Genetic Editing of HBV DNA by Monodomain Human APOBEC3 Cytidine Deaminases and the Recombinant Nature of APOBEC3G

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Abstract
Hepatitis B virus (HBV) DNA is vulnerable to editing by human cytidine deaminases of the APOBEC3 (A3A-H) family albeit to much lower levels than HIV cDNA. We have analyzed and compared HBV editing by all seven enzymes in a quail cell line that does not produce any endogenous DNA cytidine deaminase activity. Using 3DPCR it was possible to show that all but A3DE were able to deaminate HBV DNA at levels from 10^{-2} to 10^{-5} in vitro, with A3A proving to be the most efficient editor. The amino terminal domain of A3G alone was completely devoid of deaminase activity within the sensitivity of 3DPCR (~10^{-4} to 10^{-3}). Detailed analysis of the dinucleotide editing context showed that only A3G and A3H have strong preferences, notably CpC and TpC. A phylogenic analysis of A3 exons revealed that A3G is in fact a chimera with the first two exons being derived from the A3F gene. This might allow co-expression of the two genes that are able to restrict HIV-1Δvif efficiently.

Introduction

The human APOBEC3 locus on chromosome 22q13.1 encodes at least seven genes encoding cytidine deaminases with single stranded DNA substrate specificity [1]. While their precise cellular roles remain to be defined, human APOBEC3F and 3G (A3F and A3G) are so active on nascent lentiviral cDNA that they do not produce any endogenous DNA cytidine deaminase activity. Using 3DPCR it was possible to show that all but A3DE were able to deaminate HBV DNA at levels from 10^{-2} to 10^{-5} in vitro, with A3A proving to be the most efficient editor. The amino terminal domain of A3G alone was completely devoid of deaminase activity within the sensitivity of 3DPCR (~10^{-4} to 10^{-3}). Detailed analysis of the dinucleotide editing context showed that only A3G and A3H have strong preferences, notably CpC and TpC. A phylogenic analysis of A3 exons revealed that A3G is in fact a chimera with the first two exons being derived from the A3F gene. This might allow co-expression of the two genes that are able to restrict HIV-1Δvif efficiently.
sensitivity of 3DPCR it should be possible to identify low levels of editing that might not be readily picked up in phenotypic assays. Using the quail cell line QT6 that does not generate any endogenous editing background, it is shown that 6/7 APOBEC3 enzymes can deaminate a small fraction of hyperedited HBV genomes in vitro. CDD1 of A3G is totally devoid of deaminase activity to within the sensitivity of 3DPCR (~10⁻⁴). Careful phylogenetic analysis revealed that APOBEC3G is a chimera with the first two exons coming from APOBEC3F.

Results

HBV editing by monodomain A3A and A3H

3DPCR is so sensitive that it can amplify heavily APOBEC3 edited HIVΔvif genomes even from a permissive cell line such as 293T [40]. It was presumed that this resulted from clonal heterogeneity whereby a small fraction of cells are expressing high levels of A3F/G. This phenomenon has been encountered for some other cell lines, such as HuH7 (not shown). To overcome this problem we screened a number of avian cell lines because the avian genome does not encode APOBEC1 or APOBEC3 homologues [43]. By contrast, it encodes a single copy of AID and APOBEC2. The quail muscle fibroblast cell line QT6 was chosen for it is readily transfectable.

QT6 cells were transfected with the infectious molecular clone of HBV, pCayw, in the presence or absence of A3A and A3H expression plasmids, the two A3 genes not examined in our previous study [14]. Seventy-two hours post-transfection, supernatant virus was harvested and treated with DNaseI. By decreasing the denaturation temperature (Td) it was shown that 90°C was the minimal temperature allowing amplification of HBV infection alone (Figure 1A). By contrast, 3DPCR could recover HBV DNA from the A3A and A3H co-transfections at temperatures as low as 82.3 and 87.1°C respectively.

