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(54) Titre : INHIBITION DE L'INITIATION DU TRNALYS3 AMORCE DE LA TRANSCRIPTION INVERSE DANS VIH-1 PAR APOBEC3G

(54) Title: INHIBITION OF THE TRNALYS3-PRIMED INITIATION OF REVERSE TRANSCRIPTION IN HIV-1 BY APOBEC3G

(57) Abrégé/Abstract:
The present invention generally relates to the field of antiviral therapy. More specifically, the present invention relates to the inhibition of the $tRNA^{Lys3}$-primed initiation of reverse transcription in viruses by APOBEC3G. The present invention further relates to a method of treating or preventing viral infections by inhibiting $tRNA^{Lys3}$ annealing and/or priming on a viral genome thereby reducing viral replication. More particularly, the present invention relates to the use of APOBEC3G, fragments or derivatives thereof for treatment or prophylaxis of HIV-1 infection and related lentivirus infections.

ABSTRACT OF THE DISCLOSURE

The present invention generally relates to the field of antiviral therapy. More specifically, the present invention relates to the inhibition of the $tRNA^{lys3}$-primed initiation of reverse transcription in viruses by APOBEC3G. The present invention further relates to a method of treating or preventing viral infections by inhibiting $tRNA^{Lys3}$ annealing and/or priming on a viral genome thereby reducing viral replication. More particularly, the present invention relates to the use of APOBEC3G, fragments or derivatives thereof for treatment or prophylaxis of HIV-1 infection and related lentivirus infections.

TITLE OF THE INVENTION

INHIBITION OF THE $tRNA^{LYS3}$-PRIMED INITIATION OF REVERSE TRANSCRIPTION IN HIV-1 BY APOBEC3G

FIELD OF THE INVENTION

[0001] The present invention generally relates to the field of antiviral therapy. More specifically, the present invention relates to the inhibition of the $tRNA^{Lys3}$-primed initiation of reverse transcription in viruses by APOBEC3G.

BACKGROUND OF THE INVENTION

[0002] Vif (virion infectivity factor) is a 190-240 amino acid protein that is encoded by all of the lentiviruses except for equine infectious anemia virus (1-12). Vif is required for HIV-1 to replicate in certain "non-permissive" cell types, such as primary T lymphocytes, macrophages and some of T-cell lines, including H9, but is not required in other "permissive" cell types, such as SupT1 and Jurkat cells (3,5,11). The ability of Vif-negative viruses to replicate in target cells is determined by the cell producing the virus (5,12). Thus, Vif-deficient viruses produced from non-permissive cells are impaired in their ability to replicate in target cells.

[0003] Non-permissive cells have been found to contain a protein called APOBEC3G (also known as CEM-15), which prevents HIV-1 replication in the absence of Vif (13). APOBEC3G belongs to an APOBEC superfamily containing at least 10 members, which share a cytidine deaminase motif (14). These include APOBEC1 and activation-induced cytidine deaminase (AID), which have been shown to deaminate C in RNA (14) and DNA (15), respectively. It is not known if APOBEC3G can edit RNA, but several reports suggest that this protein's anti-HIV-1 activity stems from its ability to form dU by deaminating dC in the first

minus strand cDNA produced during HIV-1 reverse transcription (16-19). Vif-negative HIV-1 produced in non-permissive cells package APOBEC3G during assembly, while Vif-positive virions do not (13,16). cDNA synthesis is low in the target cell infected with Vif-negative viruses, and the minus strand cDNA made contains 1-2% of the cytosines mutated to uracil. This could allow for cDNA degradation by the DNA repair system. The coding strand found in double-stranded cDNA also contains an increase in G to A mutations that could also contribute to the anti-viral activity of APOBEC3G through mutant coding regions for viral proteins. Vif is able to bind to APOBEC3G (20), and can reduce both the cellular expression of APOBEC3G and its incorporation into virions (21). The reduction in cellular expression has been attributed to both inhibition of APOBEC3G translation and its degradation in the cytoplasm by Vif (22), and recent evidence suggests that Vif interacts with cytoplasmic APOBEC3G as part of a Vif-Cul5-SCF complex, resulting in the ubiquination of APOBEC3G and its degradation (23).

[0004] Enzymes similar to the human APOBEC superfamily are also encoded by the mouse and African green monkey (AGM) (20), and a mouse gene on chromosome 15 (murine CEM15) shows amino acid similarity and structural homology with human APOBEC3G (13,24). Vif is not present in the simple retrovirus MuLV, and Vif from HIV-1 is unable to prevent encapsidation of murine APOBEC into HIV-1, whose packaging results in severe inhibition of HIV-1 replication (20). Interestingly, while murine APOBEC is incorporated into murine leukemia virus (MLV), it appears to have little effect upon this virus's replication (16,18,20). On the other hand, the human APOBEC3G can inhibit the infectivity of different retroviruses including MLV, simian immunodeficiency virus (SIV), hepatitis C virus and equine infectious anaemia virus (EIAV) (16,18) , although at lower efficiency than for HIV-1.

[0005] The mechanism by which APOBEC3G is incorporated into Vif-

negative HIV-1 is not clear. However, a recent paper reports that mutations in either of the two active sites of APOBEC3G inhibit deoxycytidine deaminase activity to different extents, but have the same anti-viral activity (54). This latter observation implies that deoxycytidine deaminase activity of APOBEC3G may not be the sole determinant of anti-viral activity.

[0006] Therefore, there remains a need to understand the mechanism by which APOBEC3G reduces viral replication and infectivity.

[0007] There remains a need to identify novel therapeutic targets that could be used to design new drugs useful in the treatment of lentivirus infection (e.g. HIV, SIV, EIAV) as well as other viruses infection such as hepatitis C virus and MLV.

[0008] The present invention seeks to meet these needs and other needs.

[0009] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0010] Applicants have found that the incorporation of APOBEC3G into HIV-1 requires sequences found between the two zinc coordination motifs found in this protein (amino acids 104-156) and the nucleocapsid (NC) sequence in Gag. HIV-1 Gag alone among viral proteins is sufficient to package APOBEC3G into Gag viral-like particles (VLPs). Evidence is also presented that suggests that an RNA bridge between these two molecules is not involved in facilitating the Gag/APOBEC3G interaction. Moreover, it is demonstrated that APOBEC3G

prevents the proper annealing of tRNALys3 to the viral RNA genome, and also that wild-type tRNALys3 annealing and initiation of reverse transcription can be rescued with a transient exposure of the deproteinized tRNALys3/viral RNA template to NCp7.

[0011] The present invention relates to the inhibition of viral replication and infectivity by APOBEC3G, fragments or derivatives thereof through the inhibition of tRNAlys3 priming on viral genome.

[0012] In one particular embodiment, the present invention relates to the inhibition of tRNAlys3 annealing and priming on viral genome by inhibiting nucleocapsid facilitated reverse transcription. In one particular embodiment, APOBEC3G, fragments or derivatives thereof are used to treat viral infections (e.g. lentivirus, hepatitis C, MLV infections) by inhibiting tRNAlys3 annealing and priming on viral genomes.

[0013] In a more particular embodiment, the present invention relates to APOBEC3G, fragments or derivatives thereof to target nucleocapsid of HIV viruses to indirectly inhibit tRNAlys3 annealing and priming on viral genome.

[0014] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] In the appended drawings:

[0016] Figure 1 shows the incorporation of APOBEC3G into viruses or

Gag viral-like particles (VLPs). 293T cells were cotransfected with APOBEC3G expression vector and different plasmids containing wild-type or mutant HIV-1 proviral DNA. The plasmids used are listed along the top of each panel, and described in the text. 48 hours post-transfection, cells, viruses, or Gag VLPs produced by the cells were purified, lysed in RIPA buffer, and cellular and viral proteins were analyzed by Western blots. **A.** Western blots of cell lysate were probed with anti-HA (top panel), anti-β-actin (middle panel), or anti-Vif (bottom panel). **B.** Western blots of viral or Gag VLP lysates were probed with either anti-HA (upper panel) or anti-CA (lower panel). **C**. 293T cells were transfected with BH10.P-Vif- or hGag. Total cellular RNA and viral RNA were extracted, and HIV-1 viral RNA in each samples were determined by dot blot hybridization, as described in Materials and Methods. The bar graphs represent relative amount of HIV-1 viral RNA in cell lysates (upper panel) and viral lysates (lower panel), and the results are normalized to β-actin or Gag, respectively.

[0017] Figure 2 shows the interaction of APOBEC3G with wild-type or mutant Gag in the cell. 293T cells were cotransfected with APOBEC3G expression vector and different plasmids coding for wild-type or mutant Gag proteins. Interaction between Gag and APOBEC3G was measured by the ability to co-immunoprecipitate these molecules from cell lysate with anti-HA. Panel **A** graphically represents the wild-type and mutant Gag variants tested. The top drawing shows the wild-type Gag domains, with numbers representing the amino acid positions. MA,matrix domain; CA, capsid domain; NC, nucleocapsid; p6, p6 domain. **B**. Western blots of cell lysates of transfected cells were probed with anti-CA (top) or anti-HA (bottom). **C**. Western blots of anti-HA immunoprecipitates from cell lysates were probed with anti- CA (top) or anti-HA (bottom). **D.** 293T cells were cotransfected with BH10.P-.Vif- and APOBEC3G, and the cell lysates were subjected to RNase or DNase treatment, followed by immunoprecipitation with either anti-integrase (IN) or anti-HA, respectively. The immunoprecipitates were analyzed by Western blotting, using anti-CA to detect the presence of Gag in the

immunoprecipitate.

[0018] Figure 3 shows the ability of APOBEC3G to be incorporated into wild-type or mutant HIV-1. 293T cells were cotransfected with APOBEC3G expression vector and different plasmids containing wild-type or mutant HIV-1 proviral DNA. The plasmids used are listed along the top of each panel, and described in the text. A. Western blots of cell lysates were probed with either anti-HA (upper), anti-CA (middle), or anti-β-actin (bottom) B. Western blots of cell lysates of Gag VLPs produced from transfected cells were probed with either anti-HA (upper) or anti-CA (bottom).

[0019] Figure 4 shows the ability of mutant APOBEC3G to be incorporated into Gag VLPs. Plasmids coding for N- and C-terminal APOBEC3G deletion mutants were cotransfected into 293T cells with the plasmid coding for hGag. A. Graphic representation of the wild-type and mutant APOBEC3G variants tested. The filled rectangles represent the two catalytic sites in APOBEC3G, and the numbers represent the amino acid positions. B. Western blots of cell lysates probed, respectively, with anti-HA (top) and anti-β-actin (bottom). C. Western blots of lysates of Gag VLPs produced from these cells, probed, respectively, with anti-HA (top) and anti-CA (bottom). The APOBEC3G: β-actin and APOBEC3G:Gag ratios are listed at the bottom of panels B and C, respectively, and are normalized to the ratio obtained for wild-type APOBEC3G.

