Published in final edited form as:

J Mol Biol. 2014 July 29; 426(15): 2840–2853. doi:10.1016/j.jmb.2014.05.012.

APOBEC3G Inhibits HIV-1 RNA Elongation by Inactivating the Viral Trans-Activation Response Element

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Abstract

Deamination of cytidine residues in viral DNA (vDNA) is a major mechanism by which APOBEC3G (A3G) inhibits *vif*-deficient HIV-1 replication. dC to dU transition following RNase-H activity leads to viral cDNA degradation, production of non-functional proteins, formation of undesired stop codons and decreased viral protein synthesis. Here we demonstrate that A3G provides an additional layer of defence against HIV-1 infection dependent on inhibition of proviral transcription. HIV-1 transcription elongation is regulated by the trans-activation response (TAR) element, a short stem-loop RNA structure required for elongation factors binding. *Vif*-deficient HIV-1-infected cells accumulate short viral transcripts and produce lower amounts of full-length HIV-1 transcripts due to A3G deamination of the TAR apical loop cytidine, highlighting the requirement for TAR loop integrity in HIV-1 transcription. Finally, we show that free ssDNA termini are not essential for A3G activity and a gap of CCC motif blocked with juxtaposed DNA or RNA on either or 3'+5' ends is sufficient for A3G deamination, identifying A3G as an efficient mutator, and that deamination of (–)ssDNA results in an early block of HIV-1 transcription.

Keywords

HIV-1; APOBEC3G; Deamination; ssDNA; TAT

INTRODUCTION

The human *ApoBec3* locus encodes seven homologous genes expanded in tandem on chromosome 22 ^{1, 2}. Human APOBEC3 proteins catalyze deamination of cytidines in ssDNA, providing innate protection against retroviral replication and

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retrotransposition ^{2; 3; 4; 5; 6}, as well as against hepatitis B and several DNA viruses ^{7; 8; 9; 10}. The HIV-1 accessory protein Vif counteracts the cellular innate immunity elicited particularly by APOBEC3G (A3G), APOBEC3F (A3F), APOBEC3DE (A3DE) and APOBEC3H haplotype II (A3H HapII) by promoting their degradation ^{11; 12; 13; 14; 15; 16; 17} and inhibiting their enzymatic activity ¹⁸. In the absence of Vif, the restricted cellular A3G and A3F proteins inhibit HIV-1. Whereas several mechanisms have been suggested to underlie A3G antiviral activity, such as cytidine deaminase-independent inhibition of viral reverse transcription ^{19; 20; 21}, it is now widely accepted that the major antiviral activity of A3G is dC to dU hypermutation of the viral ssDNA ^{22; 23; 24; 25; 26; 27; 28}.

A3G is incorporated into the newly assembling virions as a multimer through interaction with HIV-1 RNA or 7SL RNA and the viral nucleocapsid protein ^{29; 30; 31; 32}. Following target-cell infection, the encapsidated A3G acts in the cytoplasmic reverse transcription complexes in concert with the formation of newly synthesized ssDNA. Since reverse transcription and RNase-H activities of HIV-1 are functionally uncoupled, intermittent cleavage by RNase-H leaves many RNA fragments annealed to the newly synthesized viral DNA ^{33; 34}. Hence, the activity of A3G to generate a large number of detrimental mutations, predominantly 5'CC to CU ^{24; 35; 36}, is limited to the time interval when the viral DNA remains single-stranded ³⁶. Although not determined *in vivo*, the size of these RNA-DNA duplexes *in vitro* is >100 nt in length ³³. Antiviral activity causing detrimental hypermutation in limited time requires an efficient mechanism for enzyme translocation on ssDNA and target location. Previously, we demonstrated that A3G target location is based on positionally uncorrelated nonlinear translocation on ssDNA, suggesting intersegmental transfer of the deaminase ³⁷. Although the above-mentioned A3G modes of deamination match the restrictions of catalyzing the viral DNA, it is yet unclear how A3G targets the newly synthesized viral DNA in the reverse transcriptase complexes.

Following HIV-1 infection, the viral reverse transcriptase (RT) extends the tRNA^{Lys3} annealed to the primer binding site (PBS) of the genomic RNA. RNase-H activity of RT degrades the genomic RNA template concomitant with reverse transcription. The minus-strand strong-stop DNA ((-)SSDNA) is the first ssDNA replication intermediate, which bears sequences responsible for continuation of its elongation following transfer to the 3' end of the viral RNA ³⁸. The (-)SSDNA encodes the trans-activation response (TAR) element consisting of a short stem-loop RNA structure, which is essential for viral transcript elongation. Transcription of the HIV-1 provirus starts from the repeat (R) region in the large terminal repeat (LTR) of the provirus. Binding of cellular factors, including NF-κB, Sp1, the TATA box binding protein and RNA polymerase II, to the promoter region in the LTR initiates transcription of the viral mRNAs, which are subsequently spliced and translated. The transcriptional activator Tat protein is one of the early viral proteins, which enhances transcription following binding to the TAR hairpin at the 5' end of the newly synthesized viral RNA ^{39; 40; 41}. Tat protein interacts with the TAR hairpin via a conserved 3-nucleotide (nt) pyrimidine bulge ^{42; 43} and the apical 6-nt loop, to which the transcriptional elongation factor pTEFb binds in a Tat-dependent manner ^{44; 45}. Upon Tat binding, the apical TAR loop binds several cellular factors, forming a complex that plays a pivotal role in viral transcript elongation ⁴⁶. This complex includes the kinase component of pTEFb, cyclin-

dependent kinase 9 (CDK9), which phosphorylates the C-terminal domain of RNA polymerase II, enhancing RNA elongation^{45; 47; 48; 49; 50}.

