

The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA

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High mutation frequency during reverse transcription has a principal role in the genetic variation of primate lentiviral populations. It is the main driving force for the generation of drug resistance and the escape from immune surveillance. G to A hypermutation is one of the characteristics of primate lentiviruses, as well as other retroviruses, during replication *in vivo* and in cell culture^{1–6}. The molecular mechanisms of this process, however, remain to be clarified. Here, we demonstrate that CEM15 (also known as apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G; APOBEC3G)^{7,8}, an endogenous inhibitor of human immunodeficiency virus type 1 (HIV-1) replication, is a cytidine deaminase and is able to induce G to A hypermutation in newly synthesized viral DNA. This effect can be counteracted by the HIV-1 virion infectivity factor (Vif). It seems that this viral DNA mutator is a viral defence mechanism in host cells that may induce either lethal hypermutation or instability of the incoming nascent viral reverse transcripts, which could account for the Vif-defective phenotype. Importantly, the accumulation of CEM15-mediated non-lethal hypermutation in the replicating viral genome could potentially contribute to the genetic variation of primate lentiviral populations.

HIV-1 Vif protein is required for viral replication *in vivo* and in some 'non-permissive' cells, such as peripheral blood mononuclear cells, macrophages and H9 T cells^{9–11}. The *vif*-defective viruses (Δ *vif*) from non-permissive cells cannot complete reverse transcription, or the newly synthesized DNA cannot exist in the target cells for a significant time period^{12–14}. Recently, it has been demonstrated that CEM15 is an endogenous inhibitor of HIV-1 that exists only in non-permissive cells. Its inhibitory effect on HIV-1 replication can be counteracted by HIV-1 Vif protein⁷. As Vif binds to HIV-1 RNA in the cytoplasm of virus-producing cells^{15–17}, we investigated whether CEM15, which shares significant homology with some other cytidine deaminases that edit RNA, could also edit HIV-1 genomic or spliced RNA. We have sequenced the nearly full-length genomic RNA (>98%) of HIV-1 from the Δ *vif* virions generated from H9 T cells by polymerase chain reaction with reverse transcription (RT-PCR) techniques (primer pairs are listed in Supplementary Table S1). Compared with the sequence of pNL4-3 Δ vif DNA, the change of genomic RNA in the virions is not significant. We have found an A to G change at positions 2257 and 3608, and a G to T change at position 9418 (data not shown). We have also sequenced several spliced HIV-1(NL4-3 Δ vif) RNA in H9 cells (Supplementary Fig. S1 and Table S1); however, no mutations were demonstrated.

CEM15, as well as some other homologues such as AID and APOBEC1, can function as a DNA mutator in *Escherichia coli*^{18,19}. As both CEM15 and Vif can be packaged into HIV-1 virions^{7,17}, we then investigated whether the newly synthesized HIV-1 DNA is a substrate of CEM15. We first compared the sequence of newly synthesized viral DNA in the presence or absence of Vif. The newly synthesized DNA of Δ *vif* viruses from H9 cells have G to A

substitutions in all the sequences (U3-R-U5, V3 and PR regions; primer pairs are listed in Supplementary Table S1) that we have analysed (Fig. 1a, row 2 of top panel, and data not shown). The mutation frequency at various regions is similar (data not shown). Notably, the pattern of G to A hypermutation in Δ *vif* viruses from H9 cells is consistent with the pattern of G to A hypermutation occurring during wild-type virus passage in cell culture and during *in vivo* viral growth^{3,5,6}. They both preferentially take place in a GpA or GpG dinucleotide context or in a group of G nucleotides. Besides the G to A substitution, other substitutions have also been found but with much lower frequency. Of note, as C to T substitution is not significantly higher than the rest of substitutions in the analysed regions, it has been classified into the category of 'other substitutions'. Conversely, the newly synthesized viral DNA from endogenous reverse transcription, which was driven by deoxyribonucleoside triphosphates (dNTPs) at equal amount, also has similar mutations, indicating that all the necessary factors have been packaged within the virions for this process, and that the G to A substitution is not dependent on the imbalance of dNTP pools (Fig. 1a, row 3 of top panel). The mutation frequency in viral DNA isolated from the intravirion reverse transcripts at 4 h after endogenous reverse transcription (ERT) initiation and from intracellular reverse transcripts at 12 h after infection is quite similar (data not shown). Conversely, no G to A substitution was found in Δ *vif* viruses from permissive SupT1 cells and the wild-type viruses generated from H9 or SupT1 cells (Fig. 1a, top panel, and 1b). Furthermore, we have also examined the effect of Vif on hypermutation in long-term cell culture by passing the wild-type or Δ *vif* viruses in C8166, a T-cell line that is semi-permissive for Δ *vif* viruses²⁰ and that expresses small amounts of CEM15 (ref. 7 and data not shown). We found that the G to A hypermutation in the Δ *vif* viruses is markedly higher than that in the wild-type viruses (Fig 1a, bottom panel, and 1c).

