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Comparison of G-to-A Mutation Frequencies Induced by APOBEC3 Proteins in H9 Cells and Peripheral Blood Mononuclear Cells in the Context of Impaired Processivities of Drug-Resistant Human Immunodeficiency Virus Type 1 Reverse Transcriptase Variants[▽]

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APOBEC3 proteins can inhibit human immunodeficiency virus type 1 (HIV-1) replication by inducing G-to-A mutations in newly synthesized viral DNA. However, HIV-1 is able to overcome the antiretroviral activity of some of those enzymes by the viral protein Vif. We investigated the impact of different processivities of HIV-1 reverse transcriptases (RT) on the frequencies of G-to-A mutations introduced by APOBEC3 proteins. Wild-type RT or the M184V, M184I, and K65R+M184V RT variants, which are increasingly impaired in their processivities, were used in the context of a *vif*-deficient molecular HIV-1 clone to infect H9 cells and peripheral blood mononuclear cells (PBMCs). After two rounds of infection, a part of the HIV-1 *env* gene was amplified, cloned, and sequenced. The M184V mutation led to G-to-A mutation frequencies that were similar to those of the wild-type RT in H9 cells and PBMCs. The frequencies of G-to-A mutations were increased after infection with the M184I virus variant. This effect was augmented when using the K65R+M184V virus variant ($P < 0.001$). Overall, the G-to-A mutation frequencies were lower in PBMCs than in H9 cells. Remarkably, 38% ± 18% (mean ± standard deviation) of the *env* clones derived from PBMCs did not harbor any G-to-A mutation. This was rarely observed in H9 cells (3% ± 3%). Our data imply that the frequency of G-to-A mutations induced by APOBEC3 proteins can be influenced by the processivities of HIV-1 RT variants. The high number of nonmutated clones derived from PBMCs leads to several hypotheses, including that additional antiretroviral mechanisms of APOBEC3 proteins other than their deamination activity might be involved in the inhibition of *vif*-deficient viruses.

Proteins of the APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3) family, especially APOBEC3G and, to a lesser extent, APOBEC3F, have been identified as host factors which can inhibit the replication of human immunodeficiency virus type 1 (HIV-1) and belong to an intrinsic defense system (6, 37, 57, 68, 72). They are expressed in several cell lines, as well as primary cells like $CD4^+$ T cells and macrophages (12, 57). The HIV-1 protein Vif suppresses the intrinsic antiretroviral effect by binding to APOBEC3G, which results in the degradation of APOBEC3G by the proteasome (38, 43, 58, 60). In the absence of Vif, APOBEC3G and APOBEC3F are packaged into virions through interactions with HIV-1 Gag and/or viral RNA (1, 9, 27, 28, 39, 53, 63, 68, 70).

Several antiretroviral mechanisms of APOBEC3 proteins have been proposed (reviewed in references 11 and 20). One antiretroviral mechanism of those proteins is their ability to deaminate cytosine residues in single-stranded HIV-1 DNA during reverse transcription, leading to G-to-A hypermutation in the viral plus strand (18, 35, 41, 42, 71). Here, APOBEC3F leads preferentially to G-to-A mutation in the context of GA dinucleotides (7, 37, 68), and GG dinucleotides are target sites specific for APOBEC3G, leading to GG-to-AG mutations (6, 18, 37, 68, 69). This process is time dependent, and thus, a 5′→3′ G-to-A mutational gradient is generated in the viral genome in correlation to the exposure time of single-stranded viral DNA to APOBEC3G/F (10, 61, 69). Single-stranded viral DNA is generated during reverse transcription from an RNA-DNA hybrid by the viral RNaseH domain of the HIV-1 reverse transcriptase (RT), and this activity of HIV-1 RNaseH leads to the activation of APOBEC3G (59). APOBEC3G can deaminate cytosine residues independently of the HIV-1 RT in a cell-free deamination assay (62); however, the progression of the synthesis of double-stranded viral DNA terminates the deamination process of APOBEC3 proteins, thereby resulting in the described 5′→3′ G-to-A mutational gradient.