Production of (–)SSDNA, which contains the stem and loop of the TAR element, is the first reverse transcription product exposed to A3G catalysis. The 3' dC of the three dCs located in the minus strand of the proviral DNA encodes the apical TAR loop, which can be used as a good substrate for A3G, as shown by using synthetic substrates⁵¹. Interruption of the RNA TAR loop by converting the underlined CTGGGA to A could hamper HIV-1 transcription elongation. Although conversion of this G to A has not been described before, it was previously reported that other substitutions in the TAR apical loop interrupt the binding of cellular factor, hampering HIV-1 transcription elongation, leading to inhibition of virus replication^{46; 52; 53}. A3G reduces the expression of the reporter gene regulated by a lentivirus promoter²⁴. However, it is as yet unclear whether the reduced expression is due to inhibition of transcription elongation by A3G.

Here, we demonstrate that during HIV-1 reverse transcription, A3G trapped in the virions deaminates the underlined dC in one of the two CCC motifs and in the 3' CC motif located in the (–)SSDNA. However, transition of the dC to dU does not hinder the first strand transfer during completion of the minus strand viral DNA synthesis. On the other hand, deamination of the 3' CCC in the TAR apical loop results in an early block of HIV-1 transcription, highlighting the requirement for TAR apical loop integrity for viral gene expression. Our results reveal an additional layer of defence, by which A3G inhibits HIV-1 transcription elongation, leading to accumulation of short viral transcripts and reduction of virus replication. In addition, we demonstrate that A3G deaminates ssDNA substrate, gapped by DNA:DNA or DNA:RNA duplexes at the 3'-, 5'- or 3'+5' ends, and that even a gap of 3 nt (CCC) can be targeted by the enzyme. This report reveals the mechanism of A3G activity on the reverse transcripts, elucidating it as an efficient mutator that interferes with early HIV-1 transcription.

RESULTS

Interaction of A3G with ssDNA substrates

The A3G binding ssDNA substrate was assessed by an electromobility shift assay (EMSA) using a 160-nt oligonucleotide (S₁₆₀), and S₁₆₀ annealed to short complementary oligonucleotides at the 5'-, 3'- or 3'+5' ends, leaving open ssDNA fragments of 135, 133 and 108 nt, respectively (Figure 1A). A3G molecules bind to all four free- or double-stranded blocked S₁₆₀, suggesting that blocking the 5'-, 3' or 3'+5' termini does not affect A3G tethering to ssDNA (Figure 1B). These results are in agreement with the AFM analysis performed by Lyubchenko's group⁵⁴. As with the binding, A3G deamination of a CCC motif in the S₁₆₀ substrates was not affected by annealing of short complementary oligonucleotides at the 3'-, 5'- or 3'+5' ends of the S₁₆₀ substrate (Figure 1C and D). These results are in agreement with those published by Chelico and her coworkers⁵⁵, showing that the specific activity of A3G remains roughly constant using substrates longer than 60 nt. One possible explanation is that A3G binds DNA randomly but only those molecules which encounter the target motif or are in close vicinity are engaged in deamination (see also⁵⁶)

The aforementioned results suggest that free termini are not required for A3G activity. Therefore, the deamination kinetics of a single CCC motif residing in the centre of 80-nt linear and circular substrates containing the same sequences is expected to be similar. The 80-nt linear (Figure 1E) and circular ssDNA (Figure 1F) substrates were similarly deaminated by purified A3G enzyme (Figure 1G), suggesting that A3G molecules evenly encounter the CCC motif located in the ssDNA substrates, and/or symmetrically move towards the motif (but see Chelico *et al.* ⁵⁷).

A3G targets the CCC motif embedded in ssDNA substrate blocked by complementary DNA or RNA strand

To gain insight into A3G activity during reverse transcription, we modelled the viral ssDNA by generating S₈₀ substrate containing a central CCC motif annealed to complementary deoxyribo-oligonucleotides with the 38 nt (2-nt+CCC) and 35 nt (CCC+2-nt) (Figure 2A (3,4)) sequences at the 5' and 3' ends, respectively. In addition, we constructed S₈₀ substrates annealed to complementary deoxyribo-oligonucleotides, juxtaposed to the CCC motif (Figure 2A (5,6)). Complete annealing of the oligos to the S₈₀ substrates was validated by PAGE (Supplemental Data Fig. S1 and S3, respectively). All the substrates described in Figure 2A were incubated with purified A3G (at a substrate enzyme ratio of 12.5 to 1) for 30 min at 37°C. Figures 2B and C indicate that the CCC motifs in the S₈₀ nt substrates were similarly deaminated. Hence, CCC motifs blocked with flanked DNA:DNA, which avoid A3G sliding toward the CCC motif from either direction, are equally used as a substrate for A3G, suggesting that A3G can deaminate the substrate in a bidirectional manner ^{37; 57; 58; 59}.