The 3DPCR products from the Td = 88.4°C amplification were cloned and up to 60 clones sequenced per sample. A complete collection of hypermutated sequences is available in Figures S1. The A3A and A3H enzymes were able to extensively deaminate HBV DNA (Figure 1B). Both DNA strands were heavily edited with mean editing frequencies of 20.5% and 40.1% for A3A on the minus and plus strands respectively, and 6.2% and 11.7% for A3H. All sequences were unique indicating that they represented independent molecular events. Indeed, the degree of A3A editing was among the most extensive we have observed using HBV—up to 95% of target C residues were edited whether they be on the plus or minus strands (Figure S1).

3DPCR products from the last positive amplification (Td = 90.1°C, Figure 1A) for the HBV alone control were also cloned and sequenced. No hypermutated sequences were identified at all indicating that the QT6 cell line did not generate a background editing signal (Figure 1C). To confirm this, a HIVΔvif molecular clone was transfected into QT6 cells and the resulting progeny used to infect the CEMX174 cell line. A 3DPCR cloning and sequencing analysis failed to identify any viral hypermutants (not shown), supporting the conclusion that the QT6 cell line does not generate any background signal.

Uniquely the C-domain of APOBEC3G is active

Although the literature indicates that the amino-terminal domain of A3G is without editing activity [30,31], this doesn’t formally preclude a weak-editing activity. As 3DPCR has a greater dynamic range compared to phenotypic assays, 4 logs compared to ≤1.3 logs, we addressed the question of the activity of A3G CDD1 and CDD2 by two approaches. Individual A3G N- and C-
terminal domains (CDD1 and CDD2 respectively) genes were made by deletion of exons 5–7 and 2–4 respectively compared to the parental molecule generating A3Gn and A3Gc respectively (Figure 2A). These constructs differ subtly to others [30,31,44] in that the core exons 2–4 or 5–7 are always surrounded by exons 1 and 8. Following transfection of HeLa cells the stability of the A3Gn and A3Gc was analyzed by immunofluorescence microscopy using FITC-labelled anti-V5 antibodies. Both constructs were as stable as the parental A3G. Although A3Gn showed a somewhat greater nuclear distribution compared to A3G (Figure 2B), a substantial fraction was in cytoplasm, which is where HBV capsid assembly occurs.

QT6 cells were transfected by pCayw in the presence or absence of the different expression plasmids (A3G, A3Gn, A3Gc). Seventy-two hours post-transfection, virus was harvested and treated with DNaseI. At 90.4°C it was possible to amplify the complete HBV segment alone (Figure 3A). At 89.2°C lower molecular weight bands were found for the negative control and A3Gn (Δ, Figure 3A); however cloning and sequencing revealed that these sequences contained internal deletions and no C→T transitions. By contrast, DNA was recovered between 87.9–89.2°C for the A3Gc transfection and 85.9–89.2°C for the A3G control (*, Figure 3A).

Cloning and sequencing of the 3DPCR products confirmed that these sequences were indeed hypermutated (Figure 3B, Figure S1). The average editing efficiencies for genomes recovered at the same denaturation temperature (89.2°C) were comparable for A3G (G→A 14.9%; C→T 21.7%) and A3Gc (G→A 9.5%; C→T 22.6%) indicating that the carboxy-terminal domain alone contains the editing function. To see whether the protocol could pick up differing degrees of editing a titration was performed using A3G. As can be seen form Figure 3C, the degree of editing is in proportion to the mean A3G plasmid concentration. Accordingly, it may be presumed that the 3DPCR products recovered for A3G down to 85.9°C are even more hypermutated.

To explore the hypothesis that CDD1 of A3G was slightly active uniquely in the presence of CDD2, a C291S mutant of A3G was used [15]. Cysteine 291 is one of the residues that chelates the zinc atom in CDD2 and is essential to the activity of all A3 enzymes tested to date. HBV+A3GcC291S were transfected into QT6 cells and 3DPCR performed on supernatant virus. As can be seen from Figure 4A no products were recovered at the restrictive temperature of 88°C. Products recovered at 95°C amplifications were cloned and sequenced. No excess of GC→AT substitutions was noted compared to controls (Figure 4B).