[0020] Figure 5 shows the distribution of APOBEC3G between cytoplasm and membrane. 2 μg APOBEC3G expression vector were transfected into 293T cells, or cotransfected with 2 μg of plasmids coding for wild-type or mutant hGag. Cells were lysed hypotonically in TE buffer, and the post-nuclear supernatant was resolved by the sucrose floatation assay into membrane-bound (I) and membrane-free (B) protein, as described in Materials and Methods. The left side of panels A to E show Western blots of gradient fractions probed with anti-HA,

while the right side of each panel presents these blots, as well as blots probed with anti-CA, graphically, showing the percentage of analyzed protein in each gradient fraction. ▨ and ▧ represent APOBEC3G and Gag, respectively. A. Cells are transfected with the plasmid coding for APOBEC3G alone. B-E. Cells are cotransfected with the plasmid coding for APOBEC3G and plasmid(s) coding for B. hGag, C. hGag, and Vif, D. the mutant Gag ZWt-p6.Vif-, and E. the Δ1-132 hGag. "I" and "B" at the top of panel represent interface and bottom fraction in the discontinuous sucrose gradient respectively.

[0021] Figure 6 shows the incorporation of APOBEC3G into Gag VLPs is proportional to its cellular expression. 293T cell were cotransfected with 2μg hGag and various amount of plasmid coding APOBEC3G. Western blots of cell lysate or Gag VLP lysates probed for APOBEC3G with anti-HA are shown in upper and lower blot, respectively. Bands in Western blots were quantitated, and the right panel plots the relative intensities of APOBEC3G expressed in the cell vs APOBEC3G incorporated into Gag VLPs.

[0022] Figure 7 shows the effect of Vif upon both the cellular expression of APOBEC3G and its incorporation into HIV-1. 293T cells were transfected with plasmids containing either wild-type (BH10) or Vif-negative (BH10Vif-) viral DNA, or cotransfected with these plasmids plus either plasmid alone (pcDNA3.1) or this plasmid containing APOBEC3G DNA. The plasmids used are listed along the top of each panel, and described in the text. 48 hours post-transfection, cells, or viruses produced by the cells, were lysed in RIPA buffer, and cellular and viral proteins were analyzed by Western blots. A. Western blots of cell lysates, containing similar amounts of β-actin (bottom panel) were probed, from top panel down, respectively, with anti-Vif, anti-HA, anti-CA, and anti-β actin. B. Western blots of viral lysates, containing similar amounts of CAp24 (bottom panel), were probed with either anti-HA (upper panel) or anti-CA (lower panel).

[0023] Figure 8 shows the effect of APOBEC3G upon tRNALys3 annealing to viral RNA and initiation of reverse transcription in wild-type and Vif-negative HIV-1.Total viral RNA was used in an vitro reverse transcriptase reaction as the source of primer tRNALys3 annealed to genomic RNA in vivo. A. Cartoon showing tRNA annealing and initiation of reverse transcription. This shows the annealing of the terminal 3′ 18 nucleotides of tRNALys3 to the primer binding site (PBS) on the viral RNA genome, which contains 18 complementary nucleotides. The first 6 deoxyribonucleotides incorporated (CTGCTA) during initiation of reverse transcription are underlined. B. Annealing of tRNALys3 to viral RNA. The reverse transcription reaction mix contains purified HIV-1 reverse transcriptase, 5 μM α-32P-GTP, 200 μM CTP and TTP, and 200 μM ddATP. ddATP will cause extension by reverse transcriptase to terminate after 6 bases, i.e., CTGCTA. Panel B shows the radioactive 6 base-extended tRNALys3 resolved by 1D PAGE. Lanes: 1, purified human placental tRNALys3 heat-annealed in vitro to synthetic viral genomic RNA; 2, 3, total viral RNA isolated from virions produced from cells cotransfected with the plasmid vector pcDNA3.1 and a plasmid coding for either wild-type HIV-1 (BH10, lane 2), or for Vif-negative HIV-1 (BH 10VIF-, lane 3); 4, 5, total viral RNA isolated from virions produced from cells cotransfected with the pcDNA3.1 plasmid containing the gene for APOBEC3G, and a plasmid coding for either wild-type HIV-1 (BH10, lane 4), or for Vif-negative HIV-1 (BH 10VIF-, lane 5). C. Initiation of reverse transcription. Either total viral RNA containing equal amounts of viral genomic RNA (left) or total viral RNA containing equal amounts of annealed tRNALys3 (right), as determined in panel B, were used in the reverse transcription reaction, which also contained contains purified HIV-1 reverse transcriptase and 0.16 μM α-32P-dCTP and 0.16 μM α-32P-dGTP. The viral source of primer/template RNA in the different lanes are as described in panel B, which shows the radioactive 1, 3, and 4 base extended tRNALys3 resolved by 1D PAGE. D. Quantitation of electrophoretic results. The electrophoretic bands shown in panels B and C were measured using phosphorimaging (BioRad), and graphed. Panel B, annealing; Panel C, initiation. Also plotted, using data not

shown, is the viral RNA/p24 (vRNA) and the tRNALys3 incorporated/viral RNA (tRNALys3).

[0024] Figure 9 shows the effect of increasing cellular expression of APOBEC3G upon the viral incorporation of APOBEC3G. 293T cells were cotransfected with increasing amounts of APOBEC3G DNA (pAPOBEC3G) and plasmids coding for either wild-type HIV-1 (BH10, lanes 1-5) or Vif-negative HIV-1 (BH10VIF(-), lanes 6-10). The amount of DNA transfected in the cell was kept constant by keeping the vector DNA that contains the APOBEC3G gene (pcDNA 3.1) constant. The micrograms of plasmids used for transfection are listed along the top of each panel. 48 hours post-transfection, cells, or viruses produced by the cells, were lysed in RIPA buffer, and cellular and viral proteins were analyzed by Western blots. A. Western blots of cell lysates, containing similar amounts of β-actin (bottom panel) were probed, from top panel down, respectively, with anti-HA, anti-CA, and anti-β actin. B. Western blots of viral lysates, containing similar amounts of CAp24 (bottom panel), were probed with either anti-HA (upper panel) or anti-CA (lower panel).

[0025] Figure 10. Inhibition of $tRNA^{Lys3}$ annealing and initiation of reverse transcription in Vif-negative HIV-1 is directly proportional to cellular expression of APOBEC3G. Extracellular viruses produced in the transfected 293T cells described in Figure 3 were purified by sucrose cushion centrifugation. Total viral RNA was isolated from each viral type, and used in the reverse transcription reaction to measure either $tRNA^{Lys3}$ annealing (A), or initiation of reverse transcription (B), using reaction conditions described in the Figure 2 legend. Panel A (left) shows the radioactive 6 base-extended $tRNA^{Lys3}$ resolved by 1D PAGE. The left and right sides of this gel show the effect of increasing cellular expression of APOBEC3G upon $tRNA^{Lys3}$ annealing in BH10 or BH10Vif- virions, respectively, and these results are plotted in the right side of panel A. Panel B (left) shows the radioactive 1, 3, and 4 base extended $tRNA^{Lys3}$ resolved by 1D PAGE. The left and

right sides of this gel show the effect of increasing cellular expression of APOBEC3G upon initiation of reverse transcription in BH10 or BH10Vif- virions, respectively, and these results are plotted in the right side of panel B. The micrograms of plasmids used for transfection are listed along the top of each gel.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0026] The present invention is illustrated in further details by the following non-limiting examples.

EXAMPLE 1

Experimental procedures

[0027] *Plasmid construction*— SVC21BH10.P- is a simian virus 40-based vector that contains full-length wild-type HIV-1 proviral DNA containing an inactive viral protease (D25G), and was a gift from E. Cohen, University of Montreal. SVC21BH10.FS- contains mutations at the frameshift site, i.e., from 2082-TTTTTT-2087 to 2082-CTTCCT-2087, which prevents frameshifting during the translation of Gag protein, and generates viruses that contain Gag, but not Gag-Pol (25). ZWt-p6 encodes a full-length HIV-1 genome, in which the nucleocapsid sequence has been replaced with a yeast leucine zipper domain (26). BH10.Vif-, BH10.P-.Vif-, BH10.FS-.Vif- and ZWt-p6.Vif- were generated by introducing a stop codon right after ATG of the Vif reading frame at 5043, using a site-directed mutagenesis Kit (Stratagene) with the following pair of primers: 5'-AGA TCA TTA GGG ATT TAG GAA AAC AGA TGG CAG, and 5'-CTG CCA TCT GTT TTC CTA AAT CCC TAA TGA TCT.

[0028] The human APOBEC3G cDNA was amplified from H9 mRNA by reverse transcription-PCR, using the pair of primers: 5'-GCC AGA ATT CAA GGA TGA AGC CTC ACT TCA G, and 5''-TAG AAG CTC GAG TCA AGC GTA ATC

TGG AAC ATC GTA TGG ATA GTT TTC CTG ATT CTG GAG AAT GG. The cDNA fragment was cloned into the pcDNA3.1 V5/His A vector (Invitrogen), which expresses wild-type human APOBEC3G with a fused HA tag at the C-terminus. In order to construct mutant APOBEC3G, this cDNA was PCR-amplified and digested with EcoRI and XhoI, whose sites were placed in each of the PCR primers. These fragments were cloned into the EcoRI and XhoI sites of the pcDNA3.1 V5/His A vector. We used the following primers: wild-type: forward primer: 5′-TAA GCG GAA TTC ATG AAG CCT CAC TTC AGA. reverse primer: 5'-TAG AAG CTC GAG TCA AGC GTA ATC TGG AAC. Δ1-57: 5′-TAG GCG GAA TTC ATG GTG TAT TCC GAA CTT AAG. Δ1-104: 5′-TAA GTC GAA TTC ATG GCC ACG TTC CTG GCC GAG. Δ1-156: 5′-TAA GTC GAA TTC ATG TTT CAG CAC TG TGG AGC. Δ157-384: 5′- TAG AAG CTC GAG TCA AGC GTA ATC TGG AAC ATC GTA TGG ATA TTC GTC ATA ATT CAT GAT. Δ246-384: 5′- TAG AAG CTC GAG TCA AGC GTA ATC TGG AAC ATC GTA TGG ATA CTG GTT GCA TAG AAA GCC. Δ309-384: 5′-TAG AAG CTC GAG TCA AGC GTA ATC TGG AAC ATC GTA TGG ATA GAT GCA CAG GCT CAC GTG. The resulting constructs expressing HA-tagged wild-type and mutant APOBEC3G were transfected into 293T cells.

[0029] The hGag plasmid, which encodes the HIV-1 Gag sequence, produces mRNA whose codons have been optimized for mammalian codon usage, and was a kind gift from G Nabel, NIH (27). All the N- or C- terminally deleted Gag plasmids were constructed using PCR. hGag was PCR-amplified and digested with SalI and XbaI, whose sites were introduced in each of the PCR primers. These fragments were cloned into the SalI and XbaI sites of hGag. The following primers were used to construct these deletions: Wild-type: forward primer: 5′-ATA ATA GTC GAC ATG GGC GCC CGC GCC AGC GTG. reverse primer: 5′-GAC TGG TCT AGA AGG GCC TCC TTC AGC TGG. Δ1-132: 5′-GCG GCG GTC GAC ATG CCC ATC GTG CAG AAC ATC. Δ284-500: 5′-GCG GCG TCT AGA TTA CAG GAT GCT GGT GGG GCT. Δ377-500: 5′-GCG GCG TCT AGA TTA CAT GAT GGT GGC GCT GTT. Δ433-500: 5′- GCG GCG TCT AGA TTA AAA ATT

AGC CTG TCG CTC.