To find the minimal ssDNA gap required for A3G binding and deamination, we constructed A₈₀ substrates annealed to complementary deoxyribo-oligonucleotides, leaving negative ssDNA gaps of 28, 15 and 7 nt containing a CCC motif (Figure 2D). The deamination of the underlined cytosine CCC was reduced proportionally to the gap length (Figures 2E and F).

RNase-H endonuclease cleaves the viral genomic RNA during reverse transcription, generating DNA gaps flanked by RNA:DNA duplexes. To mimic the cytoplasmic reverse transcriptase complex, we blocked the S₈₀ substrate by ribo-oligonucleotides juxtaposed to the CCC motif (Figure 3A (3,4)) and leaving a gap of 3 nt (Figure 3A(5)) flanked by the same ribo-oligonucleotide. Consistent with the result shown above in S₈₀ blocked by DNA fragments (Figures 2B and C), DNA shielded by ribo-oligonucleotide is also used as an efficient substrate by A3G (Figure 3B). Figures 3C and D show that even a gap of 3 nt flanked by ribo-oligonucleotide can be used as a substrate by A3G, albeit at low efficiency.

A3G targets terminal ssDNA hotspot during HIV-1 reverse transcription

The first step of HIV-1 reverse transcription is formation of a ~200 nt (–)ssDNA fragment (Figure 4A), which contains two putative CCC hotspots and a single CC deamination target hotspot (hs1-3). Modelling the (–)ssDNA secondary structure using the mfold program ⁶⁰ predicts that hs1 and hs2 are positioned at unique secondary structure contexts, whereas hs3 is located at the (–)ssDNA terminus (Figure 4B). A3G is incorporated into HIV-1 particles in the virus-producing cells. Following infection of the target cells, A3G restricts HIV-1 replication by deaminating the deoxycytidines in the viral minus single-strand DNA formed

during reverse transcription^{24; 35; 36}. To study A3G native activity, we utilized endogenous reverse transcription in purified wild-type (wt) and *vif*(-) HIV-1 virions produced in H9 cells endogenously expressing A3G. HIV-1 Vif protein inhibits A3G enzymatic activity, mediates A3G proteasomal degradation, and reduces its packaging into HIV-1 cores approximately 10-fold compared with *vif*(-) virions¹⁸. Deamination of hs1 and hs2 was determined by a primer extension assay using (-)SSDNA extracted from wt (A3G low) or *vif*(-) (A3G high) virions as a template for PCR. The amount of input (-)SSDNA content derived from wt or *vif*(-) virions was controlled by primers specific to sequences adjacent to hs1 or hs2 (pCTRL), whereas primers with 3'-terminal A instead of G (pG>pA) were used to indicate CCC>CCU deamination at hs1 or hs2 (Figure 4C). Excluding dNTPs in the endogenous RT reaction resulted in no apparent PCR product in either the wt or *vif*(-) viruses, indicating no background HIV-1 genomic RNA or DNA. Consistent with mfold prediction, hs1 residing in a duplex DNA stem structure was not readily deaminated by A3G, whereas hs2 residing in the (-)SSDNA loop corresponding to the RNA transcription activator (TAR) element was efficiently deaminated in *vif*(-) virions.

In order to determine whether A3G is able to deaminate the terminal cytidine of (-)SSDNA, cDNA was extracted from virions following endogenous reverse transcription and used in a primer extension assay. The primer used for Taq DNA polymerase precludes the extension of (-)SSDNA molecules with deaminated terminal cytidine (dU) (Figure 5A). The extended primer was then used as a template for PCR amplification using a forward primer specific for the extension product (pFTag) and a reverse primer specific for the (-)SSDNA (pR604). Primers specific for the (-)SSDNA (pF509 and pR604) were used for controlling the input (-)SSDNA content. PCR amplification of (-)SSDNA from both the wt and *vif*(-) viruses using the (-)SSDNA-specific pF509 primer indicated comparable (-)SSDNA input content. In contrast, the extension product-specific pFTag primer yielded 4–5-fold less PCR product of the expected length in case of (-)SSDNA derived from *vif*(-) virus, compared to the wt virus. This indicates that cytidine deamination at hs3 occurred in at least 75–80% of *vif*(-) virions. Terminal deamination was also verified by quantitative real-time PCR, corroborating a ~4-fold decrease in PCR efficiency when using (-)SSDNA from *vif*(-) virions (Figure 5B). These results show that A3G trapped in the virion mutates the cytosines located in the apical loop of TAR, and at the termini of the (-)SSDNA to uridine. We next asked what is the physiological impact caused by these mutations.

Deamination of the terminal cytosine of the (-)SSDNA does not interrupt the strand transfer

It was previously shown that A3G inhibits HIV-1 reverse transcription^{20; 24; 61}. To determine whether deamination of the terminal cytidines in the (-)SSDNA impedes reverse transcription, we used purified HIV-1 RT in an exogenous reverse transcription assay (Figure 5C). A 51-nt oligonucleotide (Table S1) comprising the sequence of the (-)SSDNA 3'-terminus (S₅₁CC) was incubated with purified A3G and then used as a primer for RT extension in the presence of dNTPs. Incubation with A3G, or using a positive control oligonucleotide with 3'-terminal UU (S₅₁CUU), did not prevent complete utilization and extension of the oligonucleotides by RT, indicating that terminal cytidine deamination does not inhibit strand transfer during HIV-1 reverse transcription. HIV-1 reverse transcriptase

(RT) can extend a G:U terminal mismatch⁶², in contrast to the high fidelity of Taq polymerase (Figure 5A).