Among all human APOBEC3 molecules, CDD1 of A3G is unique in that the alanine residue in the highly conserved HAEXnPCX2C motif is substituted by proline, a feature characteristic of the primate A3G lineage back to macaque and baboons [45]. Given the profound changes a proline residue can have on protein folding, a P→A substitution was engineered into codon 66 of the A3Gn and A3GcC291S constructs. This P66A substitution failed to recover any deaminase activity in either a mono- of double domain construct (A3GnP66A and A3GP66A/C291S, Figure 4).

Comparable A3G and A3Gc hot and cold spots

The editing frequencies of all 45 cytidine targets in minus strand HBV DNA for A3G and A3Gc in the QT6 derived experiments were compared and found to be highly correlated (Figure 5A, B), again indicating that editing specificity resided in CDD2. In order to see if the cell line used in the transfection essay could influence the site specific editing frequencies, A3G and A3Gc editing of HBV was compared in both cell lines QT6 and Huh7. Site specific editing frequencies were highly correlated between the two cell lines for both APOBEC3 constructs (Figure 5C, D) indicating that
Figure 3. Uniquely the C-terminal domain of A3G is catalytically active. A) 3DPCR amplification using an 11°C gradient in denaturation temperature across the heating block. M denotes molecular weight markers, pv the empty expression vector, the white asterisks denote hyperedited HBV DNA, while the white triangles indicate PCR products containing internal deletions (confirmed by sequencing of cloned products). B) Mutation matrices for A3G and A3Gc on QT6 cells where n indicates the number of bases sequenced. C) The inter-conversion of G→A is related to the concentration of A3G co-transfected.

doi:10.1371/journal.pone.0004277.g003

Figure 4. Proline 66 is not responsible for the inactive N-terminal Zn finger domain of A3G. A) PCR amplification of HBV DNA from a variety of co-transfection experiments at 95°C and the restrictive temperature of 88°C. M denotes molecular weight markers; C-, amplification control without DNA; C+, pCayw alone; pv the empty expression vector as negative control; A2, APOBEC2 as another negative control. Primer-dimers represent the low molecular weight band. B) Mutational analysis of the cloned 95°C PCR products and those of the positive A3G control at 88°C.

doi:10.1371/journal.pone.0004277.g004
the QT6 cell line did not introduce any bias into the editing process, which suggests that editing occurs independently of any human protein partner.

To estimate the frequency of editing, limiting dilution of first round PCR products was performed at 95°C and the restricting temperature of 89.2°C. The frequencies of hyperedited genomes were A3A ≤10^{-2}, A3G ≤10^{-3}, A3Gc ≤10^{-4}, and A3H ≤10^{-5} (data not shown). The values are lower limits in that not all the cells were doubly transfected by pCayw and the A3 expression vector. The results show that the A3Gn domain increases the efficiency of A3G editing, while A3A turns out to be particularly efficient (Figures 1A and 3A).

Dinucleotide context of HBV editing by six A3s

As there was no difference between site specific A3G editing of HBV in the QT6 and Huh7 cell lines, the dinucleotide contexts for G→A hypermutants can be compared using data sets for A3A and A3H derived from QT6 and A3B, A3C, A3F ad A3G derived from Huh7. As mentioned above, we were unable to recover any A3DE hyperedited HBV genomes. In view of the general excess of G→A hypermutants over C→T hypermutants, only the former, which reflect editing of the minus strand were considered. Sequences with >80% of cytidine residues edited were excluded from the analysis in order not to mask any dinucleotide preferences. Obviously, the greater the degree of editing, the more the observed and expected dinucleotide frequencies converge. Three distinct patterns were apparent. A3G showed a strong and significant preference for CpC while A3H showed a predilection for TpC (Figure 6A). Both showed a strong aversion for GpC and ApC. The remaining four A3s showed weaker dinucleotide biases with A3F even showing a slight, but significant bias in favour of GpC.

