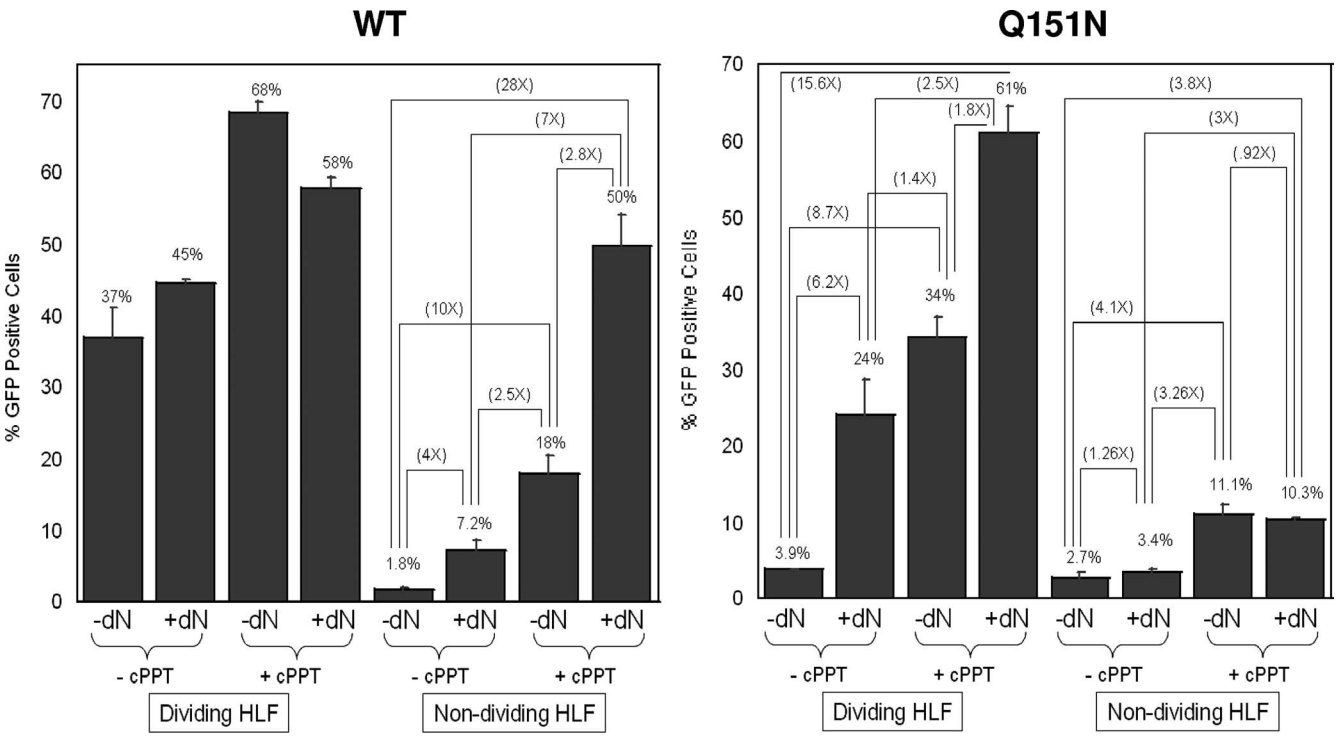

FIG. 4. Effect of cPPT and dN treatment on the transduction of WT and Q151N HIV-1 vectors in HLFs cultured in the presence or absence of 10% serum. HLFs cultured with 10% (dividing) or with 0.1% (nondividing) serum for 3 days were transduced with the (−) or (+) cPPT WT or Q151N vector (2.8×10^5 pg p24), and the transduction was determined by FACS for GFP expression at 48 h posttransduction. The 2.5 mM dN treatment was performed at 5 h before transduction as previously described (12). Data are presented with standard deviation of the mean.

tion of dN treatment and cPPT insertion resulted in an overall 28-fold increase of transduction efficiency in serum-starved HLFs and recovered the transduction level observed for dividing HLFs, while dividing HLFs gained only a small improvement by the combination of dN treatment and cPPT (37% to 58%) (Fig. 4).

The large impact (10×) of cPPT in serum-starved/nondividing HLFs compared to that of the dN treatment (4×) may be due to the additional nuclear targeting function of cPPT that dN treatment cannot mechanistically improve. However, the fact that the dN treatment further enhanced transduction of the (+) cPPT vector (2.8×) implies that cPPT alone may not be able to reach to the maximum replication kinetics. Importantly, the significant transduction enhancement by the dN treatment, which can be mimicked by cPPT, can be seen for serum-starved HLFs containing reduced dNTP concentration but not for dividing HLFs. Overall, the data shown in Fig. 4 imply that the cPPT, together with dN treatment, can improve WT vector transduction efficiency in serum-starved HLFs with limited dNTP pools.

We also tested whether the cPPT can improve the transduction efficiency of mutant vectors (only the Q151N vector is discussed) in serum-starved HLFs compared to that in HLFs cultured with 10% serum. We also employed treatment with dNs (2.5 mM). As shown in Fig. 4, the (−) cPPT Q151N vector displayed a transduction efficiency ~1.4 times lower (2.7%) for serum-starved HLFs than for normally cultured HLFs (3.9%). However, this reduced transduction capability of the (−) cPPT vector in serum-starved HLFs could be partially rescued by dN treatment (1.26×) and cPPT insertion (4.1×). Interestingly, dN treatment had a minimal effect on the transduction of the (+) cPPT vector in serum-starved HLFs (0.92×), and cPPT improved vector transduction in the serum-starved and dN-treated HLFs by threefold. The combination of dN treatment and cPPT insertion resulted in an overall 3.8-fold increase of transduction efficiency in serum-starved HLFs and failed to recover transduction levels observed for dividing HLFs, while the Q151N vector in dividing HLFs gained a 15.6-fold improvement by the combination of dN treatment and cPPT (3.9% to 61%).

This indicated to us that nondividing HLFs, even with dN treatment, may not provide the optimal environment to observe cPPT enhancement for vector harboring kinetically defective RT. This is most likely due to the dNTP binding defect of the mutant RTs becoming rate limiting during reverse transcription in nondividing HLFs with intracellular dNTP concentration below functional levels.

The data presented in this report support that the cPPT sequence can improve the transduction efficiency of HIV-1 vectors, particularly when proviral DNA synthesis becomes kinetically restricted by mutant RTs or limited cellular dNTP pools. This role of cPPT appears to be mechanistically connected with accelerated proviral DNA synthesis by having two (+)-strand DNA synthesis replication origins, resulting in fast completion of the full-length viral genome compared to what is seen for a single-replication initiation in the absence of cPPT.

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