Deamination of a cytosine residue in the TAR loop impedes the elongation of viral RNA transcripts

Next, we asked whether transition of G to A in the TAR apical loop could hamper Tat function in viral RNA transcription elongation. We infected SupT1 (A3G-negative) and H9 (A3G-positive) cells with *vif*(-) HIV-1 produced in HEK293T, at a multiplicity of infection of 0.2. Total genomic DNA was isolated five days post infection, two fragments mapping to the first 350 nt of HIV-1 genome and to the beginning of the *gag* gene were amplified and sequenced. Chromatogram analysis at positions complementary to underlined 31GGG33 in viral genomic RNA shows that, C to T transition in H9 cells are 3.9-fold higher than in the SupT1 proviral DNA (Figure 7A). Sequencing of *gag* region containing the hotspot for A3G deamination at position complementary to 970GGG972 in the viral RNA shows that C to T transition in H9 cells are 47.7-fold higher than the SupT1 proviral DNA (Figure 7B). The TAR loop region in the H9 and SupT1-proviruses contained CCC, while in H9 cells 10.4% of the underlined C (position complementary to 31G in the viral RNA) and about 25.6% of the C complementary to 970G in the *gag* region were mutated to T in H9 cells. This analysis suggests that the endogenous A3G incorporated into *vif*(-) virions produced in H9 cells deaminates the CCC motif resides in the TAR apical loop following infection of the target cells, impairing the TAR function in the newly produced proviruses.

The GGG sequence in the 6-nt TAR apical loop was shown to be essential for binding of the protein assembly required for HIV-1 transcription elongation⁴⁶. We asked whether transition of the underlined GGG to A in the TAR apical loop could hamper Tat function in viral RNA transcription elongation. To determine whether A3G inhibits viral transcription elongation, we assessed the accumulation of short nascent viral transcripts in infected cells. TZM-bl cells were infected with *vif*(-) viruses produced in HEK293T cells co-transfected with plasmids expressing A3G or an empty vector. Northern blot analysis, using PAGE that precludes the migration of large RNA molecules, shows that TZM-bl cells synthesized short viral transcripts following infection with *vif*(-) HIV-1 containing A3G (Figure 6A). On the other hand, cells infected with HIV-1 devoid of A3G did not synthesize these short transcripts. These cells, in contrast, expressed the full-length viral genome as shown by RT-PCR using primers specific for *gag*, *tat* and *env* genes (Figure 6B and Supplemental Data Table S2). The number of large transcripts was dramatically reduced in cells infected with A3G-containing virus. These results indicate that deamination of the viral (-)ssDNA causes an early block in HIV-1 transcription, highlighting the requirement for TAR apical loop integrity in HIV-1 transcription.

DISCUSSION

Reverse transcription and the endonuclease activity of RNase-H are uncoupled, leaving viral RNA fragments annealed to the cDNA^{33; 34}. Considering the hallmark of reverse transcription, A3G can progress on the newly synthesized linear ssDNA annealed to RNA islands, mainly by sliding, micro-jumping and intersegmental transfer^{37; 55; 63}. We used

synthetic oligonucleotides annealed to short DNA or RNA fragments to define the A3G properties allowing efficient catalysis of the newly synthesized HIV-1 DNA. A single CCC motif embedded in 160-nt or 108-nt gapped substrates were deaminated at similar rates (enzyme and substrate concentrations were constant), suggesting that only the A3G molecules encounter the substrate in close vicinity to the motif participating in cytosine transition. On the other hand, deamination of short ssDNA with a CCC motif, gapped between RNA:DNA or DNA:DNA duplexes, is proportional to the length of the ssDNA (28 to 3 nt gaps), indicating that the level of A3G activity is determined by the number of A3G/ssDNA encounters, which is proportional to the available length of substrate and the length accessible for bidirectional sliding (and/or micro-jumping) toward the motif^{54; 64}. However, a gap of 3 nt does not allow sliding and the catalysis is probably directed by a single A3G/ssDNA encounter, which follows by substrate dissociation, pointing at a distributive mode of action.

It is now widely accepted that the major antiviral activity of A3G is dC to dU hypermutation of the viral ssDNA^{22; 23; 24; 25; 26; 27; 28}. Viral cDNA containing uracils could lead to degradation of the cDNA or to insertion of adenines into the genomic strand, thereby leading to strand-specific C/G-to-T/A transition mutations (G-to-A hypermutations). Previously, it was well documented that deamination of cDNA transcripts by A3G (and others A3 proteins) results only in hampering the function of translated proteins by insertion of non-desired stop-codons, or by encoding non-functional proteins²⁴. This could be beneficial to the host immune system that can activate HIV-1-specific (HS) CD8⁺ cytotoxic T lymphocytes (CTLs)⁶⁵. Here, we demonstrate that A3G inhibits the elongation of viral RNA, blocking replication, which leads to a decrease in virus production^{66; 67}. Considering the hallmark of innate immunity exerted by A3G against HIV-1 replication, the inhibition of viral transcription reduces the total number of viral genomes produced by the cells and therefore prevents the production of mutated viruses with fitness to diverse environmental conditions^{68; 69}.