We then examined the effect of CEM15 on G to A substitution. 293T cells, which are permissive cells and in which no endogenous CEM15 is expressed⁷, were transfected with wild-type or Δ *vif* HIV-1 DNA in the presence or absence of CEM15. After 48 h, the viruses in the supernatants were then allowed to infect C8166 cells. We found that the newly synthesized DNA of Δ *vif* viruses with CEM15 contained markedly more G to A hypermutations than that in the newly synthesized DNA from Δ *vif* viruses without CEM15, or from wild-type viruses (Fig. 2a, top panel, and 2b). Again, the endogenous reverse transcripts of Δ *vif* viruses with CEM15 also contained G to A hypermutations (Fig. 2a, row 5 of top panel). Similar phenomena were also found in the newly synthesized viral DNA of Δ *vif* viruses that were generated from the SupT1 T cells containing retrovirally transduced CEM15 (Fig. 2b).

However, it is clear that the G to A hypermutation still occurs in the presence of the *vif* gene when the viruses replicate in the cell culture for several passages^{3,21}. We then examined the effect of CEM15 on the mutation frequency of wild-type viruses in long-term cultures. The CEM15 gene was transduced into SupT1 cells by a Moloney murine leukaemia virus (MMLV) retroviral vector. The wild-type viruses were then passaged in the SupT1 cells, with or without CEM15 expression. After four passages, the viral DNA was amplified and sequenced. We found that wild-type viruses passaged in SupT1 cells containing CEM15 have significantly more G to A hypermutations in their genomes than those in SupT1 cells without CEM15 (Fig. 2a, bottom panel, and 2c). This result further demonstrated that CEM15 is able to induce G to A hypermutation and could be responsible, at least in part, for the hypermutation in the long-term culture of wild-type viruses. Less Vif incorporation into virions in chronic infection might decrease its counteracting effects on CEM15 in the virions (Fig. 2d)²². It is notable that CEM15 does not significantly affect the dNTP pools in SupT1 cells and C8166

cells (Supplementary Fig. S2), excluding the possibility that CEM15 induces the imbalances in the dNTP pools in cells.

To verify that CEM15 has cytidine deaminase activity, glutathione *S*-transferase (GST)-CEM15 fusion protein was purified from *E. coli*. Figure 3 illustrates that CEM15 indeed has cytidine deaminase activity that can be inhibited with tetrahydrouridine (THU). By sequence alignment, it is predicted that CEM15 contains two zinc finger domains⁷. The similar zinc finger domains in other cytidine deaminases have a principal role in cytidine deaminase activity²³. To verify that the zinc finger domains in CEM15 are important for cytidine deaminase, several mutations at zinc finger domains were generated. Figure 3b demonstrates that the mutants at either zinc domains have significantly decreased cytidine deaminase activity. To determine whether purified GST-CEM15 contains the enzymatic activity to edit viral RNA, DNA, or DNA-RNA duplexes, PCR-amplified DNA, *in vitro*-transcribed RNA, or *in*

vitro reverse-transcribed RNA-DNA duplexes were incubated with GST-CEM15, respectively. All the nucleic acids were generated from the same HIV-1 fragment (nucleotides 2134-2594). After incubation, the DNA, RNA or RNA-DNA duplexes were used as the templates for PCR or RT-PCR amplification. The PCR products were then inserted into the pGEM-T vector and sequenced. No mutation was found (data not shown). It is probable that, similar to APOBEC1, CEM15 requires additional factor(s) to perform editing^{23,24}.