[0030] *Cells, transfections and viruses purification*— HEK-293T cells were grown in complete DMEM plus 10% fetal calf serum (FCS), 100 Units of penicillin and 100μg of streptomycin per ml. For the production of viruses, HEK-293T cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Supernatant was collected 48 hours post-transfection. Viruses were pelleted from culture medium by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 hour. The viral pellets were then purified by centrifugation in a Beckman SW41 rotor at 26,500 rpm for 1 hour through 15% sucrose onto a 65% sucrose cushion. The band of purified virus was removed and pelleted in 1X TNE in a Beckman Ti45 rotor at 40,000 rpm for 1 hour.

[0031] *Viral RNA isolation and quantification*— Total cellular and viral RNA was extracted using guanidinium isothiocynate, and the relative amount of HIV-1 viral RNA was quantified by dot blot hybridization, as previously described (28). Variable known amounts of BH10 plasmid were used as a standard, and each sample of total cellular or viral RNA was blotted onto Hybond N+ nylon membranes (Amersham Pharmacia), and was probed with a 5′ ^{32}P- end-labelled 30-mer DNA probe specific for the sequence from nt 2211 to nt 2240 of the HIV-1 genome. Experiments were done in triplicate. The amounts of HIV-1 viral RNA per sample were analyzed using phosphor-imaging (BioRad), and the relative amount of viral RNA in cell lysates and virus preparations was determined.

[0032] *Protein Analysis*— Cellular and viral proteins were extracted with RIPA buffer (10 mM Tris, pH 7.4, 100mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin A, 100 mg/ml PMSF). The cell and viral lysates were analyzed by SDS PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham

Pharmacia). Western blots were probed with monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepto Metrocs Inc.), HA (Santa Cruz Biotechnology Inc.), and β-actin (Sigma), or with Vif-specific polyclonal antiserum #2221 (NIH AIDS Research and Reference Reagent Program). Detection of proteins was performed by enhanced chemiluminescence (NEN Life Sciences Products), using as secondary antibodies anti-mouse (for capsid and β-actin) and anti-rabbit (for HA and Vif), both obtained from Amersham Life Sciences. Bands in Western blots were quantitated using UN-SCAN-IT gelTM automated digitizing system.

[0033] *Immunoprecipitation assay*— 293T cells from 100 mm plates were collected 48 hours post transfection, and lysed in 500 µl TNT buffer (20mM Tris-HCl pH 7.5, 200mM NaCl, 1% Triton X-100). Insoluble material was pelleted at 1800 X g for 30 minutes. The supernatant was used as the source of immunoprecipitated Gag/APOBEC3G complexes, Equal amounts of protein were incubated with 30 µl HA-specific antibody for 16 hours at 4^{o}C, followed by the addition of protein A-Sepharose (Pharmacia) for two hours. For a Western blot of different cell lysates, 500 µg of lysate protein was used for immunoprecipitation from each lysate, while for different nuclease experiments on the same lysate sample, approximately 200 µg of lysate protein was used for immunoprecipitation. Lysate protein was determined by the BioRad assay. The immunoprecipitate was then washed three times with TNT buffer and twice with phosphate-buffered saline (PBS). After the final supernatant was removed, 30 µl of 2X sample buffer (120 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, and 0.02% bromphenol blue) was added, and the precipitate was then boiled for 5 minutes to release the precipitated proteins. After microcentrifugation, the resulting supernatant was analyzed using Western blots. In the DNase and RNase treatment assay, the cell lysates were pre-treated with 20 µg DNase or RNase before the immunoprecipitation, as previously described (29).

[0034] *Subcellular fractionation and sucrose floatation assay*— Cells were lysed 48 hours post-transfection at 4°C by dounce homogenization in 1.0 ml hypotonic TE buffer (20 mM Tris-HCl, pH 7.4, 1mM EDTA, 0.01% β-mercaptoethanol), supplemented with protease inhibitors cocktail ("Complete", Boehringer Manheim). The cell homogenate was then centrifuged at 1500 x g for 30 minutes to remove nuclei and unbroken cells. 0.5 ml of the resulting supernatant (S1) was mixed into 3 ml of final 73% sucrose. 7 ml of 65 % sucrose in TNE (20 mM Tris pH 7.8, 100 mM NaCl, 1mM EDTA) were layered on top of the 73% sucrose, and 1.5 ml of 10% sucrose was layered on top of the 65% sucrose. The gradients were then centrifuged at 100,000 x g in a Beckman SW55 Ti rotor overnight at 4°C. 2 ml fractions were collected, diluted with 10 ml TNT, and each fraction was centrifuged at 100,000 X g at 4°C for 1 hour. The pellets from each fraction were dissolved in SDS sample buffer, and analyzed by SDS-PAGE and Western blotting.

[0035] Measuring $tRNA^{Lys3}$ annealing to viral RNA and the initiation of reverse transcription.— Total viral RNA isolated from virus produced in transfected 293T cells was used as the source of a primer tRNA-template complex in an *in vitro* reverse transcription reaction, and used to measure both the amount of extendable tRNALys3 annealed to viral RNA, and the ability of this annealed tRNA to initiate reverse transcription, as previously described (1, 2). Briefly, total virus RNA was incubated at 37°C in 20 mlof RT buffer (50mM Tris-HCl[pH7.5], 60mM KCl, 3mM $MgCl_2$, 10mM dithiothreitol) containing 50 ng of purified HIV RT, 10U of RNasin, and various radioactive α-32P- deoxynucleotide triphosphates (dNTPs). The extension product was ethanol precipitated, resuspended, and analyzed on 6% polyacrylamide-7M urea-1 X tris-borate-EDTA. Initiation from unextended $tRNA^{Lys3}$ was measured in the presence of the first base incorporated, dCTP, while initiation from 2 base-extended tRNALys3 ($tRNA^{Lys3}$-CT) was measured in the present of the 3rd base incorporated, dGTP. To measure total $tRNA^{Lys3}$ annealing to viral RNA (which includes both unextended and 2-base-extended forms of

tRNA^{Lys3}), the reaction mixture contained 200 μM dCTP, 200 μM dTTP, 5 μCi of α-32P-dGTP(0.16μM), and 50μM ddATP. In some experiment, NCp7 (obtained from Rob Gorelick, NIH Frederick) was incubated with total viral RNA for 30min at 37°C in RT buffer, and removed by protinase K digestion and phenol-cholroform extraction as described previously (1), followed by initiation of reverse transcription.

[0036] Nucleocapsid protein— Recombinant HIV-1 nucleocapsid protein (NCp7) composed of 55 amino acids, was expressed in bacteria as previously described, and was obtained from Rob Gorelick. The primer/template complex was pre-incubated with with 10 pmolar NCp7 in RT buffer at 37°C for 30min. The NCp7 was then removed by proteinase K digestion and phenol-chloroform extraction. Then reverse transcription was initiated through the addition of RT, and the reaction was incubated for 30 minutes, and then analyzed by 1D PAGE. The results indicate that the reduced initiation of reverse transcription seen in Vif-negative viruses produced from 293T cells expressing APOBEC3G is rescued 40-70% when the total viral RNA is transiently exposed to mature nucleocapsid protein. Exposure to nucleocapsid of the total viral RNA isolated from wild-type viruses produced in APOBEC3G-expressing cells has no effect upon initiation of reverse transcription.

EXAMPLE 2

Incorporation of APOBEC3G into Gag VLPs

[0037] 293T cells were co-transfected with a plasmid coding for human APOBEC3G containing a C-terminal HA tag, and plasmid containing wild type or mutant HIV-1 proviral DNA. BH10.Vif- and BH10.P-.Vif- both contain a stop codon immediately after the initiation ATG codon of the Vif reading frame, and BH10P- contains an inactive viral protease. hGag contains a humanized HIV-1 Gag gene

(i.e., codon usage optimized for translation in mammalian cells (27)), and only wild type HIV-1 Gag and Gag VLPs are produced (25). The cell lysates of transfected cells were analyzed by Western blots (Figure 1A), using anti-HA (top panel), anti-β-actin (middle panel) and anti-Vif (bottom panel) antibodies as probes. Vif is detected only in cells transfected with BH10. In cells producing virions or Gag VLPs lacking Vif, APOBEC3G is is strongly expressed, while in cells producing BH10, very little APOBEC3G is seen in the cytoplasm. The viruses produced from these cells were analyzed by Western blotting (Figure 1B), using anti-HA (top panel) and anti-CAp24 (bottom panel). While no APOBEC3G is seen in wild-type BH10, it is found in virions not expressing Vif. These results also indicate that Gag alone is sufficient among the viral proteins for facilitating APOBEC3G incorporation. Our results also confirm previous observations of a diminished presence of APOBEC3G in both the cytoplasm and in virions in the presence of Vif expression, and this has been shown to be due to the Vif-induced polyubiquination of APOBEC3G, and subsequent degradation by the proteosome (22,23,30-32)

[0038] As well as lacking coding sequences downstream of Gag, the RNA coding for hGag has the 5′ RU5 and leader sequence of the viral RNA replaced with a CMV promoter. Therefore, it is not expected that hGag VLPs will specifically package this RNA, which lacks viral packaging signals. This suggests that APOBEC3G incorporation into these particles occurs independently of viral genomic RNA packaging. To further confirm this, total RNA was extracted from cells cotransfected with APOBEC3G and either BH10.P-.Vif- or hGag, and from the virions produced from these cells. Viral mRNA in the cells and viruses were quantified by dot blot, using a ^{32}P-labelled DNA probe specific for the p6 coding sequence, which is present in both BH10.P-.Vif- and hGag RNA. The ratios for viral RNA: β-actin in the cytoplasm, and viral RNA:Gag in virions, is presented graphically in Figure 1C. Although cytoplasmic expression of viral genomic RNA is strong in cells expressing hGag (top panel, Figure 1C), the genomic RNA/Gag in hGag VLPs is reduced to approximately 15% of that found in BH10.P-.Vif-, (bottom

panel, Figure 1C). This reduced incorporation of viral RNA does not, however, affect APOBEC3G incorporation into hGag VLPs (panel B), indicating that APOBEC3G incorporation into virions occurs independently of viral RNA incorporation.