Thus, it is conceivable that the HIV-1 RT is influencing the G-to-A mutation frequency induced by APOBEC3 proteins. Here, we investigated the impact of HIV-1 RT variants differing in their processivities, defined as the number of nucleotides added to a template prior to dissociation of the enzyme from its template, on the G-to-A mutation frequencies induced by APOBEC3 proteins. We hypothesized that reduced processivities lead to an increase of the G-to-A mutation frequencies

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induced by APOBEC3 proteins, because the time period during which the target viral DNA remains single stranded is prolonged. The rate of polymerization correlates with the processivity (3, 56). Very recently, it has been shown that defects in the rate of polymerization and processivities of M184 variants of HIV-1 RT are both due to defects in deoxynucleoside triphosphate (dNTP) utilization (16). For our approach, we have chosen the RT variants carrying the M184V, M184I, or the K65R+M184V mutations, which confer resistance to certain nucleoside/nucleotide RT inhibitors. Viruses harboring these mutations at codon 184 of the RT are resistant to the RT inhibitors lamivudine and emtricitabine (8, 54, 55). The M184V mutation causes a decrease of the processivity (3, 56), which is even further impaired by RT variants carrying the M184I mutation (3). The additional introduction of the K65R mutation, which confers resistance to tenofovir and abacavir (47), further reduces the processivity of the M184V RT variant (13, 67).

The deaminase activities of APOBEC3G/F and the G-to-A mutation frequencies and pattern have predominantly been studied in T-cell lines, partially using exogenous APOBEC3G/F (18, 35, 41, 42, 68, 71). Only recently has the deaminase activity of endogenous APOBEC3G of several T-cell lines and primary $CD4^+$ T cells been compared with exogenous APOBEC3G derived from APOBEC3G-transfected epithelial cell lines, showing that endogenous APOBEC3G expresses a significantly lower deaminase activity than exogenous APOBEC3G (64). In our study, we determined the G-to-A mutation frequencies of endogenous APOBEC3 proteins in peripheral blood mononuclear cells (PBMCs) in comparison to these mutation frequencies in H9 cells in the context of *vif*-negative viruses harboring different RT variants in order to gain more insight into the deamination process in physiological target cells of HIV-1.

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MATERIALS AND METHODS

Plasmids. The RT mutations M184V and M184I were separately introduced into the molecular HIV-1 clone pNL4-3 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH], Bethesda, MD, from Malcolm Martin) as previously described (21). The HIV-1 clone pNL4-3Δvif was kindly provided by Klaus Strebel, NIH, NIAID (25). The M184V and M184I mutations were introduced into this plasmid by restriction with Sph1 and Age1 (New England Biolabs, Frankfurt, Germany), and subsequent ligation using 1 U T4 DNA ligase (Invitrogen, Karlsruhe, Germany). The K65R mutation was introduced into pNL4-3_{M184V} and pNL4-$3\Delta vif_{M184V}$ by site-directed mutagenesis (QuikChange XL site-directed mutagenesis kit; Stratagene, La Jolla, CA) using the mutagenesis oligonucleotides sdm K65R (5′-CTCCAGTATTTGCCATAAAGAGAAAAGACAGTACTAAATGGAG-3′) and sdm K65Rrc (5′-CTCCATTTAGTACTGTCTTTTCTCTTTATGGCAAATACTGGAG-3′) (introduced mutation is underlined) following the manufacturer's instructions. The mutations were confirmed by sequence analysis.