The lack of any strong preference for TpC by A3F was surprising as it is a frequent finding in the HIV-1 literature [4,8,46–48]. In view of this, we generated comparable hyperedited data sets for HIVΔvif and the same 6 A3 constructs co-transfected into QT6 cells. Supernatant HIV was then used to infect CEMX174 cell line. DNA was recovered after 18 h and 3DPCR performed on a region corresponding to the hypervariable V1V2 regions of env, the restrictive temperature being 78°C (Figure 7A). 3DPCR products amplified at 78°C were cloned and sequenced. Large numbers of hypermutants were obtained all with G→A mutation frequencies >100 in excess of the control (Figure 7B, Figure S2). The dinucleotide frequencies associated with deamination on the minus strand (G→A on the plus strand) were analyzed in a comparable manner to the HBV hypermutants, that is those with >80% editing were eliminated from Figure 6B. Five of the six A3s showed a strong and significant preference for TpC.

Figure 5. Human A3G and A3Gc hot and cold spots are highly correlated. A) Individual editing frequencies across the minus strand target cytidine residues for A3G and A3Gc co-transfections of Huh7 cells. These represent the fraction of A3 edited sequences bearing an edited C at a given position. B–D) Highly correlated site-specific editing frequencies for A3G, A3Gc/HBV co-transfections of QT6 and Huh7 cells. The site-specific editing frequencies calculated for a given data set are correlated with the site-specific editing frequencies from another data set at the level of each C residue. The identities of the data sets are given on the x and y-axes. Least-mean squared fits to the data are also given. doi:10.1371/journal.pone.0004277.g005
while only A3G showed any specificity for CpC indicating that dinucleotide preferences may result from the presence of hot spots within a target sequence (Figure 6B). This time, A3F showed an aversion for GpC unlike its behaviour on a HBV template (Figure 6A). Nonetheless, the preference of A3G for CpC and A3H for TpC remained.

Human A3C showed the least polarized dinucleotide editing preference on either an HBV of HIV template. The A3C sequence is unique in encoding a serine at position 188 as opposed to an isoleucine residue conserved among all other A3 deaminases, APOBEC2, AID and even APOBEC1 [1]. A S188I mutant of the A3C-V5 tagged construct was generated and found to deaminate HBV in an indistinguishable manner (frequency, dinucleotide context) compared to the parental sequence without (data not shown).

Exon based phylogenies for A3s

The modular nature of the human A3s as well as extant phylogenic studies have shown that recombination can occur between CDD1 and 2 [49]. As the exon/intron structures for CDD1 and CDD2 are strictly comparable there is the possibility of recombination between exons within CDDs. An exon-based phylogenic analysis showed that the mainly non-coding exons 1 and 8 are, perhaps not surprisingly, fixing mutations faster than the coding exons 2+5, 3+6 and 4+7, which themselves showed a generally congruent topology (Figure 8). The obvious exception is A3G shown in red. Here, exons 1+2 strongly cluster with those of A3F. As A3F exons 1, 2, 3 and 4 always cluster with those of A3B and A3DE it would appear that A3G exon 2 is anomalous. Indeed the coding regions of the first two exons of A3F and A3G differ by 1 base (0.6%, or a single amino acid residue Δ~2%) while exons 3 and 4 differ by 121 nucleotides (~30%, or 62 amino acid residues, Δ~47%) and 2 indels. The 5’ breakpoint may map as far as 6.5 kb upstream of exon 1 (not shown). This event seems to exist as far back as the Macaca and Cercopithicus monkey lineages (see Supplementary table 3 in [50]).