EXAMPLE 3

The nucleocapsid sequence within Gag is required for the viral packaging of APOBEC3G

[0039] A series of Gag deletion constructs were used to identify the motif within Gag involved in the incorporation of APOBEC3G into viruses. These constructs are shown in Figure 2A. 293T cells were cotransfected with APOBEC3G and wild-type or mutant Gag constructs, and cells were lysed in RIPA buffer. Western blots of cell lysates (Figure 2B) were probed with anti-CA (upper panel) or anti-HA (lower panel). The first lane represents cells transfected with hGag alone. All Gag mutants were expressed at similar levels in the cytoplasm, except for the 378-500 construct. This Gag has NC, p1 and p6 deleted from the C-terminus, and is expressed 2-3 fold higher than full-length Gag.

[0040] Most of these mutant Gag molecules are impaired in their ability to form extracellular particles due to the absence of membrane- or RNA-binding regions. We have therefore investigated the interaction between APOBEC3G and mutant Gag species using immunoprecipitation to detect cellular complexes. The presence of both Gag and APOBEC3G in the cell lysate was first analyzed by Western blots probed with anti-CA (Figure 2B, upper panel), and anti-HA (Figure 2B, lower panel). The Gag:APOBEC3G ratios, listed at the bottom of panel B, normalized to the hGag:APOBEC3G ratio, are similar for all mutant Gag species expressed, except for Δ378-500, which shows a higher expression of Gag. APOBEC3G in each cell lysate was then immunoprecipitated by anti-HA, and the

presence of both Gag and APOBEC3G in the immunoprecipitate was analyzed by Western blotting, using anti-CA (Figure 2C, upper panel), and anti-HA (Figure 2C, lower panel). The Gag:APOBEC3G ratios, listed at the bottom of panel C, normalized to the hGag:APOBEC3G ratio, indicate no change in the association of Gag with APOBEC3G with removal of the N-terminal MA sequences (Δ1-132), and a small decrease (12%) with removal of the C-terminal p1/p6 sequences (Δ433-500). However, a C-terminal deletion of Gag which also included NC (Δ378-500) resulted in a >95% reduction in the interaction of Gag with APOBEC3G, even though the expression of this mutant Gag is greater in the cell lysate than seen for hGag (Figure 2B). A larger C-terminal Gag deletion (Δ284-500), in which p2 and the C terminal region of capsid (including the MHR domain) have been further removed, also prevented interaction with APOBEC3G. These data suggest that nucleocapsid sequences within Gag are responsible for the interaction between APOBEC3G and Gag The small decrease in the Gag:APOBEC3G ratio found with removal of the p1/p6 sequences might reflect an altered conformation affecting the neighboring NC binding site in Gag.

[0041] Both Gag nucleocapsid (33) and members of the APOBEC family, including APOBEC3G (14), can bind to RNA, so that the interaction demonstrated between Gag and APOBEC3G could be mediated by an RNA bridge. However, the data in Figure 2D suggests that an RNA bridge is not likely. 293T cells were cotransfected with BH10.P-.Vif- and APOBEC3G, and the cell lysates were subjected to RNase or DNase treatment, followed by immunoprecipitation with either anti-integrase (IN) or anti-HA, respectively. The immunoprecipitates were analyzed by Western blotting, using anti-CA to detect the presence of Gag in the immunoprecipitate. The left side of panel D shows the effects of DNase and RNase upon the immunoprecipitation of Gag with anti-IN, which reacts with GagPol. We have previously reported that anti-IN will not immunoprecipitate Gag in the presence of RNase (29), and the results on the left side of panel D repeat those results. The right side of panel D shows a similar

experiment in which APOBEC3G is immunoprecipitated with anti-HA, and the coimmunprecipitation of Gag is determined. It can be seen that exposure of the immunoprecipitate to either RNase or DNase does not affect the coimmunprecipitation of APOBEC3G with Gag. While this suggests the lack of an RNA or DNA bridge between these two molecules, we cannot eliminate the possibility that a small RNA bridge may be protected from RNase digestion by the two proteins.

[0042] The requirement for nucleocapsid sequence is further shown in Figure 3, in which the nucleocapsid sequence in HIV-1 has been replaced with a yeast leucine zipper domain to allow for protein/protein interactions (plasmid ZWt-p6.Vif-). It has previously been shown that the parental plasmid, ZWt-p6, can efficiently produce extracellular viruses (26). Another mutant, BH10.FS-.Vif-, in which frame shift sequence had been changed to produce only Gag, was used as a control. 293T cells were cotransfected with APOBEC3G and mutant HIV-1 plasmids, and expression of APOBEC3G in cells were analyzed by Western blots, probed with anti-HA, anti-CA, and anti-β-actin (Figure 3A). The results show that similar amounts of APOBEC3G were efficiently produced in all the cells transfected with Vif- constructs (Figure 3A, upper panel, lanes 2, 4 and 6), whereas cellular APOBEC3G was severely reduced if the viral constructs produced Vif (Figure 3A, upper panel, lanes 1, 3 and 5). The absence or presence of Vif had no effect upon cellular Gag levels (Figure 3A, middle panel). The ability of the viruses to package APOBEC3G was then assessed by Western blots of viral lysates probed with anti-CA (Figure 3B, lower panel) or anti-HA (Figure 3B, upper panel). The results show that BH10.FS-.Vif- can package APOBEC3G as efficiently as BH10.P-. On the other hand, the ability of ZWt-p6.Vif- to incorporate APOBEC3G is reduced 90% compared with BH10.FS-.Vif-. These data demonstrate that while the leucine zipper motif can functionally replace nucleocapsid for Gag multimerization and virus assembly, it cannot replace its ability to facilitate APOBEC3G incorporation.

EXAMPLE 4

Sequences in APOBEC3G required for its incorporation into Gag VLPs

[0043] 293T cells were cotransfected with hGag and a plasmid coding for wild-type or N- or C-terminal-deleted APOBEC3G tagged with HA. These constructs are shown graphically in Figure 4A. APOBEC3G has sequence homology with APOBEC1, and contain two or one active site regions, respectively, (H-X-E-$(X)_{24-30}$-P-P-X-X-C) containing a zinc coordination motif. The cytoplasmic expression and viral incorporation of the different APOBEC3G variants was determined by Western blots probed with anti-HA and anti-β-actin for cells (Figure 4B) or anti-HA and anti-CA for viruses (Figure 4C). The mutant APOBEC3G:β-actin ratio in the cell lysates, or APOBEC3G:Gag ratio in the viral lysates, are normalized to a ratio of 1.0 for wild-type APOBEC3G, and are listed at the bottom of each panel. As shown in Figure 4C, deletion of the N-terminal 104 amino acids or the C-terminal 157-384 amino acids does not affect the ability of APOBEC3G to be packaged into Gag VLPs, whereas the deletion of the N-terminal 156 amino acids abolishes its incorporation into viruses. This result indicates that amino acids 104-156, found in the N-terminal portion of a linker sequence between the two zinc coordination motifs in APOBEC3G, are required for its incorporation into Gag VLPs.

[0044] All C-terminal APOBEC3G deletions shown in Figure 4 show reduced expression in the cell lysate (10-20% of wild-type (Figure 4B). This may be due to intracellular degradation since it has been reported that N-terminal fragments of APOBEC3G are inherently unstable (34). Interestingly, the viral content of these N-terminal fragments is >60% of wild type APOBEC3G, i.e., does not reflect their low cytoplasmic expression. Thus, the removal of the C-terminal regions of APOBEC3G appears to result in a significant decrease in its

concentration in the total cell lysate without a similar quantitative decrease in its incorporation into Gag VLPs. This suggests that the decreased APOBEC3G pools are not the source of viral APOBEC3G. The floatation gradients of post-nuclear supernatant, as shown in Figure 5 below, indicate that almost all cytoplasmic APOBEC3G interacts with Gag and moves to the membrane. However, we have recently observed that >80% of APOBEC3G is found in the nucleus (data not shown), so the decreased expression of C-terminally truncated APOBEC3G in cell lysate might involve primarily nuclear APOBEC3G, and not affect the cytoplasmic pools. The cellular source of viral APOBEC3G is currently being investigated, and might be similar to the cellular origins of viral GagPol (35) and viral LysRS (36,37). Both of these molecules are rapidly incorporated into Gag particles, and appear to come from cytoplasmic pools of newly-synthesized molecules. The alternative explanation that the C-terminally truncated APOBEC3G interacts with Gag more efficiently than wild-type Gag is not likely, since, as shown in Figure 6 below, increasing concentrations of wild-type APOBEC3G in the cytoplasm interact efficiently with Gag.

EXAMPLE 5

Effect of Gag expression upon the intracellular distribution of APOBEC3G

[0045] 293T cells were transfected with the plasmid coding for APOBEC3G alone, or co-transfected with this plasmid and plasmids coding for mutant forms of hGag in the presence or absence of Vif. Transfected cell were lysed in hypotonic buffer, and, after a low-speed centrifugation to remove broken cells and nuclei, the post-nuclear supernatant was resolved on sucrose gradients into membrane-free and membrane-bound protein, as described previously (35). Gradient fractions were analyzed by Western blots, probed with anti-HA or anti-CA antibody. As shown in Figure 5A, in the absence of Gag, >90% APOBEC3G is present near the bottom of the gradient, i. e., in the cytoplasmic fraction (lanes 5 and 6). However, in the presence of Gag (Figure 5B), >90% of APOBEC3G is localized in the membrane-bound protein near the top of the gradient at the 10%/65% sucrose interface, reflecting a similar intracellular distribution for Gag (35). If Vif is also expressed, the APOBEC3G remains in the cytoplasm at reduced levels (Figure 5C). When cells express both APOBEC3G and the mutant Gag species, ZWt-p6. Vif-, the majority of APOBEC3G remains in the cytoplasm even though most Gag is found at membrane (Figure 5D). When cells are transfected with a mutant Gag that can no longer bind to membrane (Δ1-132), but that retains the ability to bind to APOBEC3G, the APOBEC3G remains in the cytoplasm (Figure 5E). These data indicate that binding to Gag transports most cytoplasmic APOBEC3G to the membrane during viral assembly. This interaction is efficient, since when cells are cotransfected with the hGag plasmid and increasing amounts of the plasmid expressing APOBEC3G, the amount of APOBEC3G incorporated into viruses is proportional to the amount of APOBEC3G expressed in the cell (Figure 6).

EXAMPLE 6

Implication of APOBEC3G interaction with Gag

[0046] Applicants have shown that Gag alone among viral proteins is sufficient for the incorporation of APOBEC3G, and deletion analysis shows that Gag nucleocapsid and amino acids 104-156 in APOBEC3G are required for the Gag/APOBEC3G interaction. Figure 2C shows that the cytoplasmic interaction between Gag and APOBEC3G requires NC sequences. The requirement for Gag nucleocapsid suggests a direct interaction of this Gag domain with APOBEC3G, but could also reflect a requirement for either Gag multimerization or for an RNA bridge binding the two proteins. The fact that the Gag/APOBEC3G interaction is still detected after Rnase A treatment (Figure 2D) suggests that Gag multimerization is not required for the interaction. Furthermore, Gag multimerization is not sufficient for the incorporation of APOBEC3G into viral particles. Thus, experiments with ZWt-p6.Vif-, a virus in which the nucleocapsid sequence has been replaced with a yeast leucine zipper responsible for facilitating protein interactions, show that the resulting extracellular Gag particles produced do not incorporate APOBEC3G (Figure 3B), i. e., the presence of NC is still required. This indicates that, while the incorporation of APOBEC3G into Gag VLPs is proportional to its expression in the cell (Figure 6), APOBEC3G is not randomly incorporated into Gag VLPs or virions. The simple production of viral particles does not ensure a random incorporation of APOBEC3G. On the other hand, the fact that APOBEC3G is incorporated into virions with diverse Gag sequences, including HIV-1, MLV, SIV, and EIAV (16,18) suggests some common property of Gag NC other than sequence similarity is required. This feature could be common structural motifs, or it could be their common ability to bind RNA.