Virus stocks. Amounts of 3×10^6 293T cells were separately transfected with pNL4-3 or pNL4-3Δvif encoding wild-type RT or the M184V, M184I, or K65R+M184V variants, respectively. After 48 h, supernatants were collected, centrifuged at $2{,}000 \times g$ at room temperature for 5 min to pellet cell debris, and finally digested with 10 U DNase I/500 μl (Boehringer, Mannheim, Germany) at 25°C for 30 min to avoid possible carryover of plasmids. Virus stocks were stored at −80°C. For characterization, viral RNA of each virus stock was isolated by using a NucleoSpin RNA virus kit (Macherey Nagel, Dueren, Germany) according to the manufacturer's instructions. cDNA synthesis was performed by using random hexamers and Moloney murine leukemia virus RT (Invitrogen) according to the manufacturer's instructions. To evaluate the virus titer, quantitative real-time PCR was performed with the reaction mixture containing 10 μl cDNA, 1× ROX PCR buffer (46), 3.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.4 μM each of the oligonucleotides pol 2981 (5′-TCAGTACAATGTGCTTCCACAGG-3′) and pol 3206 rc (5′-TTTGTCTGGTGTGGTAAATCCCCAC-3′), with *Taq* DNA polymerase in a final volume of 50 μl. Amplification was performed for 50 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Applied Biosystems, Foster City, CA). The oligonucleotides were based on the HIV-1 NL4-3 and HIV-1 HXB2 sequences (GenBank accession numbers AF324493 and K03455, respectively) and synthesized by biomers.net (Ulm, Germany).

Cells and infections. The T-cell lines H9 and MT4 were maintained under standard conditions. Briefly, cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, 170 mM penicillin, and 40 mM streptomycin at 37°C and 5% CO_2. PBMCs were isolated from buffy coats obtained from healthy donors (Transfusionsmedizin, Suhl, Germany) by using a Ficoll-Hypaque (Sigma, Munich, Germany) gradient. After isolation, cells were washed twice, counted, and transferred into complete RPMI 1640 medium at a density of 3×10^6 cells/ml. PBMCs were stimulated with 3 μg/ml phytohemagglutinin (Sigma). An amount of 10 U/ml interleukin-2 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Hoffmann-La Roche, Inc.) was added after 24 h to the cell culture and maintained in the medium. After an additional 48 h, the phytohemagglutinin was removed by washing the cells twice. H9 cells (1×10^6) or PBMCs (3×10^6) (72 h after isolation) were infected with each virus stock by spinoculation (48), washed twice, and resuspended in 1 ml medium. At 36 to 48 h postinfection, the supernatants were harvested, centrifuged, and used for a second round of infection, which was carried out as described above. Cells were harvested 6 h later and stored at −20°C until further analyses. To test the replication abilities of the different virus variants, 1×10^6 H9 cells and MT4 cells were infected by spinoculation with the same quantity of all HIV-1 NL4-3 variants as defined by HIV-1 RNA copy numbers, i.e., 1×10^9 HIV-1 RNA copies were used. Cells were washed twice after infection, supernatants were collected daily or every second day for 5 days after infection, and an in-house p24 enzyme-linked immunosorbent assay was performed as previously described (45).

Viral DNA isolation and sequences. Total DNA of infected cells was isolated by using a NucleoSpin blood kit (Macherey-Nagel) in accordance with the manufacturer's instructions, and DNA was eluted in 130 μl elution buffer (5 mM Tris-HCl, pH 8.5). The DNA was controlled in several ways. First, part of the β-actin gene was amplified to control for the presence of cellular DNA. Briefly, PCR was performed using 1× PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 0.4 μM of each of the oligonucleotides bact 532 (5′-CCATCTACGAGGGGTATGC-3′) and bact 728 rc (5′-CGTGGCCATCTCTTGCTC-3′) (β-actin; GenBank accession number X00351), and *Taq* DNA polymerase in a final volume of 50 μl. DNA was amplified in 35 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s) with a termination of 5 min at 72°C in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). Second, PCR for the discrimination between *vif*-positive and *vif*-negative DNA was performed using the same conditions described above and the HIV-1 oligonucleotides int 4962 (5′-GGTGAAGGGGCAGTAGTAATAC-3′) and vpr 5685 rc (5′-CCTAAGTTATGGAGCCATATCCTAG-3′). All amplification products were analyzed by gel electrophoresis. Third, M184V, M184I, and K65R allele-specific real-time PCRs were conducted to exclude contaminations between the variants used, as previously described (2, 46). The K65R allele-specific PCR was performed accordingly using the primers pol 2835 rc (5′-GTGGTATTCCTAATTGAACTTCCC-3′) and IN K65 (5′-TCCAGTATTTGCCATAAAGIA-3′) or IN K65R (5′-CCAGTATTTGCCATAAAGIG-3′), respectively (I, inosine). Fourth, contamination by full-length HIV-1 plasmids was ruled out by amplification of the plasmid-specific sequence spanning parts of the HIV-1 5′ long terminal repeat (LTR) and the cloned human genomic DNA. The PCR was carried out according to the conditions described above by using the oligonucleotide LTR 611 (5′-AGTCAGTGTGGAAAATCTCTAGC-3′) plus the pNL4-3-genomic rc (5′-TCTTGTGGTATCAGAGTAGAGG-3′) and an annealing temperature of 55°C. All oligonucleotides were synthesized by biomers.net.