The CEM15 mutants were then co-transfected with pNL4-3Δ*vif* into 293T cells to test their functional activities. After 48 h, the viruses in the supernatants were collected. The virions, normalized for quantity, were then used to infect C8166 cells or HLCD4-CAT cells. Twelve hours after infection, the newly synthesized DNA in C8166 cells was extracted, followed by PCR amplification and sequencing. The lysate of HLCD4-CAT cells was collected for CAT

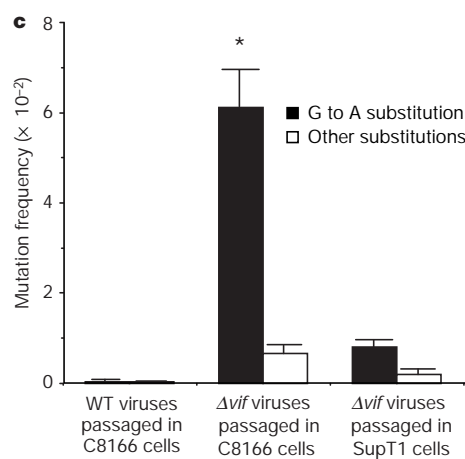
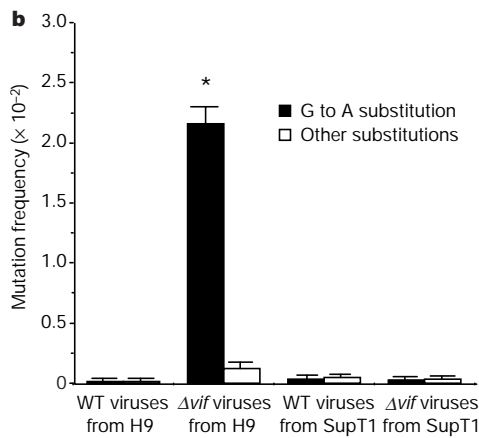
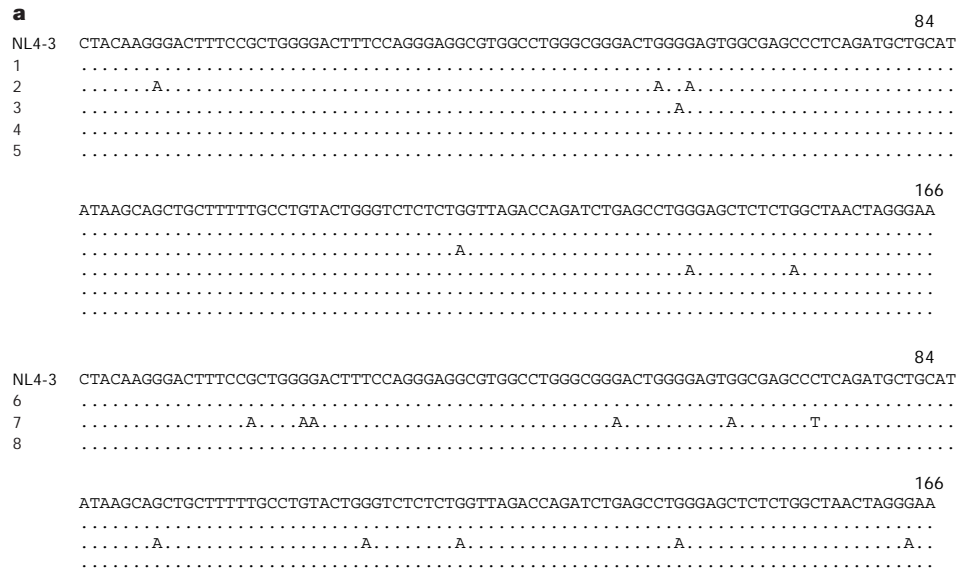