We demonstrated that the endogenous A3G trapped in the virions mutates at least two out of the three motifs residing in the (–)SSDNA, including the CCC motif residing in sequences transcribed to the apical TAR loop of 6 nt and the 3' CC (hs-3) located in the (–)SSDNA (Figures 4 and 5). The 5' terminus of the genomic RNA is terminated by a single G⁷⁰, but the (–)SSDNA terminus is occupied by a CC motif, because reverse transcriptase can add a cytosine templated by the 7-methylguanosine cap^{71; 72}. Although A3G mutates the 3' CC to UC at the terminus of the (–)SSDNA, this mutation does not hamper the (–)SSDNA transfer. Moreover, our results strongly suggests that A3G deaminates a motif residing at the 3' terminus of the SSDNA, indicating that there is no “dead zone” for A3G activity, as suggested before by Chelico and coworkers using synthetic oligonucleotides^{37; 57; 58}, but see Iwatani *et al.*⁵⁹.

Here, we demonstrate that A3G elicits an additional anti-HIV-1 defence underlying inhibition of viral transcript elongation. Conversion of C to U in the cDNA (hs-2) guides G to A replacement in the apical TAR loop, leading to impeded viral RNA transcript elongation. TAR RNA consists of highly stable, nuclease-resistant stem-and-loop structure. Mutations which destabilize the TAR-RNA structure, abolish Tat function in viral

transcripts elongation^{53; 73}. The integrity of the apical TAR is crucial for viral RNA transcription elongation^{45; 74; 75} as several cellular “TAR loop factors”, namely CDK9 and CycT1, which are components of the P-TEFb, form a complex with the Tat protein⁴⁶ bound to the conserved TAR stem bulge. The indispensability of TAR loop integrity was demonstrated previously by replacing each of the three guanosines and the other three residues comprising the TAR loop³⁹. The G-to-A conversion resulting from deamination by A3G is sufficient to hamper TAR function, representing a post-integration phenomenon. This mechanism can contribute to the production of aborted infected cells, which contain provirus but do not express viral proteins^{76; 77}. Note that the GGG motif is a phylogenetically conserved element residing in the apical TAR loop of several primate viruses, such as HIV-1, SIV cpz, SIV smm, SIV agm, SIV mac and HIV-2^{78; 79}.

Considering the hallmark of A3G (and probably A3F) as a factor that can inactivate the viral DNA by mutational insertion, it could also contribute to viral fitness^{68; 69}. However, stalling viral transcript elongation reduces virus replication and minimizes the possibility of mutated virus production, emphasizing the importance of developing efficient anti-Vif drugs.

In summary, we demonstrated that the characteristics of A3G allow efficient catalysis of (–)SSDNA. A3G mutates the underlined CCC_{hs2} in the (–)SSDNA to U, which causes a G-to-A mutation in the apical TAR RNA. Mutations in the apical TAR loop hamper Tat functions and stall transcription elongation, suggesting a major mechanism by which A3G inhibits virus production.

MATERIAL AND METHODS

Cells

Cutaneous T cell lymphoma H9 cells, T lymphoblastic leukemia SupT1 cells and TZM-bl cell lines were provided by the National Institutes of Health (NIH) AIDS Reagent Program [Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH, USA], and were grown in RPMI 1640 (Biological Industries, Beit Haemek, Israel), and HEK293T cells were grown in DMEM (Biological Industries, Beit Haemek, Israel). Media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine (Biological Industries, Beit Haemek, Israel).

Deamination assay

A3G was purified from HEK293T cells transfected with pcDNA3.1-A3G-myc-His₆ vector as previously described by Nowarski *et al.*³⁷. Deamination reactions were performed in a total volume of 10 µl in 25 mM Tris, pH 7.4, 0.1 µg/µl BSA and 1 fmol/µl ssDNA substrate with CCC deamination motif (Integrated DNA Technologies) at 37°C (standard conditions). Kinetic assays were performed at [E] ≪ [S] so that the overall product formation fell below 15% of the substrate. The reaction was terminated by heating to 95°C for 5 min following immediate cooling on ice. One µl of the reaction mixture was used for PCR amplification with Redmix (Larova) in a total volume of 20 µl. PCR products (10 µl) were incubated with the *Stu*I restriction enzyme (NEB, Fermentas) for 1 h at 37°C, to cleave the deaminated

product (CCT:GGA). The analyses of the S₈₀ were carried out as described above. Substrates blocked with long complementary DNA fragments could not be amplified by PCR (due to higher T_m than the primers); therefore, Klenow DNA polymerase was used to close the gaps. As the Klenow DNA polymerase leaves CCU:GGA in the deaminated product, which cannot be cleaved by *Sst*I or any restriction enzyme, *Apa*I was selected to cleave solely the non-deaminated motif CCC:GGG (A₈₀). Note that the CCC motif is located in the middle of the substrate, yielding similar fragments following cleavage with the restriction enzyme. Restriction was verified by a positive-control substrate. Restriction products were loaded onto gels and separated by 14% polyacrylamide gel electrophoresis (PAGE). Gels were stained with SYBR gold nucleic acid stain (Molecular Probes) diluted 1:10,000 in 0.5 M Tris-borate-EDTA buffer (TBE, pH 7.8), visualized by blue light (460 nm), captured by LAS-3000 (FUJIFILM) and analyzed by densitometry using ImageJ image processing and analysis software.