To examine the G-to-A mutation frequencies of viral DNA extracted from infected H9 cells or PBMCs, high-fidelity PCR was performed either by using a Triple Master PCR system (Eppendorf) or an EasyA kit (Stratagene). A part of

the HIV-1 *env* gene was amplified by using the oligonucleotides BamHI/EcoRI env 7045 (5′-GGATCCGAATTCCTGCCAATTTCACAGACAATGC-3′) and ApaI/XhoI env 7552 rc (5′-GGGCCCCTCGAGCACATTTGTCCACTGATG GGAGG-3′) following the manufacturer's instructions. Amplicons were purified by using a NucleoSpin extract II kit (Macherey-Nagel), eluted in 20 μl elution buffer (5 mM Tris-HCl, pH 8.5), and cloned into the TA cloning vector provided in a StrataClone PCR cloning kit (Stratagene) in accordance with the manufacturer's instructions. Plasmids were isolated and sequenced by using the oligonucleotides M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′), a BigDye terminator cycle sequencing kit, version 3.1, and an ABI 3100 sequencer (Applied Biosystems). Sequences were analyzed with Edit View 3.7 NT (Applied Biosystems) and Vector NTI suite 7 (Invitrogen). G-to-A mutations were analyzed by using the program Hypermut 2.0 that is freely available at http://www.hiv.lanl.gov/content/sequence/HYPERMUT /hypermut.html (52).

Statistical analysis. Differences in the G-to-A mutation frequencies were analyzed for statistical significance by using the two-tailed Mann-Whitney test. A P value of <0.05 was considered a significant difference between two groups.

RESULTS

Replication kinetics of HIV-1 NL4-3 and HIV-1 NL4-3Δvif harboring different RT variants in H9 and MT4 cells. To determine the impact of differences in the processivities of HIV-1 RT on the G-to-A mutation frequencies induced by APOBEC3 proteins, we introduced several mutations into the RT gene of the HIV-1 clones pNL4-3 and pNL4-3Δvif. Virus stocks of all variants were generated and tested with regard to their replication abilities. First, we infected nonpermissive H9 cells, which express APOBEC3G/F and, therefore, inhibit the replication of HIV-1 NL4-3Δvif variants. None of the HIV-1 NL4-3Δvif viruses replicated in H9 cells, whereas HIV-1 NL4-3, HIV-1 NL4-3_{M184V}, and HIV-1 NL4-3_{M184I} showed similar replication abilities (Fig. 1A). Next, we infected permissive MT4 cells, which do not express APOBEC3G/F, and thus, both *vif*-positive and *vif*-negative HIV-1 variants could replicate in those cells (17). The replication abilities of all eight HIV-1 NL4-3 and Δ*vif* variants were similar in MT4 cells (Fig. 1B). As expected, HIV-1 NL4-3 variants were able to replicate in both cell lines, whereas HIV-1 NL4-3Δvif variants could only replicate in MT4 cells.