Figure 1 G to A hypermutation in viral DNA of Δvif viruses from non-permissive cells or semi-permissive cells. **a**, The DNA sequence alignments in the U3-R region. The clones with the most-identical sequences from the total clones ($n = 8$) are listed. The newly synthesized viral DNA are listed in the top panel. The rows are: (1) wild-type viruses from H9 cells to infect C8166 cells; (2) Δvif viruses from H9 cells to infect C8166 cells; (3) Δvif viruses from H9 cells to process ERT; (4) wild-type viruses from SupT1 cells to infect C8166 cells; (5) Δvif viruses from SupT1 cells to infect C8166 cells. After two passages, the viral DNA in C8166 cells was sequenced (bottom panel). The rows are: (6) wild-type

viruses; (7) Δvif viruses; (8) Δvif viruses passed in SupT1 cells. **b**, Comparison of mutation frequency of G to A in the newly synthesized DNA of viruses generated from H9 or SupT1 cells (301 nucleotides in U3-R-U5 region, $n = 8$). The G to A mutation frequency of Δvif viruses from H9 cells to infect C8166 cells is significantly higher than that of others (asterisk, $P < 0.001$, *t*-test). WT, wild type. **c**, Comparison of the G to A mutation frequency in the viral DNA after passage in C8166 cells ($n = 8$). The G to A mutation frequency of Δvif viruses passed in C8166 is significantly higher than that of wild-type viruses (asterisk, $P < 0.001$, *t*-test).

assay after 48 h. Figure 4 shows that, compared with wild-type CEM15, CEM15 mutants that contain less cytidine deaminase activity could not decrease the infectivity of Δvif viruses in permissive HLC4-CAT cells (Fig. 4a). Furthermore, the frequency of G to A hypermutation in the newly synthesized DNA induced by the mutants is also much lower than that induced by wild-type CEM15 (Fig. 4b). These data suggest that CEM15 induces G to A hypermutation in the newly synthesized viral DNA through its cytidine deaminase activity.

Our data have indicated that the genomic RNA of Δvif viruses from non-permissive cells does not contain the G to A substitution, and CEM15 does not cause a significant imbalance of dNTPs in the

cells. We have also found that, in the presence of CEM15, the product of endogenous reverse transcription of Δvif viruses at high and equal concentration of dNTPs (1 mM) contains the G to A hypermutation. Furthermore, the hypermutation preferentially takes place in a GpA or GpG dinucleotide context or in a group of G nucleotides, which does not occur when the imbalance of the dTTP/dCTP ratio induces dC to dT substitution. On the basis of these observations, we propose that CEM15 may directly deaminate dC in the newly synthesized viral DNA and convert it to dU. The dC to dU conversion in the minus strand DNA of Δvif viruses would further be base-pair-matched by dA during plus-stranded viral DNA synthesis, generating G to A hypermutation in the newly