[0047] However, the data presented here, while not eliminating the existence of an RNA bridge facilitating the interaction between Gag and

APOBEC3G, does not favor the prime importance of such a bridge. The RNA producing hGag does not contain viral genomic RNA packaging signals. The hGag VLPs produced, while containing only 14% as much viral genomic RNA as virions containing wild-type Gag (Figure 1C), do efficiently package APOBEC3G (Figure 1B). This indicates that APOBEC3G packaging occurs independently of HIV-1 viral genomic RNA, and supports an earlier finding that used a UV crosslinking assay to demonstrate that APOBEC3G bound specifically to apoB mRNA and UA rich RNA, but not to HIV-1 RNA (14). A unique role for cellular RNA in facilitating an APOBEC3G/Gag interaction is also not supported by the data. The ability to immunoprecipitate a cytoplasmic Gag/APOBEC3G complex is only slightly diminished upon prior treatment with RNase A (10-14% decrease), while the immunoprecipitation of a Gag/GagPol complex is completely inhibited by a similar RNase A treatment (Figure 2D). However, we cannot eliminate the possibility that RNA bridging Gag and APOBEC3G isn't protected from RNase digestion by these proteins.

[0048] Although the RNA-binding region(s) within APOBEC3G are not known, they have been mapped in the related family member APOBEC1 to its single zinc coordination motif (38,39). APOBEC3G binds to zinc *in vitro*, and has an RNA binding capacity similar to APOBEC1 (14). Amino acids 104-156 in APOBEC3G are required for this molecule's incorporation into Gag VLPs, yet lay outside either zinc coordination motif, which does not support a major role for RNA in the Gag/APOBEC3G interaction. There also does not appear to be any local cluster of basic amino acids within amino acids 104-156 which could contribute to the non-specific binding of RNA. We observe little or no effect on APOBEC3G incorporation into virions with the removal of either zinc coordination motif (Figure 4C).

[0049] The data presented in the middle panel in Figure 3A do not show a difference in Gag levels in Vif+ or Vif- cells expressing APOBEC3G, i. e.,

while the cellular expression of APOBEC3G is decreased in Vif- cells, Gag does not decrease. In fact, while the presence of Vif in non-permissive cells alters the cytoplasmic distribution of APOBEC3G, it does not alter the cytoplasmic distribution of Gag. This is shown in Figure 5, panels A-C. APOBEC3G in the post-nuclear supernatant is found primarily in the cytoplasm of non–permissive cells (Figure 5A). In cells also expressing Gag, almost all of it is carried to the membrane in the absence of Vif (Figure 5B), but wild-type Gag does not carry APOBEC3G to the membrane in the presence of Vif (Figure 5C). It can also be seen that the cellular distribution of Gag between membrane and cytoplasm is unaltered whether Vif is present or not. The ability of Gag to alter the cytoplasmic distribution of APOBEC3G depends upon Gag's ability to interact with either cell APOBEC3G (Figure 5D, in which the mutant Gag species ZWt-p6.Vif- is expressed), or with membrane (Figure 5E, in which the Δ1-132 mutant Gag species, which lacks membrane-binding sequences, is expressed.)

[0050] The data in Figures 3 and 5 suggest that little, if any, Gag is associated with the Vif/APOBEC3G complex. Although immunofluorescence studies showed a colocalization of Gag and Vif in the cell (40), cosedimentation studies indicated an interaction of Vif only with some early viral assembly intermediates, and the presence of Vif in mature virions remains controversial (41-48). In insect cells infected with baculovirus expressing Gag and Vif, it was estimated that there were 70 Vif molecules per 2000 Gag molecules in extracellular Gag particles, or one molecule of Vif for every 30 molecules of Gag (49). If single Gag molecules bound to Vif at this same ratio within an APOBEC3G/Vif/Gag complex destined for degradation in the proteosome, this would account for only 3.5% of Gag molecules produced, and a change in Gag distribution in the cell would not be detectable by our Western blot assay.

[0051] Alternatively, the formation of an APOBEC3G/Vif/Gag complex may be prevented by overlapping binding sites. While the ability to

coimmunoprecipitate Gag and Vif from cell lysates has met with varying degrees of success (50,51), the in vitro interaction between Vif and Gag has been used to map interacting sites on these two molecules (49). These results indicate that the Vif binding sites on Gag include the C terminal of NC (including the second zinc finger), the spacer peptide sp2, and the N terminal region of p6. Since NC is involved in binding to both Vif and APOBEC3G, the latter two molecules might compete for binding to Gag. Similarly, the APOBEC3G binding sites for Vif and Gag have been estimated to include amino acids 54-124 for Vif (34), and amino acids 104-156 for Gag, as reported herein. The lack of formation of a Gag/Vif/APOBEC3G complex could therefore also be due competitive binding between Gag and Vif for sites on APOBEC3G, or to conformational restraints preventing both molecules binding to APOBEC3G.

[0052] Most cytidine deaminases act as homodimers or homotetramers (52,53). It has been reported for APOBEC1 that small N- (10 amino acids) or C- (10 amino acids) terminal deletions reduce RNA editing, RNA binding, and homodimerization activities (53). Similarly, it has been reported for APOBEC3G that N- and C-terminal deletions which do not eliminate either active site still destroy enzyme activity, and that this is due to inhibition of APOBEC3G dimerization (54). We show here that larger N- and C-terminal deletions of APOBEC3G can still be packaged into HIV-1 (Figure 4), which suggests that neither APOBEC3G dimerization, nor its binding to RNA is required for this process.

[0053] It is not clear if the deoxycytidine deaminase activity of APOBEC3G is the sole determinant in inhibiting HIV-1 replication. For example, while two reports have indicated that mutations in either active site result in similar losses of both deoxycytidine deaminase activity and anti-viral activity (16,17), a more recent paper reports that mutations in either active site inhibit deoxycytidine deaminase activity to different extents, but have the same anti-

viral activity (54). This latter observation implies that deoxycytidine deaminase activity of APOBEC3G may not be the sole determinant of anti-viral activity. It is possible that the interaction of APOBEC3G with nucleocapsid might result in the inhibition of viral functions associated with nucleocapsid. For example, Gag nucleocapsid sequences facilitate $tRNA^{Lys3}$ annealing to viral genomic RNA (55), which could explain the observation that deproteinized viral RNA (which contains primer $tRNA^{Lys3}$ annealed to viral genomic RNA) extracted from Vif-negative HIV-1 produced in non-permissive cells shows a decreased ability to support reverse transcription *in vitro* compared to the same RNA extracted from similar virions produced in permissive cells (8). Alternatively, this observation might reflect the presence in non-permissive cells of other anti-HIV-1 factors yet to be discovered.

EXAMPLE 7

Inhibition of primer $tRNA^{lys3}$ function in HIV-1 by APOBEC3G

[0054] The initiation of reverse transcription in HIV-1 requires $tRNA^{Lys3}$ as a primer, and this tRNA is packaged into the virus during its assembly. $tRNA^{Lys3}$ is annealed to a region near the 5' end of the viral RNA termed the primer binding site (PBS), and used to prime the reverse transcriptase-catalyzed synthesis of minus strand cDNA, the first step in reverse transcription. We have previously reported that Vif-negative virions produced from H9 cells, a non-permissive cell line, have approximately 50% reduced annealing of primer $tRNA^{Lys3}$, and >90% reduction in initiation of reverse transcription, compared to Vif-positive virions (8). The implication of these results is that even if some $tRNA^{Lys3}$ is annealed to the viral genome, it is not placed properly to initiate reverse transcription. We have reported a similar situation when comparing $tRNA^{Lys3}$ annealing to the viral RNA genome in wild- type vs protease-negative HIV-1 (56). In that report, annealing and initiation of reverse transcription in the protease-negative virus were rescued

through the transient addition of mature HIV-1 nucleocapsid (NCp7) to the viral RNA/primer $tRNA^{Lys3}$ template used to measure these parameters. Both Gag (55, 56, 57) and mature nucleocapsid (NC) (58, 59) have been shown to facilitate the annealing of $tRNA^{Lys3}$ to viral RNA, *in vitro* and *in vivo*. The data presented herein indicate that APOBEC3G is incorporated into HIV-1 through its interaction with Gag NC, and it is therefore possible that APOBEC3G might inhibit $tRNA^{Lys3}$ annealing through its binding to NC.

[0055] 293T cells were transfected with plasmids containing BH10 or BH10Vif- DNA, or cotransfected with either of these plasmids plus a plasmid coding for APOBEC3G. The extracellular viruses were isolated, and protein composition of the different cell lysates and the virions produced from these cells is show in the Western blots in Figure 7, A and B, respectively. The panels, moving down from the top panel, are probed, respectively, with anti-Vif, anti-HA (which detects APOBEC3G tagged with HA), anti-capsid (CA), and anti-β-actin. Using aliquots of cell lysates containing equal amounts of β-actin (Figure 7A, panel 4), these results show that cells expressing BH10Vif- viral proteins contain the normal pattern of viral Gag and capsid proteins (Figure 7A, panel 3), but lack Vif (Figure 7A, panel 1). Vif facilitates the proteosomal degradation of APOBEC3G (23), and as previously described, the absence of Vif in the cell results in a higher cellular concentration of APOBEC3G (Figure 7A, panel 2). The results shown in Figure 7B represent Western blots of lysates of viruses produced from these cells, and show that in the presence of cellular APOBEC3G, but the absence of cellular Vif, the virions produced contain increased amounts of APOBEC3G.

[0056] The different types of viruses were purified the sucrose ultracentrifugation, and total viral RNA was isolated. This RNA was analyzed by dot-blot hybridization with probes specific for $tRNA^{Lys3}$ or viral genomic RNA, as previously described (60). The ratios of $tRNA^{Lys3}$:genomic RNA were then determined, and these ratios, normalized to BH10 virions produced from 293T

cells not expressing APOBEC3G, are plotted in Figure 8D. The results indicate that no difference in $tRNA^{Lys3}$ incorporation into virions exists in the different viral samples.