Discussion

HBV is sensitive to low frequency editing by 6/7 human APOBEC3 cytidine deaminases. The finding of A3A hyperedited HBV genomes is novel and contrasts with a negative report [51]. Those for A3H confirm a recent study [24] and show that all the monodomain A3 enzymes are able to edit HBV and HIV cDNA. Indeed, A3A and A3H are some of the most efficient cytidine deaminases that we have worked with [24]. As the hypermutant frequencies were in the ~10^{-2} to 10^{-5} range, deamination may represent an insufficient selection pressure to cause the virus to adapt, unlike the lentiviruses that evolved a vif gene. While APOBEC3s can be found in the viral HBcAg capsid [14,15], packaging is inefficient, certainly with respect to the HIV-1Δvif/ΔA3G as reference. This follows from a consideration of the intravirion enzyme concentration: the inner HBcAg core volume is ~9 zl [52]. Ignoring the excluded volume due to the HBV genome, a single molecule of any packaged A3 deaminase translates into [A3]≥200 μM. Given that A3G hyperediting of ssDNA [IC_{50}~0.2, [53]] was observed in vitro with an enzyme concentration of ~2 μM, once even a single molecule is packaged within the HBcAg capsid, hyperediting is inevitable. Accordingly, packaging must be inefficient.

A similar argument goes for HIV where the core volume is ~90 zl [54]. Ignoring the excluded volume of 2 genomic RNAs, one molecule of A3G translates into an intravirion [A3G]>18 μM. As Pathak et al. estimated that on average there are 7 A3G molecules

Figure 6. Cytidine deamination dinucleotide context varies as a function of the target sequence. A) Cytidine editing of the minus DNA strand of HBV by six A3s, the horizontal line indicating the expected values based on the sequence composition of the target. B) Cytidine editing of the minus DNA strand of HIV by six A3s, the horizontal line indicating the expected values based on the sequence composition of the target. Asterisks denote statistically significant deviations from the expected value (p<0.001).

doi:10.1371/journal.pone.0004277.g006
supports the conclusions based on phenotypic analyses of A3G and A3H, which showed any strong dinucleotide specificity (Figure 6A). By contrast the HIV/A3 literature describes A3F as having a bias for the CpC dinucleotide (Figure 6B and [4,8,16–48]). The dinucleotide composition of the minus strand of the HBV segment analysed is 20.5% TpC, 31.9% CpC, 25.0% GpC and 22.7% ApC. By comparison, that for the HIV minus strand is even more polarized with 38% TpC, 16% CpC, 18% GpC and 28% ApC. It is possible that the HIV locus, with a greater proportion of TpC encodes a few hotspots allowing A3A, A3B, A3F and A3G editing at this dinucleotide to become significant.

When editing HBV cDNA, only A3G (double domain) and A3H (mono-domain) showed any strong dinucleotide specificity (Figure 6A). In contrast to the HIV/A3 literature describes A3F as having a bias for the TpC dinucleotide [4,8,16–48]. The dinucleotide composition of the minus strand of the HBV segment analysed is 20.5% TpC, 31.9% CpC, 25.0% GpC and 22.7% ApC. By comparison, that for the HIV minus strand is even more polarized with 38% TpC, 16% CpC, 18% GpC and 28% ApC. It is possible that the HIV locus, with a greater proportion of TpC encodes a few hotspots allowing A3A, A3B, A3F and A3G editing at this dinucleotide to become significant.

The mammalian APOBEC3 locus has undergone expansion presumably from a single gene. The presence of 1–6 APOBEC3 genes among rodent, carnivore and perissodactyl lineages, comprising both mono- and double domain molecules, all point towards extensive gene conversion [60,61]. Given that exon/intron structures are highly comparable among A3 genes there was the possibility of recombination between exons. From a phylogenetic consideration of the different exons it would appear that A3G is a chimera of at least three different segments (exons 1+2, exons 3+4 and exons 5–8; Figure 8).

The ensemble of the A3 phylogenetic trees can be resumed in a schematic form where colour reflects the deep phylogenetic clustering into three or four groups (Figure 9). Other recombination events are evident, for example, i) the A3A first exon stands out by coding for 10 distinct residues as opposed to 6 highly conserved residues for A3B-G; ii) A3H is unusual in that the first exon is non-coding (see NM_181773); iii) it could be argued that A3C arose from a duplicated A3DE or A3F gene with subsequent deletion of exons 2–4; iv) the discordant arborescence of A3A, A3B and A3G in exons 6 and 7, all of which are supported by good bootstrap values, is tell tale. If true, the latter example would mean that A3G has an even more complex evolutionary history. With this latter example in mind, it is worth noting that the present 7 gene locus in not invariant. A human haplotype dominant in Papua New-Guinea is lacking the A3B gene, whereas others have coevolved or been lost and gain of the A3H gene [62].