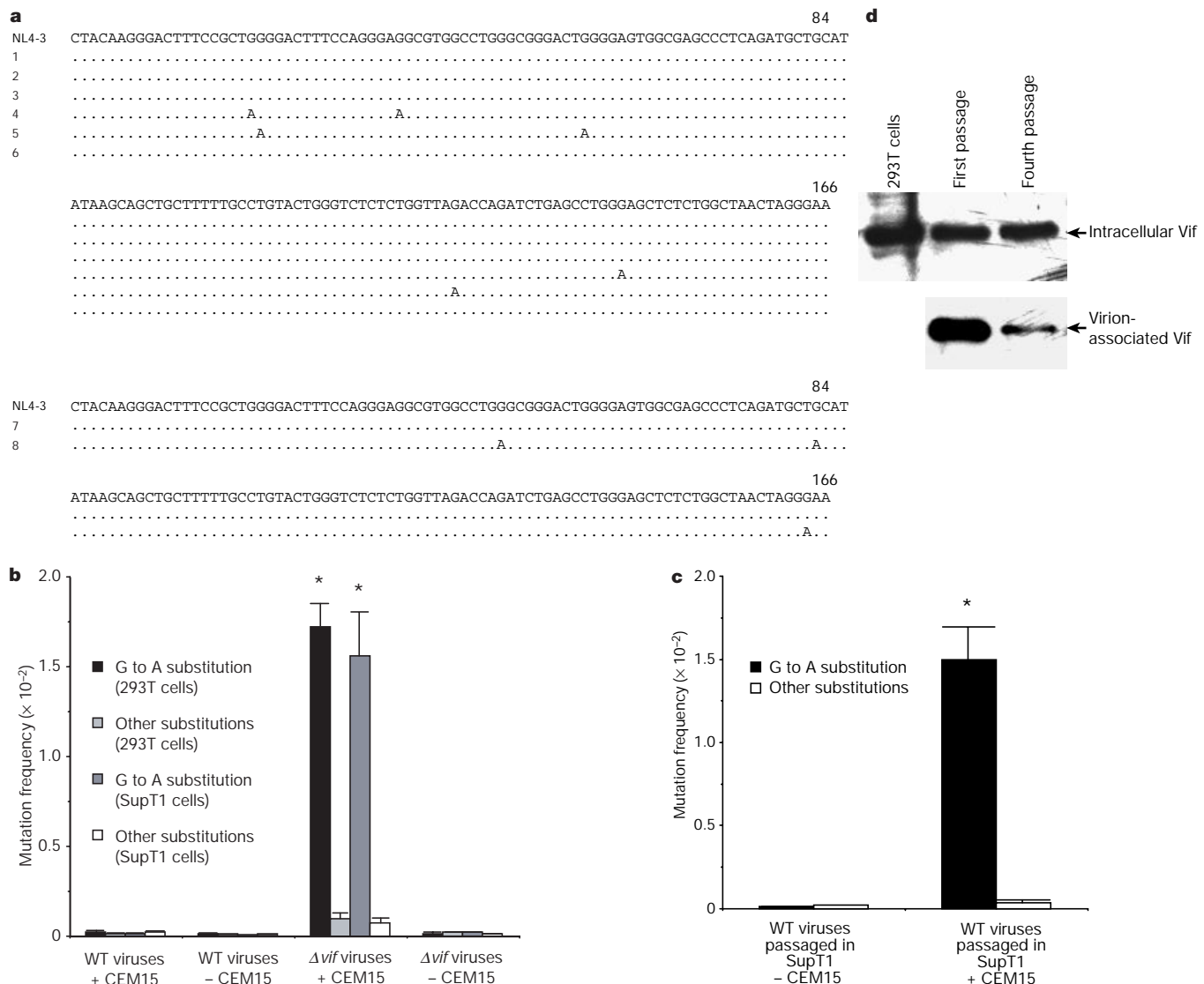


Figure 2 G to A hypermutation in newly synthesized DNA of viruses generated from cells containing CEM15. **a**, DNA sequence alignment in HIV-1 U3-R region. The clones with the most-identical sequences from the total clones ($n = 8$) are listed. Viruses were from 293T (top) cells or passaged in SupT1 (bottom) cells. In the top panel, the rows are: (1) wild-type viruses, with CEM15, to infect C8166 cells; (2) wild-type viruses, with CEM15, to process ERT; (3) wild-type viruses, without CEM15, to infect C8166 cells; (4) Δvif viruses, with CEM15, to infect C8166 cells; (5) Δvif viruses, with CEM15, to process ERT; (6) Δvif viruses, without CEM15, to infect C8166 cells. In the bottom panel, the rows are: (7) wild-type viruses, without CEM15; (8) wild-type viruses, with CEM15. **b**, Comparison of G to A mutation frequency in newly

synthesized DNA (301 nucleotides in U3-R-U5 region, $n = 8$). The G to A mutation frequency of Δvif viruses from 293T or SupT1 cells containing CEM15 is significantly higher than that of others (asterisk, $P < 0.001$, t -test). **c**, Comparison of G to A mutation frequency in the viral DNA after passage ($n = 8$). The G to A mutation frequency of wild-type viruses passaged in SupT1 cells containing CEM15 is significantly higher than that in SupT1 cells without CEM15 (asterisk, $P < 0.001$, t -test). **d**, The wild-type viruses, after various passages in SupT1 cells containing CEM15, were purified²⁹. After normalization with HIV-1 p24, the virion-associated Vif protein was detected by western blot¹⁵.

synthesized viral DNA. If integrated, the Δvif proviral DNA containing the lethal hypermutation might encode 'pre-mature stop' or mutated viral proteins, therefore generating defective viruses. Conversely, the deaminated Δvif viral DNA may become unstable in the cytoplasm. One of the possibilities is that the uracil-DNA glycosylase (UDG) enzyme, which has been packaged into the HIV-1 virions, could excise the dU from the DNA. Accordingly, a break point in the minus strand of viral DNA might occur, which may no longer be able to serve as the template for full-length viral DNA synthesis and thus would gradually be degraded.