Annealing oligonucleotides to substrate

The sequence of S₈₀ and A₈₀ substrates used for deamination and the respective complementary DNA or RNA oligonucleotide used to block the substrate at the 5' and 3' ends are listed in Supplemental Data Table S1. The A₈₀ substrate (25 pmol) was incubated with the respective oligonucleotides (30 pmol) along with 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂ and 1 mM dithiothreitol (DTT) at 95°C for 5 minutes and gradually cooled down to 25°C over a period of 40 min. The substrate (open) and the hybridized substrate were analyzed by PAGE (Supplemental Data Fig. S1).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were annealed by heating to 95°C and slow cooling to room temperature for 1 h. One pmol of oligos was incubated with A3G at the indicated E/S ratios for 10 min at 37°C in EMSA buffer: 25 mM Tris, pH 7.4, 50 mM NaCl, 0.1 µg/µl BSA and 10% (v/v) glycerol, in a 10-µl reaction volume. Samples were immediately resolved by 6% native PAGE, stained with SYBR Gold (Molecular Probes) and visualized by LAS-3000 (FUJIFILM).

Preparation of circular ssDNA molecules

The S₈₀ linear oligo (20 pmol) was incubated for 60 min at 37°C in PNK buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM ATP and 10 mM dithiothreitol) containing 10 U of polynucleotide kinase (Thermo Scientific) in 20 µl. Following incubation, the reaction volume was increased to 40 µl by adding KCl and Tris to final concentrations of 10 mM and 50 mM, respectively, and 100 U of *CircLigase*TM (EPICENTRE, Biotechnologies), and incubated for 60 min at 60°C. Digestion of the non-circularized molecules was carried out by incubation with 20 U of exonuclease I (New England Biolabs) in MgCl₂ at a final concentration of 5 mM for 60 min at 37°C. The reactions were inactivated at 80°C for 10 min before separating the circular oligonucleotides on an 8 M urea-20% acrylamide denaturing gel (Supplemental Data Fig. S2).

Analysis of terminal cytidine deamination in HIV-1 (–)ssDNA

Wild-type or *vif*(–) HIV-1_{HXB2} viruses were produced in H9 cells endogenously expressing A3G, harvested and concentrated as described³⁷, and suspended in PBS. Endogenous reverse transcription reactions were performed in viral particles equivalent to 10 ng p24 (CA) protein, 0.2 mM dNTPs, 10 mM MgCl₂, 10 mM Tris (pH 7.4), 20 µg ml^{–1} BSA and 0.006% (v/v) Triton X-100. Following 1 h incubation at 37°C, samples were treated with 50 µg ml^{–1} RNase-A (Sigma) for 30 min at 37°C, DNA was extracted with phenol:chloroform (1:1) and purified using QIAquick PCR purification kit (Qiagen). (–)ssDNA (approximately 0.02 fmol) was added to a PCR-containing template, which is complementary to the “R” and the 3′ end of the viral RNA (gDNA oligonucleotide) (10 fmol), pFTag and pR604 primers (1 pmol), 0.2 mM dNTPs, buffer S and 0.2 U Taq polymerase (PeqLab), in a 20 µl reaction volume, and subjected to the following PCR program: melting at 94°C for 3 min, followed by 33 cycles of melting at 94°C for 15 s and polymerization at 64°C for 40 s. PCR products were resolved by PAGE (10%) and stained with SYBR Gold. Real-time qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7700 PCR machine (Applied Biosystems).

The “primer extension” products achieved by using recombinant reverse transcriptase were resolved by denaturing PAGE, transferred to a Hybond N nylon membrane (GE Healthcare) using a semi-dry transfer apparatus (Biorad) and UV-crosslinked at 312 nm for 15 min. Following blocking with 5% skim milk, the membrane was treated with horseradish peroxidase-conjugated streptavidine (Jackson) for 20 min at room temperature, washed 4 times with TBS, pH 7.4, and visualized by enhanced chemiluminescence.

Analysis of viral RNA transcripts

HEK293T cells were transfected with pSVC21 vector expressing *vif*(–) HIV-1_{HXB2}⁸⁰. These cells were co-transfected with plasmid expressing A3G⁸¹ or with empty pcDNA3 vector (control). The ratio between the viral-expressing vector and plasmid-expressing A3G or control was 9:1. Viruses were harvested forty hours post-transfection, and used to infect TZM-bl⁸² indicator cells. Cells were harvested 48 h post-infection and the total RNA was extracted using an EZ-RNA II Total RNA Isolation Kit (Biological Industries, Beit Haemek).

Northern blot—Twenty micrograms of the total RNA were separated on 15% urea-PAGE, transferred to Amersham HybondTM -N⁺ nylon membrane (GE Healthcare) and hybridized with a mixture of two synthetic oligonucleotide probes (listed in Supplemental Data Table S2) labeled with biotin (500 ng each). Hybridization was performed overnight in PerfectHybTM Plus Hybridization buffer (Sigma) at 42°C. Following stringent washes according to manufacturer’s instructions, the membrane was blocked with streptavidin blocking buffer (10 mM Tris pH 7.4, 100 mM NaCl, 3% BSA, 0.1% Tween 20, 1% SDS) for 1 h and then exposed to streptavidin-HRP conjugate (Jackson) for 30 min. Following the ECL reaction, the signals were captured by LAS-3000 (FUJIFILM).