[0057] To study *in vivo* $tRNA^{Lys3}$ annealing to viral RNA and the ability of the annealed $tRNA^{Lys3}$ to initiate reverse transcription, total viral RNA was isolated and used as the source of the primer $tRNA^{Lys3}$ annealed to viral genomic RNA *in vivo*, in an *in vitro* reverse transcription assay. The assumption that the annealed primer tRNA in the total viral RNA reflects its annealed configuration *in vivo* rests upon several pieces of evidence. Earlier studies have reported that the annealed primer tRNA in retroviruses is thermally stable (61), and we have similarly found that in the reverse transcription reaction buffer, the primer $tRNA^{Lys3}$ bound to the viral RNA template is very heat-stable, dissociating only at temperatures above 70°C (unpublished data). Second, unannealed $tRNA^{Lys3}$ added to viral RNA under reverse transcription reaction conditions at 37°C will not anneal to the genomic RNA (65, 63). Third, the amount of $tRNA^{Lys3}$ annealed to viral RNA, in wild-type viruses, as measured by this method, is proportional to the amount of $tRNA^{Lys3}$ packaged into the virion (60). Fourth, the different degrees of inhibition of $tRNA^{Lys3}$ annealing produced in virions containing wild type or mutant Gag (62) must reflect what had occurred in the virus since the total viral RNA used in the *in vitro* reverse transcription reaction has been deproteinized. Fifth, although the total viral RNA used has been deproteinized, it has been shown that only a transient exposure of NC to total viral RNA is required to produce long-term effects upon $tRNA^{Lys3}$ annealing to viral RNA (56). Sixth, a mutant $tRNA^{Lys3}$ with an altered anticodon sequence (SUU to CUA) is an efficient primer for reverse transcription *in vitro* when it is heat- annealed to genomic RNA. However, while this mutant tRNA is packaged into HIV-1 *in vivo*, it does not act as a primer tRNA in our RT assay using total viral RNA unless we first heat-denature the total viral RNA and allow the tRNA to anneal back to the genomic RNA (63).

[0058] Figure 8A shows the 3´ terminal 18 nucleotides of $tRNA^{Lys3}$ annealed to a complementary region near the 5´ terminus of viral RNA known as the primer binding site (PBS). Also shown are the first 6 dexoynucleotides added to the 3´ terminus of $tRNA^{Lys3}$ during the initiation of reverse transcription, in the order 5´CTGCTA 3´. Earlier work has indicated that in the virus, approximately 80% of the $tRNA^{Lys3}$ annealed to the vRNA is present in unextended form, while the remainder is present as a 2 base extended form, i. e., tRNA-CT (64). To measure the amount of extendable $tRNA^{Lys3}$ annealed to the vRNA, the reverse transcription reaction mix contains the total viral RNA, reverse transcriptase, 5 μM α-^{32}P-GTP, 200 μM CTP and TTP, and 200 μM ddATP. The ddATP will cause extension by reverse transcriptase to terminate after 6 bases. Figure 8B shows the radioactive 6 base extended $tRNA^{Lys3}$ resolved by 1D PAGE. Lane 1 represents purified human placental $tRNA^{Lys3}$ heat-annealed *in vitro* to synthetic viral genomic RNA, and extended by 6 bases. Lanes 2 and 3 use as the source of primer/template total viral RNA isolated from virions produced from cells cotransfected with the plasmid vector pcDNA3.1 and a plasmid containing either wild-type HIV-1 (BH10), or for Vif-negative HIV-1 (BH 10VIF-) DNA. In lanes 4 and 5 the sources of total viral RNA are virions produced from cells cotransfected with the pcDNA3.1 plasmid containing the gene for APOBEC3G and a plasmid containing the DNA for either BH10 or BH 10VIF-. These results indicate that $tRNA^{Lys3}$ annealing is reduced approximately 50% when Vif-negative virions are produced from 293T cells expressing APOBEC3G (lane 5).

[0059] However, the ability of this annealed $tRNA^{Lys3}$ to initiate reverse transcription (i. e., incorporate the first nucleotide, dCTP) is inhibited even further. We have previously shown that equal amounts of annealed $tRNA^{Lys3}$ may have different abilities to initiate reverse transcription (56). In that work, it was shown that $tRNA^{Lys3}$ annealed to the viral genome in protease-negative HIV-1 has its ability to incorporate the first dCTP reduced 2/3 compared to $tRNA^{Lys3}$ annealed in protease-positive viruses. This difference, however, is only seen when using low

amounts of dCTP (0.16 μM dCTP); at higher concentrations of dCTP (5 μM), this difference is obliterated. The reverse transcription reactions shown in Figure 8C measure the initiation of reverse transcription, using either equal amounts of genomic RNA (left side) or equal amounts of annealed $tRNA^{Lys3}$ (right side), as determined in panel A. The reactions contain 0.16 μM α-^{32}P-dCTP and 0.16 μM α-^{32}P-dGTP. While the α-^{32}P-dCTP measures the incorporation of the first dCTP onto unextended $tRNA^{Lys3}$ *in vitro*, the α-^{32}P-dGTP incorporation gives a measure of the ability of the annealed $tRNA^{Lys3}$ to incorporate the first two bases, C and T, *in vivo*.The four base- extended $tRNA^{Lys3}$ seen represents $tRNA^{Lys3}$-CTGC. dGTP incorporation is not sensitive to the same concentration range to which dCTP is sensitive, i.e., dGTP incorporation is the same at 0.16μM or 5.0μM (56). Thus, at 5μM dCTP and dGTP, 60-80% of the signal represents unextended $tRNA^{Lys3}$ (56), but when these nucleotides are both at 0.16μM, incorporation of the first dCTP is sub-optimal. The three $tRNA^{Lys3}$-extension bands are shown in Figure 8C, and all were used together as a measure of initiation of reverse transcription. It can be seen that when equal amounts of total viral RNA are used (left side, panel C), initiation is not detected in BH10Vif- viruses produced from cells expressing APOBEC3G (lane 5). When equal amounts of primer $tRNA^{Lys3}$ in the total viral RNA are used in the reaction (right side, panel C), initiation is reduced in BH10Vif- viruses produced from cells expressing APOBEC3G (lane 5) to to <10% that found for wild-type virions. The electrophoretic bands from the right side, panel C were quantitated by phosphorimaging (BioRad), and the results are plotted in Figure 8D.

[0060] As shown in Figures 9 and 10 the inhibition of initiation of reverse transcription in BH10Vif- viruses produced in non-permissive 293T cells is dependent upon the amount of APOBEC3G DNA transfected into the cell. 293T cells were transfected with plasmids containing BH10 or BH10Vif- DNA, or cotransfected with either of these plasmids plus increasing amounts of pcDNA 3.1 plasmid containing DNA coding for APOBEC3G (pAPOBEC3G) plasmid and

decreasing amounts of the same plasmid not carrying the APOBEC3G gene (pcDNA 3.1), so as to keep the total amount of transfected pcDNA 3.1 plasmid equal. Lysates of cells and the extracellular viruses produced from them were analyzed by Western blots as shown in Figure 9, A and B, respectively. In Figure 9A (cell lysates), the panels, moving down from the top panel, are probed, respectively, with anti-HA, anti-CA, and anti-β-actin. In Figure 9B (viral lysates), the upper and lower panels are probed with anti-HA and anti-CA, respectively. Using aliquots of cell lysates containing approximately equal amounts of β-actin. These results show that while cells cotransfected with both HIV-1 DNA and increasing amounts of pAPOBEC3G show an increase in APOBEC3G in the cell, this increase in much larger when the viruses are not able to express Vif (Figure 9A). Figure 9B shows that the amount of APOBEC3G incorporated into the virus is proportional to the amount expressed in the cell.

[0061] Total viral RNA was also isolated from these different virions, and used as the source of primer $tRNA^{Lys3}$/viral RNA template in an *in vitro* reverse transcription reaction. The initiation of reverse transcription was measured in the presence of 0.16μM α-^{32}P-CTP and α-^{32}P-GTP, and the 1, 3, and 4 base $tRNA^{Lys3}$ extension products, shown in Figure 10A, were resolved by 1D PAGE as described for Figure 8C. Lane 1 represents purified human placental $tRNA^{Lys3}$ heat annealed to viral RNA *in vitro*, and serves as a size marker. The electrophoretic bands were quantitated by phosphorimaging (BioRad), and the results, plotted in Figure 10B, show that, while the expression of APOBEC3G has no effect upon initiation of reverse transcription in wild-type HIV-1 (lanes 2-6), the initiation of reverse transcription is completely inhibited in virions lacking Vif (BH10Vif-) at higher levels of APOBEC3G expression (Figure 10, lanes 7-11). These results suggest that the incorporation of APOBEC3G into HIV-1 inhibits proper $tRNA^{Lys3}$ annealing to the viral RNA genome.

EXAMPLE 8

Rescue of APOBEC3G-induced inhibition of tRNALys3-primed initiation ofreverse transcription by nucleocapsid

[0062] Recombinant HIV-1 nucleocapsid protein (NCp7) was obtained from Rob Gorelick. The total viral RNA was pre-incubated with with 10 pmolar NCp7 in reverse transcription buffer at 37°C for 30min. The NCp7 was then removed by proteinase K digestion and phenol-chloroform extraction. This RNA was then used as the source of primer/template in the reverse transcription reaction, and the $tRNA^{Lys3}$ extension products were analyzed by 1D PAGE. The results indicate that the reduced initiation of reverse transcription seen in Vif-negative viruses produced from 293T cells expressing APOBEC3G is rescued 40-70% when the total viral RNA is transiently exposed to mature nucleocapsid protein. Exposure to nucleocapsid of the total viral RNA isolated from wild-type viruses produced in APOBEC3G-expressing cells has no effect upon initiation of reverse transcription.

[0063] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. A method of treating or preventing viral infections by inhibiting $tRNA^{Lys3}$ annealing and/or priming on a viral genome thereby reducing viral replication.

2. A purified polypeptide comprising amino acids 104-156 of APOBEC3G having the ability, when introduced in a viral particle, to inhibit $tRNA^{Lys3}$ annealing and/or priming on a viral genome, thereby reducing viral replication.