Previous work has found that G to A hypermutation cannot be reproduced in a cell- and virus-free reverse transcription reaction when the concentrations and ratios of dNTPs are similar to those in living cells. When the dTTP/dCTP ratio rises to 10,000 to 1, dC to dT substitution occurs²⁵. Furthermore, there is evidence suggesting that G to A hypermutation occurs in thymidine-treated U937-2 cells²⁶. Here, we have not only indicated that G to A hypermutation occurs in the newly synthesized DNA of Δvif viruses generated from the non-permissive cells, but we have also

shown that CEM15 is responsible for this phenomenon. Furthermore, we have demonstrated that CEM15 induces non-lethal hypermutation in the viral DNA of wild-type viruses passaged in a long-term culture. Therefore, we propose that CEM15 could be responsible for the hypermutation observed *in vivo* and in cell culture³⁻⁶.

It has been reported that interferon-inducible, double-stranded RNA-specific adenosine deaminase can also extensively edit the

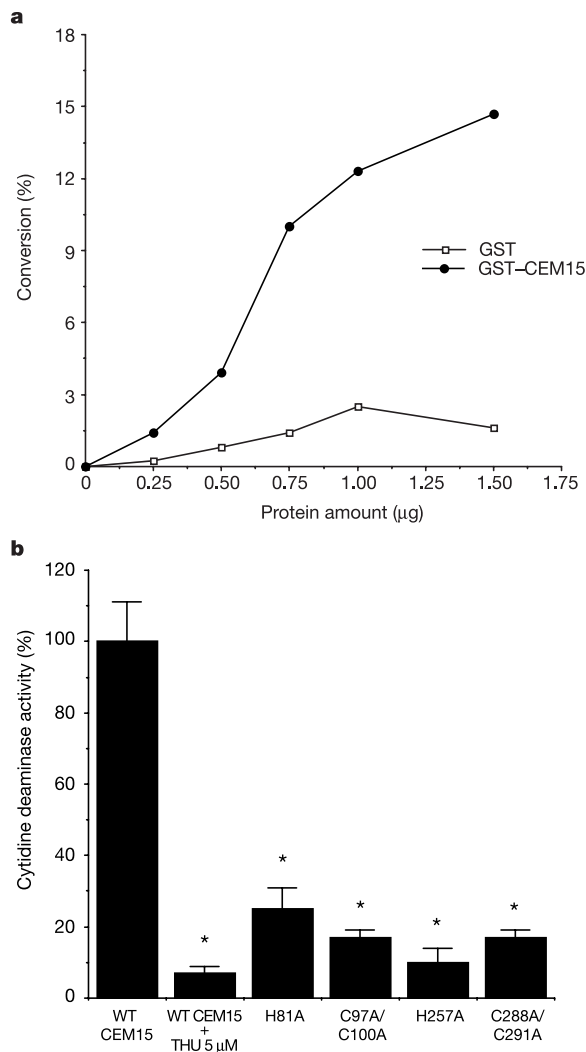


Figure 3 CEM15 has cytidine deaminase activity *in vitro*. **a**, GST-CEM15, but not GST alone, converts deoxycytidine to deoxyuridine *in vitro* in a concentration-dependent manner. This figure represents five independent experiments. **b**, Mutations at zinc finger domains significantly decrease cytidine deaminase activity (asterisk, $P < 0.001$, *t*-test). THU significantly inhibits cytidine deaminase activity (asterisk, $P < 0.001$, *t*-test).