Interestingly, A3F and A3G are precisely the cytidine deaminases that are most active on HIV-1Δvif in phenotypic assays [5,6,8,46]. As the promoters are highly similar, it is expected that their expression would be coordinated and hence they could restrict HIV together, if given the chance. In this context it is worth noting that not only A3F and A3G expression was surprisingly coordinated, the two deaminases formed heterodimers [8]. A3G exon 2 is homologous to exon 5 [Jarmuz]. In the recently reported crystal structures for the CDD2 domain of A3G, exon 2 is part of a homodimerization interface (Harris & Goodman). In view of the recombinant nature of A3G, this might contribute to efficient A3F and A3G heterodimer formation.

Two New World monkey (NWM) sequences formally annotated as A3G could well be A3F-G chimeras as they were generated by screening genomes with the A3G sequence [50]. As it is a chimera, blast searching alone might well generate a recombinant even though the A3G of the genome in question might not have exons 1+2 akin to A3F. Both of these NWM sequences lack the singular HPE motif in the CDD1 of all other A3Gs. While not definitive, it urges caution. Three ribbon sequences annotated as A3G are
Figure 8. Phylogenic analysis of A3 exons indicates past intergenic recombination. All Neighbor-Joining trees are drawn to the same scale. Numbers at branching nodes are percentages of 1000 bootstrap replications - only bootstrap values > 40 are given. A3H was excluded from the exon 1 and 8 trees for lack of significant homology. A3B was excluded from the exon 1 tree because it is only 71 bases long and led to an even less robust tree. Nonetheless, as it differs from the A3DE sequence by only 2 bases, it is shown in parentheses. The anomalous A3G sequences are highlighted in red. Another case of possible recombination is highlighted in sky blue as the differing (3A-3, 3B-6, 3G-6) and (3A-4, 3G-7) 3B-7) topologies are supported by strong bootstrap values. After gap stripping, the lengths of each alignment were as follows: exon 1, 89 bp; exons 2+5, 135 bp; exons 3+6, 256 bp; exons 4+7, 113 bp and exon 8, 240 bp.

doi:10.1371/journal.pone.0004277.g008
clearly A3F-G chimeras – they were compiled by screening of genomic DNA while the break points map to exon boundaries [65]. That three subspecies of gibbon ape each harbour a different A3G chimera is not impossible, although parsimony suggests this to be implausible.

Given the extensive gene conversion and near identity of some exons, for example A3A-4 and A3G-7 (Figure 8), it is understandable that computer programs are unable to always identify correctly an APOBEC3 gene. The above examples are not, unfortunately unique. For example, the *Macaca mulatta* entry XM_001094452, ostensibly for A3G, actually encodes three CDDs. By comparison with the human A3 sequences, it is trivial to show that CDD1+2 are those of a *bona fide* macaque A3F gene while the third domain corresponds to CDD2 of the macaque A3G gene. The *Macaca mulatta* A3D entry XM_001094328 misidentifies the initiator methionine codon – as the coding region of exon 1 is only 6 amino acid residues long, the program presumably missed it. Consequently the A3D entry starts with a conserved Met residue that is actually within the second exon of the human orthologue. Equivalents for A3A and A3B are not annotated in the macaque genome via a NCBI search although it is trivial to find them with a Blast search. A few analogous errors can be found among the annotated genomic chimpanzee A3 sequences.

Apart from these *in silico* issues, some rather mundane issues come to the fore. Given the near identity of some exons across A3 deaminases, for example A3A exon 4 and A3G exon 7 or A3A exon 3 and A3B exon 6, ambiguities may arise when probing Northern and Southern blots with near-complete cDNAs. The same goes for *in situ* RNA hybridization using complete cDNAs. Polyclonal sera could possibly pick up more than one APOBEC3 protein. Depending on the region targeted, there is even the chance that a monoclonal antibody might show a little cross-reactivity.