Application number numéro de demande: 2467312

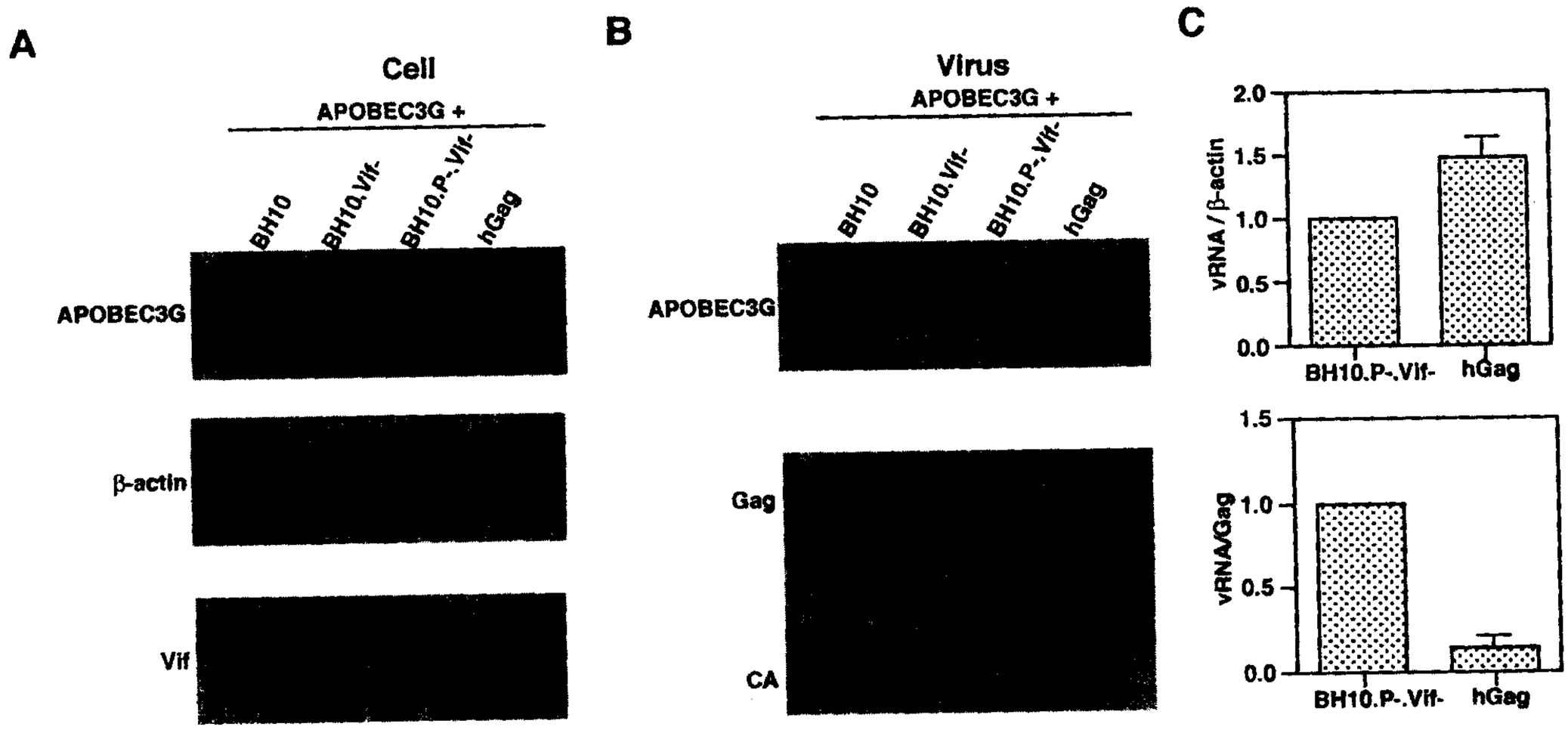
Figures: 1a, 1b, 2b, 2c, 3a, 3b, 4b, 4c, 5, 6, 7, 8b, 8c, 9a, 9b, 10a, 10b

Pages:

Drawings

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)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APOBEC3G +
BH10
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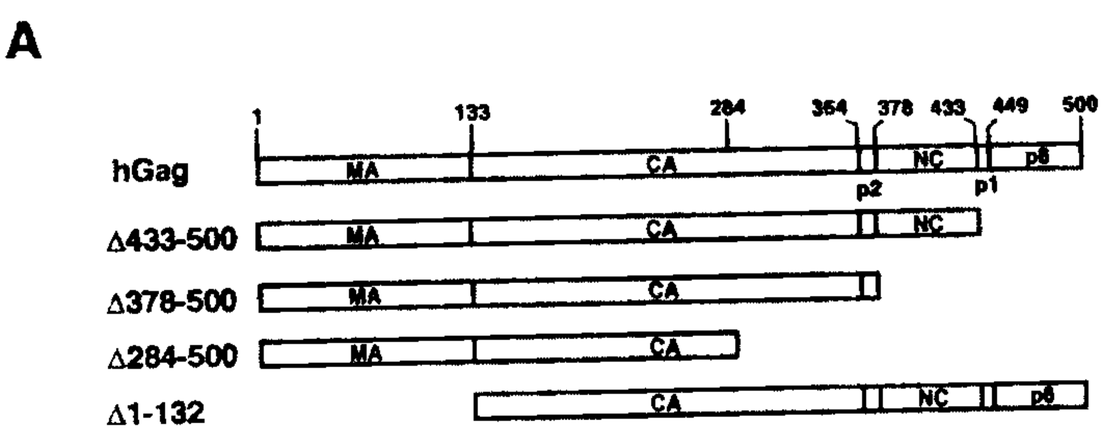
Figure 1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Δ1-132
B
APOBEC3G +
hGag
hGag
Δ1-132
Δ284-500
Δ378-500
Δ433-500
Gag
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Gag
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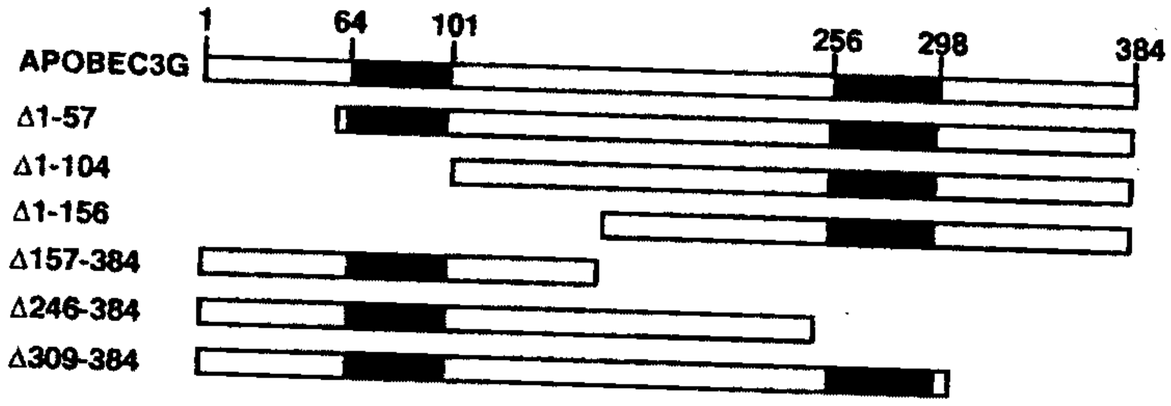
RNase
DNase
Gag

Figure 2

A


1
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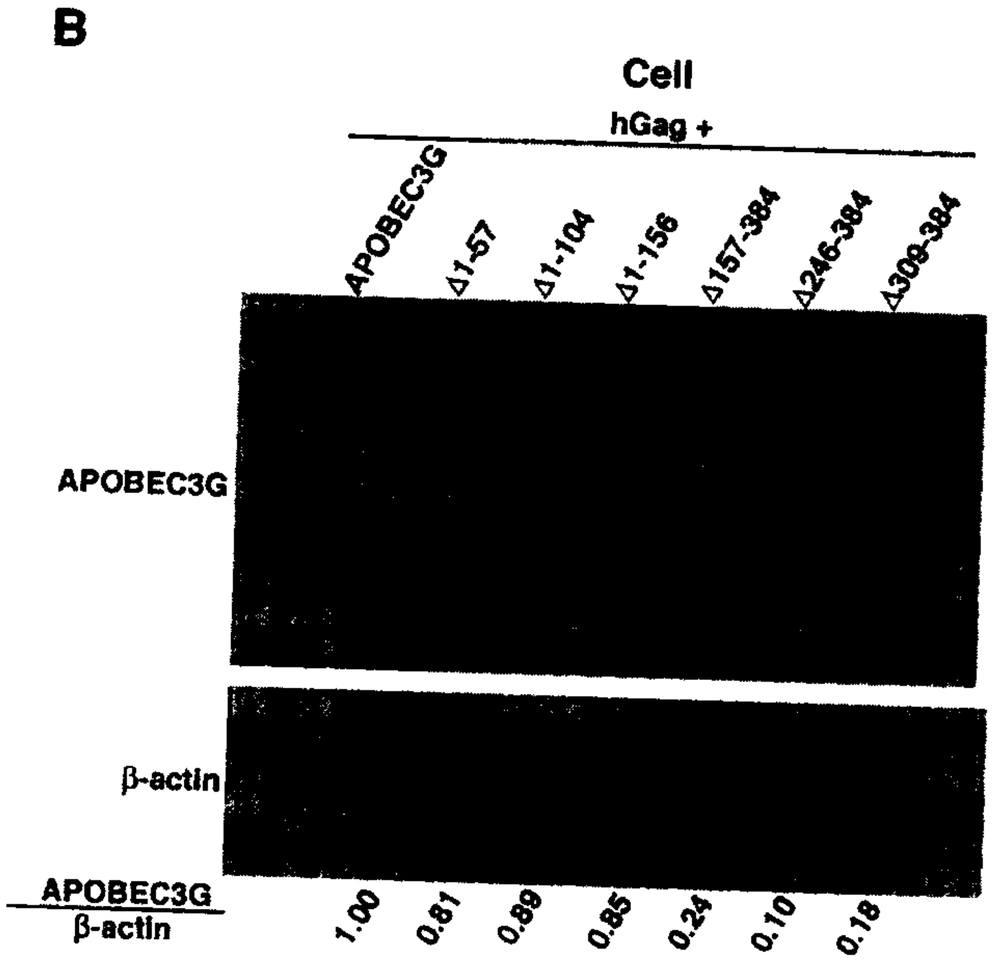
Δ1-156
Δ157-384
Δ246-384
Δ309-384