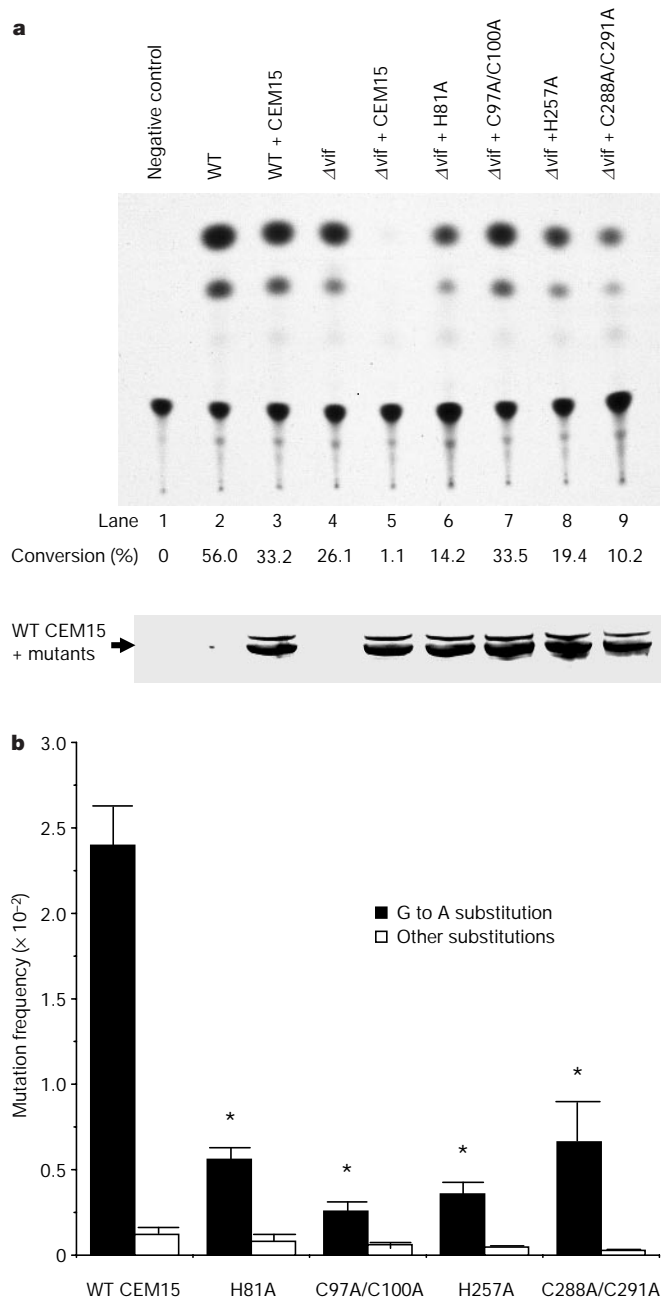


Figure 4 CEM15 without cytidine deaminase activity can neither inhibit the infectivity nor induce hypermutation in the newly synthesized DNA of Δvif viruses. pNL4-3 or pNL4-3 Δvif were transfected into 293T cells with CEM15 or CEM15 mutants. **a**, The viruses were used to infect HLCD4-CAT cells. CAT assays were performed. This figure represents three independent experiments. **b**, The newly synthesized viral DNA in C8166 cells was amplified by PCR and sequenced. The G to A mutation frequency of Δvif viruses from 293T cells containing mutant CEM15 genes is significantly lower than that containing wild-type CEM15 (asterisk, $P < 0.01$, *t*-test).

RNA genome of some RNA viruses in virus-producing cells^{27,28}. We have now demonstrated that G to A hypermutation in newly synthesized reverse transcripts might be induced by a similar host defence factor. Further investigations are needed to determine the precise molecular mechanism(s) of this unique interaction of primate lentiviruses with the host cells. □

Methods

Plasmid constructions, mutagenesis and protein purification

The CEM15 gene with a Flag-tag sequence at its 3' terminus was amplified from the messenger RNA of H9 cells through RT-PCR, and the sequence was confirmed. It was then cloned into various vectors: pCDNA3 for transfection into 293T cells; pGEX for GST fusion protein expression; and pSLX-CMV for retroviral (MMLV) transduction into SupT1 cells. CEM15 mutations were generated by a PCR-based mutagenesis approach¹⁵. The GST, GST-CEM15 and other GST fusion CEM15 mutant proteins were produced according to previously described methods¹⁵.