RT-PCR—Total RNA preparations (described above) were treated with RQ1 RNase-free DNase (Promega) according to the manufacturer’s protocol. cDNAs were generated from 1

µg of total RNA using MasterScript RT (5PRIME). Following reverse transcription, 1 µl of the cDNA was amplified using RedMix (LAROVA) with a specific primer set for each gene (Supplemental Data Table S2). The amplification protocol included 1 cycle of initial activation at 95°C for 3 min, 29 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s and 1 cycle of terminal extension at 72°C for 5 min. Ten microliters of the PCR product were separated on a 1.5% agarose gel and stained with ethidium bromide. The primers listed in Supplemental Data Table S2 represent the *gag*, *tat* and *env* genes of HIV-1, while the *actin* gene used as a control.

Sequencing of proviral DNA

H9 and SupT1 cells (5×10^6) were infected with *vif*(-) HIV-1 produced from HEK293T cells at moi 0.2. Genomic DNA was isolated five days post-infection using EZ-DNA reagent (Biological Industries, Beit Haemek). The first 359 nucleotides of the HIV-1 DNA starting from the transcription initiation point and nucleotides 345 to 1014 of HIV-1 *gag* were amplified using specific primers (Supplemental Data Table S2) and the DNA was sequenced using the reverse primer (340_359hivrna, *gag* reverse). Sequencing reactions were run on an ABI 3700 DNA analyzer (PE Applied Biosystems, performed at the Center for Genomic Technologies, The Hebrew University).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Amnon Hizi for providing purified HIV-1 RT protein and Joy Lengyel and Ming Li for A3G plasmid DNA constructs and sharing unpublished EMSA data. We thank Dr. Cheryl Balshayi for critical reading of this manuscript. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: anti-APOBEC3G C-terminal from Dr. Jaisri Lingappa, anti-APOBEC3G from Dr. Warner C. Greene. This work was carried out in the Peter A. Krueger Laboratory with the generous support of Nancy and Lawrence Glick, and Pat and Marvin Weiss.

FUNDING

This work was supported by the National Institutes of Health grant [P01 GM091743 to R.S. Harris, with a subaward to M.K.] and the United States-Israel Binational Science Foundation (BSF). R.N. was a fellow of the Clore Scholars Programme.

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Research Highlights

- APOBEC3G mutates viral ssDNA during reverse transcription in *vif*-deficient HIV-1.
- CC motif in ssDNA terminus is efficiently deaminated by A3G.
- A CCC motif juxtaposed by ribo- or deoxy-oligonucleotide is deaminated by A3G.
- Mutations in the apical TAR loop stall transcription elongation inhibiting virus production.

Figure 1.

Deamination and binding of A3G to ssDNA substrate is bidirectional and terminus-independent. (A) 160-nt substrates were left as ssDNAs, or annealed with short complementary oligonucleotides of 25 nt to the 5' terminus, 27 nt to the 3' terminus or to both termini of the 160-nt substrate, leaving a gap of 108 nt. (B) All substrates (5 pmol) were incubated with purified A3G (1 pmol) and analyzed by electrophoresis mobility shift assay (EMSA). The arrow indicates the A3G bound substrate; C denotes control without A3G, T denotes test sample with A3G. (C) These substrates (5 pmol) were also incubated for 20 min with purified A3G (0.4 pmol) and the deamination products were determined as described previously³⁷. Reactions were terminated by boiling; substrates were amplified by PCR, cleaved with *StuI* restriction enzyme and separated on PAGE gels. (D) The percentage of the deaminated products was calculated and plotted. PAGE gel showing deamination reaction of purified A3G on linear (S_{80}) substrate (E) and deamination of purified A3G on circular substrate (F). Linear (◆) and circular (■) substrates consisting of identical 80-nt sequences were incubated with purified A3G and samples were taken at the indicated time intervals. Reactions were terminated by boiling; substrates were amplified by PCR, cleaved with *StuI* restriction enzyme and separated on PAGE gels. The percentage of the deaminated products was calculated and plotted (G). ND: Non-deaminated substrate; D: Deaminated product; NC: Negative control; PC: Positive control.

Figure 2.

A3G targets the CCC motif embedded in ssDNA substrates juxtaposed with complementary DNA. (A) The schematic representation of the S₈₀ substrates:

- **a and d1** S₈₀ with CCU used as positive control.
- **a and d2** S₈₀ with CCA used as negative control.
- **a3** S₈₀ with CCC juxtaposed with 38 nt of ssDNA at the 5' termini.
- **a4** S₈₀ with CCC juxtaposed with 35 nt of ssDNA at the 3' termini.
- **a5 and d3** S₈₀ with CCC juxtaposed with 40 nt of ssDNA and RNA at the 5' termini, respectively.
- **a6 and d4'** S₈₀ with CCC juxtaposed with 37 nt of ssDNA and RNA at the 3' termini, respectively.
- **a7** S₈₀

(B) The substrates described in (A) (5 pmol) were incubated for 30 minutes with purified A3G (0.4 pmol) and the deamination products were determined as described previously³⁷. Reactions were terminated by boiling; substrates were amplified by PCR, cleaved with *StuI* restriction enzyme and separated on PAGE gels (deamination converts the motif in the S₈₀ substrate into *StuI*-cleavable). (C) The percentages of the deaminated products were calculated and plotted. (D) The schematic representation of A₈₀ substrates annealed with short complementary deoxyribo-oligonucleotides (see Supplemental Data Table 1) leaving

gaps of 28, 15, 7 nt. (E) These substrates (5 pmol) were incubated for 20 minutes with purified A3G (0.4 pmol) and the deamination products were determined. Reactions were terminated by boiling, the gaps in the substrates were filled by Klenow DNA polymerase, and the substrates were cleaved with *ApaI* restriction enzyme and separated on PAGE gels. (F) The percentages of the deaminated products were calculated and plotted. C denotes control, T denotes test sample.