B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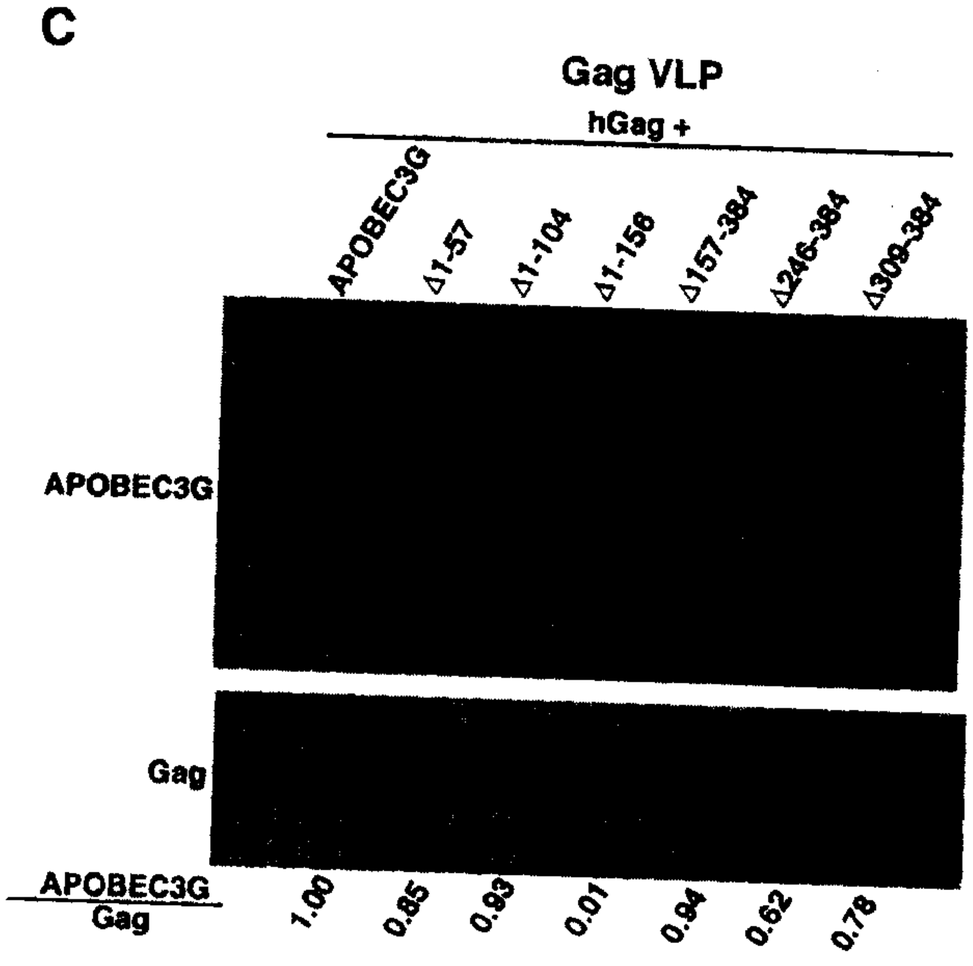
C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hGag +
APOBEC3G
Δ1-57
Δ1-104
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APOBEC3G
Gag
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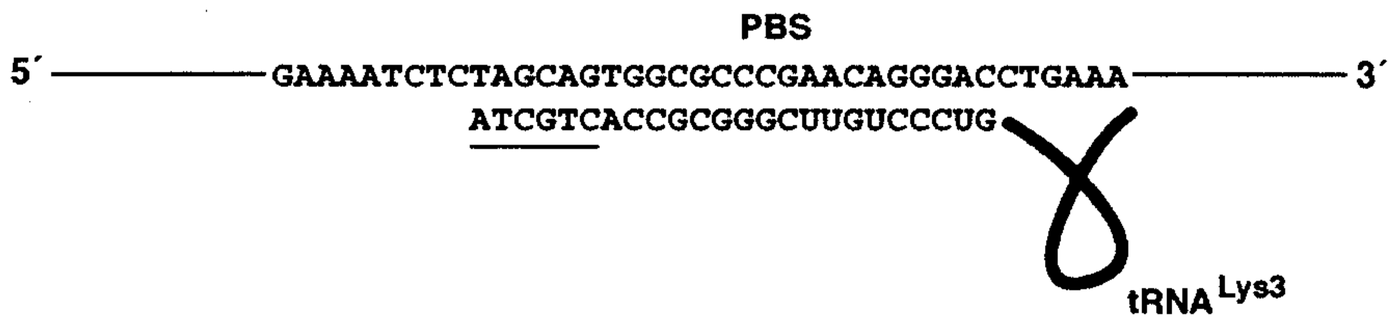
0.78

Figure 4

A



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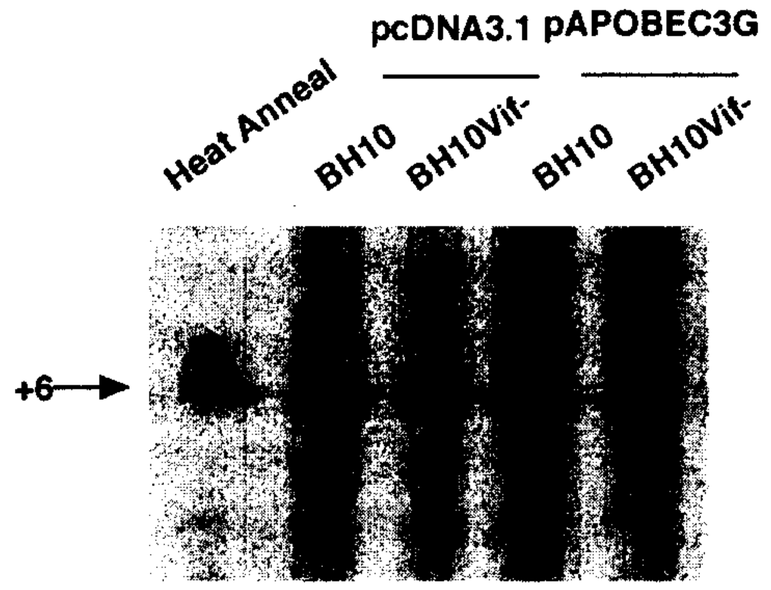

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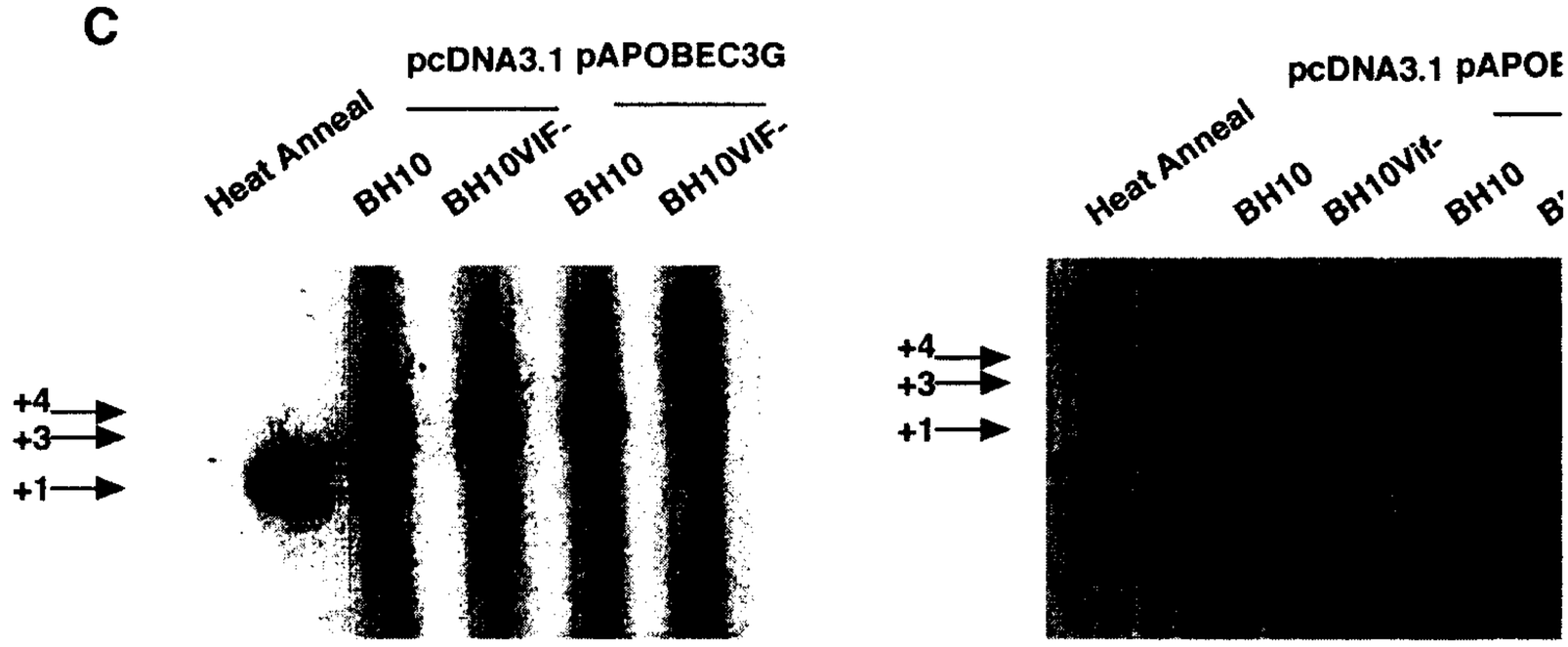

D

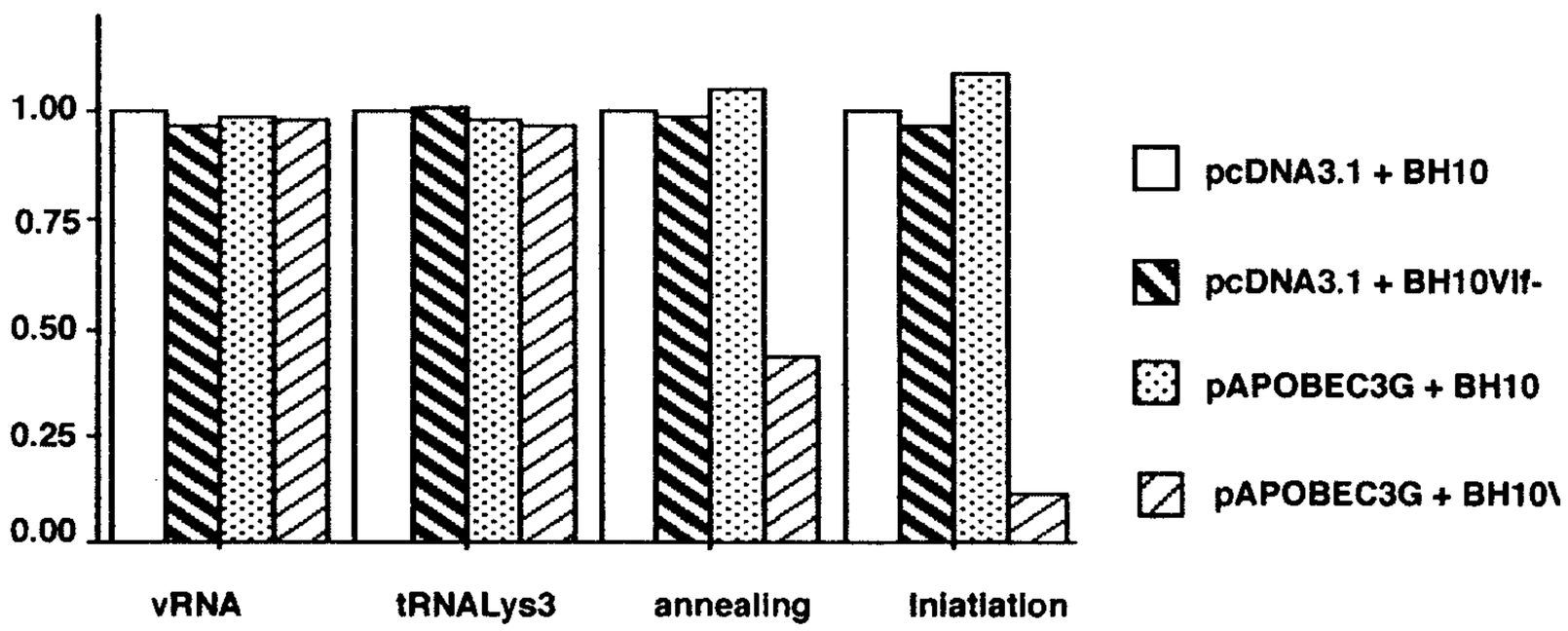

Figure 8