Transfections

The 293T or T-lymphoid cells (H9 or SupT1) were transfected with pNL4-3 or pNL4-3Δvif (2 μg), plus various plasmids (0.75 μg pCDNA3-CEM15 or its mutants), with FuGene 6 transfection reagents (Roche). The viruses in the supernatants were collected after 48 h. Of note, pNL4-3Δvif was constructed in our laboratory and has been described previously¹⁵.

Intracellular reverse transcription

Wild-type or Δvif viruses (10 ng of p24 equivalents), generated from either permissive cells or non-permissive cells, were treated with RQ1 DNase (5 U) (Promega) at 37 °C for 1 h and then allowed to infect C8166 T cells. Twelve hours after infection, the infected cells (in 150 μl TN buffer) were treated again with DNase (5 U) and incubated at 37 °C for 1 h. After washing, the newly synthesized viral DNA was extracted from the cells and amplified by PCR²⁰.

Endogenous reverse transcription

The wild-type or Δvif viruses (75 ng of p24 antigen equivalents) were concentrated by ultracentrifugation, followed by DNase treatment at 37 °C for 1 h. After washing off DNase, the viruses in the TN buffer were incubated with 1 mM dNTPs, 2.5 mM MgCl₂, and 15 μg ml⁻¹ melittin at 37 °C for 4 h^{20,29}. The newly synthesized DNA was isolated and amplified with PCR. The PCR products were inserted into the pGEM-T vector and individual clones were sequenced and analysed.

Viral passage experiments

The retroviral vectors (MMLV) carrying the CEM15 gene (pSLX-CMV-CEM15) or the vector alone were first transduced into SupT1 T cells, following methods described previously¹⁵. The naive C8166 or G418-resistant SupT1 T cells or C8166 cells were infected by wild-type or Δvif viruses (0.1 ng of p24 antigen equivalents), which were generated from 293T cells through transfection with pNL4-3 or pNL4-3Δvif. The infected T cells were then cultured in RPMI1640 medium plus 10% fetal bovine serum. After 2 weeks, the progeny viruses in the supernatants were allowed to infect C8166 or SupT1 cells, with or without CEM15, respectively. This procedure was repeated two or four times. The viral DNA in the final round of infected cells was extracted and amplified by PCR, followed by insertion into pGEM-T vector. The individual clones were then analysed by sequencing.

DNA PCR, RT-PCR and DNA/complementary DNA sequencing

The viral DNA was extracted from viruses or infected cells and PCR was performed, as described previously²⁰. The viral RNA was extracted from viruses or infected cells through RNeasy Mini Kits (Qiagen). DNase treatment was performed to eliminate any contaminating DNA. The RT-PCR was performed with ready to go RT-PCR beads (Amersham-Pharmacia). The PCR products were inserted into the pGEM-T vector and individual clones were sequenced. The mutation frequency (*f*) was calculated as: (number of substitutions)/(number of target nucleotides).

Cytidine deaminase assay

A method described previously³⁰ was performed to determine the cytidine deaminase activity of CEM15, with some modifications. Briefly, GST-CEM15 at various amounts was incubated with 4 μCi ³H-deoxycytidine (23 Ci mmol⁻¹; Moravsek) and 250 μM cytidine in a total volume of 10 μl Tris-HCl buffer (45 mM, pH 7.5). After incubation at 37 °C for 4–6 h, the reactions were quenched by adding 2 μl of 10 μg μl⁻¹ each of deoxycytidine and deoxyuridine. Four microlitres of sample was then added onto a silica-polyester TLC plate (Sigma-Aldrich). The plate was developed in 10:1:0.5 (v/v) ethyl acetate:methanol:trichloroacetic acid (6 M) for 2–3 h. The deoxycytidine and deoxyuridine bands were visualized by exposure to 254 nm ultraviolet light and cut separately into scintillation fluid for ³H-isotope quantifications.

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

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