The study shows that while six human APOBEC3 deaminases can edit HBV albeit at low frequencies, the degree of nascent cDNA editing can be as great as 90%. Recently the A3A, A3C and A3H deaminases have been shown to be active against human papillomavirus DNA in benign and pre-cancerous lesions [64]. Transcriptionally active HPV genomes are in the form of mini-papillomavirus DNA in benign and pre-cancerous lesions [64–70]. Indeed, transgenic mice with AID under the control of a hepatotropic promoter give rise to HCCs, paralleling earlier findings for APOBEC1 transgenic mice [71,72]. While efficient mutators, especially A3A, their effects are surely countered by highly efficient DNA repair machinery and so it may be extremely difficult to identify hyperedited human genes precisely for this reason. From this perspective, hyperedited HBV or dsDNA virus genomes could be seen as a surrogate marker for these powerful mutators especially when associated with predisposing or pre-neoplastic lesions like cirrhosis and low and high-grade intraepithelial lesions of the cervix.

### Materials and Methods

#### Plasmids

Human A3A cDNA was obtained from Bryan Cullen, A3G from Naveenan Navaratnam and A3DE from Michael Malim. The remaining human APOBEC3 cDNAs were obtained from an IMAGE clones cDNA library and were sub-cloned in the pcDNA3.1D/V5-His-TOPO expression vector (Invitrogen) as previously described [14]. Construction of A3Gn (N-terminal region or CDD1) was obtained by deleting exons 5–7 from the A3G clone, while A3Gc (C-terminal region or CDD2) was obtained by deleting exons 2–4 (Figure 2A). Plasmid pCayw allows expression of pre-genomic HBV RNA under control of the powerful cytomegalovirus immediate early promoter (CMV-IE) and was provided by Frank Chisari (Scripps Research Institute). The HIV-1 infectious molecular clone pNL4.3 plasmid has already described [73].

#### Cells, transfections and infections

Virus stocks were made following transfection of the quail muscle fibroblast cell line QT6. Briefly, 10^7 QT6 cells were co-transfected with equal amounts (2 μg) of the pCayw or pNL4.3 and A3 expression plasmids using FuGENE6 (Roche). In both
experiments, viruses were harvested 72 hours post-transfection, treated with DNaseI (10 U/ml, 1 mM MgCl2, 1 h/37°C). HIV virus stock was titrated with a p24 Elisa kit (PerkinElmer) and was used to infect H9 cells for 18 h. Total DNA was extracted using the MasterPure™ complete DNA and RNA purification kit (Epicentre). QT6 cells were maintained in HAM’s F10 medium, supplemented with 1% chicken serum, 10% FCS, 5% tryptose-phosphate, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin while H9 cells were maintained in DMEM medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Immunofluorescence

HeLa cells were grown to a density of 5 × 10^5 cells per dish and transfected with 0.4 μg of pcDNA6-mAPOBEC using FuGENE 6 (Roche). After 48 hours, the cells were washed twice with PBS, fixed for 45 minutes in a 50:50 methanol/ethanol mix. As primary antibodies, a mouse monoclonal antibody specific for the V5 epitope tag (Invitrogen) was used at a 1:200 dilution for 1 hour at 37°C. Cells were washed twice with PBS, and fluorescein isothiocyanate-conjugated anti-mouse antibody was used as secondary antibody (Sigma) at a dilution 1:200 for 30 minutes at 37°C. Cells were then washed three times in PBS, then incubated with 0.1 μg/ml of 4’, 6-diamino-2-phenylindole (DAPI) for 10 min. at room temperature to counter stain nuclei. Immunofluorescence was observed by microscopy (Zeiss).