Figure 3.

A3G targets the CCC motif embedded in ssDNA substrate juxtaposed by complementary RNA (A). Schematic representation of the S₈₀ substrates. (B) These S₈₀ substrates (5 pmol) were incubated for 30 minutes with purified A3G (0.4 pmol). Reactions were terminated by boiling, the DNA molecules were amplified by PCR in the presence of RNase A (40 µg/ml), cleaved with *StuI* restriction enzyme and separated on PAGE gels. (C) S₈₀ substrate was annealed with short complementary ribo-oligonucleotides, leaving 3-nt gaps, and incubated with A3G. (D) The percentage of the deaminated products was calculated and plotted. D denotes the deaminated product, ND denotes the non-deaminated substrate, PC denotes positive control and NC denotes negative control. Asterisk denotes the S₈₀ juxtaposed with RNA.

Figure 4.

Deamination hotspots in the viral strong-stop ssDNA. (A) Scheme of the endogenous reverse transcription assay in which the viral (-)SSDNA is generated inside HIV-1 virions by reverse transcription of genomic RNA (gRNA). (B) Predicted secondary structure of HIV-1 (-)SSDNA as calculated by mfold⁶⁰. Shown is the 3'(-)SSDNA portion corresponding to nucleotides 1–113 of HIV-1 RNA (GenBank: AF033819). Putative A3G target deamination hotspots are indicated as hs1–3. (C) (-)SSDNA produced by endogenous reverse transcription was extracted from HIV-1 wild-type (wt) or *vif*⁻ (ΔV) virions and used as template in a primer extension assay to assess cytidine deamination at hs1 and hs2. Primers specific for sequences adjacent to hs1 or hs2 were used to control input (-)SSDNA (pCTRL), whereas primers with 3'-terminal A instead of G (pG>A) were used to indicate CCC>CCU deamination at hs1 or hs2 (black line in (B) illustrates pG>A for hs2).

Figure 5.

A3G targets terminal (-)SSDNA during HIV-1 reverse transcription. (A) Top, scheme of the polymerase extension assays. The (-)SSDNA, which was synthesized by endogenous reverse transcription in the presence of encapsidated A3G, was extracted and served as an initial primer for DNA Taq polymerase extension using a complementary DNA template (gDNA). The non-complementary AAA at the 3'-terminus of the gDNA template prevents its extension. Bottom, polymerase extension assay using (-)SSDNA extracted from wild-type (wt) or *vif*⁽⁻⁾ (ΔV) viruses following endogenous reverse transcription and PCR amplification using the pF509-pR604 (-)SSDNA-specific primers (pF509), or pFTag-pR604 extension-specific primers (pFTag). PC, a positive control oligonucleotide with the expected extension target sequence. DNA sizes are indicated on the left (bp). (B) Quantification of polymerase extension products by real-time PCR. (-)SSDNA extracted from HIV-1 wt or ΔV viruses was used as a template in a primer extension assay as described in (B). Values are shown relative to the wt (-)SSDNA which was set to 1. Data are represented as mean ± S.D. from three independent experiments performed in duplicate. (C) Exogenous reverse transcription was performed with purified recombinant HIV-1 reverse transcriptase and A3G using biotinylated (b) 51-nt oligonucleotides containing the 3'-terminal CC (complementary to the viral "R" region in the viral RNA genome) or UU. RT extension products were resolved by denaturing PAGE (right) and the biotin-labeled oligonucleotides (b) were detected. Oligonucleotide size is indicated (nt). Arrow indicates the desired RT product.

Figure 6.

A3G hampers transcription elongation of HIV-1. (A) TZM-bl cells were infected with vif(–) HIV-1 harvested from HEK293T cells co-transfected with A3G or empty vector. Cells were harvested 48 hours post-infection, the total RNA was extracted, separated on urea-15% PAGE, and subjected to northern blot analysis with probes corresponding to the TAR region. Duplicates are indicated as 2,3 and 4,5 for infection with virus empty or containing A3G, respectively. (B) The total RNA extracted from these cells was used to prepare cDNA, which was amplified by specific primers to reveal *gag*, *tat* and *env* transcripts. Primers specific to amplify *actin* were used as controls.

Figure 7.

Graph representing the ratios of C to T mutation in H9- and SupT1-derived HIV-1 proviral DNA. (A) Transition of C to T in 10–129 nt; (B) 854–973 nt of the HIV-1 genome.

Genomic DNA was extracted, PCR-amplified and sequenced, and the chromatograms were analyzed as follow: i) All the C positions in the 120 nt amplicon were analyzed and the T/C ratios were determined. ii) The average of all these T/C ratios was calculated (T/C_{avg}). iii. This value was subtracted from all the T/C ratios in the amplicon. The figures A and B show the positive T/C values in the C positions.