PCR amplification, cloning and sequencing

All samples were digested with proteinase K (0.1 mg/ml, Eurobio) in lysis buffer at 65°C for 15 min. Total nucleic acids were extracted using the MasterPure™ complete DNA and RNA purification kit (Epicentre). A fragment just 5’ to the HIV X gene was chosen as described [14]. In order to increase sensitivity and specificity, hot start PCR was performed with primers 5’HBVOut and 3’HBVOut while primers 5’HBVin and 3’HBVin were used for the second round and were already described [14]. G→A hypermutated genomes were recovered using differential DNA denaturation PCR (3DPCR) in a two round procedure [40]. The first reaction involved standard amplification, the reaction parameters were 95°C for 5 min., followed by 35 cycles (95°C for 30 s., 50°C for 30 s., and 72°C for 1 min.) and finally for 10 min. at 72°C for the first round. Differential amplification occurred in the second round using the equivalent of 0.2 μl of the first round reaction as input. Differential amplification was performed by using an Eppendorf gradient Mastercycler S programmed to generate a 4–12°C gradient in the denaturation temperature. Gradient cycling conditions were, from 95°C to 81°C for 5 min., followed by 35 cycles (93–81°C for 30 s., 50°C for 30 s., and 72°C for 1 min.) and finally 10 min. at 72°C. First round PCR gave a 294 bp product, while second round PCR yielded a 213 bp fragment.

The HIV-1 env V1V2 region was amplified by a 3DPCR procedure, the first reaction PCR was amplified using primers SK122out 5’ TACCCACARCCCGAACCCGAGAA and SK123out, 5’ TTGTGAATGAAAAAYYAG (R = A, G and Y = C, T). Parameters were 95°C for 5 min., followed by 35 cycles (95°C for 30 s., 50°C for 30 s., and 72°C for 1 min.) and finally for 10 min. at 72°C. Selective 3DPCR was performed at the second round using primers SK122/Rin 54 AARCCCTAARCCCATRTRA and SK123/Yin 5’ TAATGTATGGAAATTGGYTTEAA with the following conditions: 77°C to 93°C for 5 min., followed by 35 cycles (77–93°C for 30 s., 50°C for 30 s., and 72°C for 1 min.) and finally for 10 min. at 72°C. The last PCR product yielded a 304 bp fragment. PCR products were purified from agarose gels (Qiaex II kit, Qiagen, France) and ligated into the TOPO TA cloning vector (Invitrogen, France). After transformation of Top10 Blue cells, up to 40 clones were picked. Sequencing was performed using BigDye Terminator V1.1 (Applied Biosystems).

Primers used for the construction of A3Gn plasmid were E1 and E4 respectively 5’ CACCATGAAAGCCTACTTCAAGAAC and 5’ GGTTTTCGTATTCTGAGAATCTCCCCAGCATG where the initiator methionine codon is underlined, while for A3Gc plasmid construction primers used were E5 and E8 respectively, 5’ CACCATGAAAGCCTACTTCAAGACCAGTGCAGATGATCACCAGCCA and 5’ CCATTCTCCAGAATGAGAAGAAC. The buffer conditions for amplifications were 2.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 200 μM of each dNTP, 100 μM of each primer, and 2.5 units of Pfu polymerase (Promega) in a final volume of 50 μl. PCR conditions were: 95°C for 5 min., followed by 20 cycles (95°C for 30 s., 55°C for 30 s., and 72°C for 30 s.) and finally for 10 min. at 72°C. PCR product was purified and cloned in pcDNA3.1D/V5-His-TOPO.

Phylogenetic analyses

The APOBEC3 sequences used in the alignment were: A3A NM_145699; A3B NM_004900; A3C NM_014568; A3D NM_152426; A3F NM_001006666; A3G NM_021822; and A3H NM_181773. All sequences were aligned using CLUSTALX [74]. Trees were constructed by the Neighbor-Joining method in MEGA (Version 3.1) [75].

Supporting Information

Figure S1
Found at: doi:10.1371/journal.pone.0004277.s001 (0.11 MB RTF)

Figure S2
Found at: doi:10.1371/journal.pone.0004277.s002 (0.09 MB RTF)

Author Contributions

Conceived and designed the experiments: SWH JPV. Performed the experiments: MH DG RS CR. Analyzed the data: SWH JPV. Wrote the paper: SWH JPV.

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