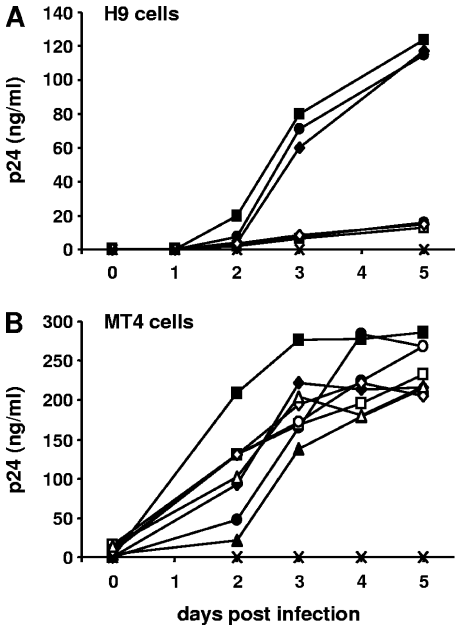

FIG. 1. Replication kinetics of HIV-1 NL4-3 and HIV-1 NL4-3Δvif harboring different RT variants in H9 and MT4 cells. Shown are the results of a representative example of three independent experiments. Supernatants from uninfected cells served as negative controls and are indicated by crosses. (A) H9 cells were infected with HIV-1 NL4-3 (black squares), HIV-1 NL4-3_{M184V} (black circles), HIV-1 NL4-3_{M184I} (black diamonds), HIV-1 NL4-3Δvif (open squares), HIV-1 NL4-3Δvif$_{M184V}$ (open circles), and HIV-1 NL4-3Δvif$_{M184I}$ (open diamonds). (B) MT4 cells were infected with all six HIV-1 variants listed above, HIV-1 NL4-$3_{K65R+M184V}$ (black triangles), and HIV-1 NL4-3Δvif$_{K65R+M184V}$ (open triangles). Supernatants were collected daily or every second day and assayed for viral p24 antigen by enzyme-linked immunosorbent assay.

Lack of differences in total mutation frequencies of HIV-1 NL4-3 carrying different RT variants in infected H9 cells and PBMCs. It has been reported that the processivities of HIV-1 RT variants are inversely correlated to their fidelities, i.e., slower enzymes introduce fewer mutations (reviewed in reference 51). To account for those differences in mutation frequencies in the following experiments with the HIV-1 NL4-3Δvif viruses, we investigated the frequencies of mutations of the HIV-1 NL4-3 variants containing *vif*. Thus, the impact of APOBEC3 proteins on the mutation frequencies is precluded. Two independent experiments were performed using H9 cells, and another two independent experiments with PBMCs from donors A and B, and the results are summarized in Fig. 2 and Table 1. In H9 cells infected with HIV-1 NL4-3, HIV-1 NL4-3_{M184V}, and HIV-1 NL4-3_{M184I}, no mutations in the *env* sequences were observed in approximately 50% (48 to 57%) of clones. The majority of mutated clones harbored only one mutation (36 to 39%), clones carrying two or three mutations were infrequently observed, and the maximum of four mutations was only detected in one of 80 clones (Fig. 2A). G-to-A mutations were observed in seven clones, each carrying one G-to-A mutation. There were no significant differences observed between the mutation frequencies regarding the three different viral variants. In total, 53 mutations in 80 clones were observed; 7 of 53 mutations were G-to-A changes (Fig. 2B). Remarkably, A-to-G mutations were the most-frequently detected (24/53), followed by T-to-C mutations (12/53) (Fig. 2B).

Similar results were obtained from the infection of PBMCs from donors A and B with those viruses. Forty-six to 83% of clones did not show any mutation, 16 to 27% harbored one mutation, and 9 to 21% carried two or three mutations (Fig. 2A). In comparing the mutation frequencies of the four viral variants HIV-1 NL4-3, HIV-1 NL4-3_{M184V}, HIV-1 NL4-3_{M184I}, and HIV-1 NL4-$3_{K65R+M184V}$, no significant differences were observed. In addition, the mutation frequencies in H9 cells (0.13% ± 0.04% [mean ± standard deviation]) and PBMCs (0.20% ± 0.11%) revealed no significant differences (Table 1). In total, 43 *env* clones derived from infected PBMCs were analyzed and 36 mutations were detected. As seen in *env* sequences derived from infected H9 cells, A-to-G transitions were frequently detected (Fig. 2B). More than 50% of all mutations were G-to-A changes (19/36) (Fig. 2B). However, this includes one single clone obtained from PBMCs infected with HIV-1 NL4-3_{M184V}, which, in contrast to the other 35 clones, exhibited G-to-A hypermutation. In this clone, we detected 11 G-to-A mutations; 7 of those occurred within GG dinucleotides (GG to AG), which is the target site specific for

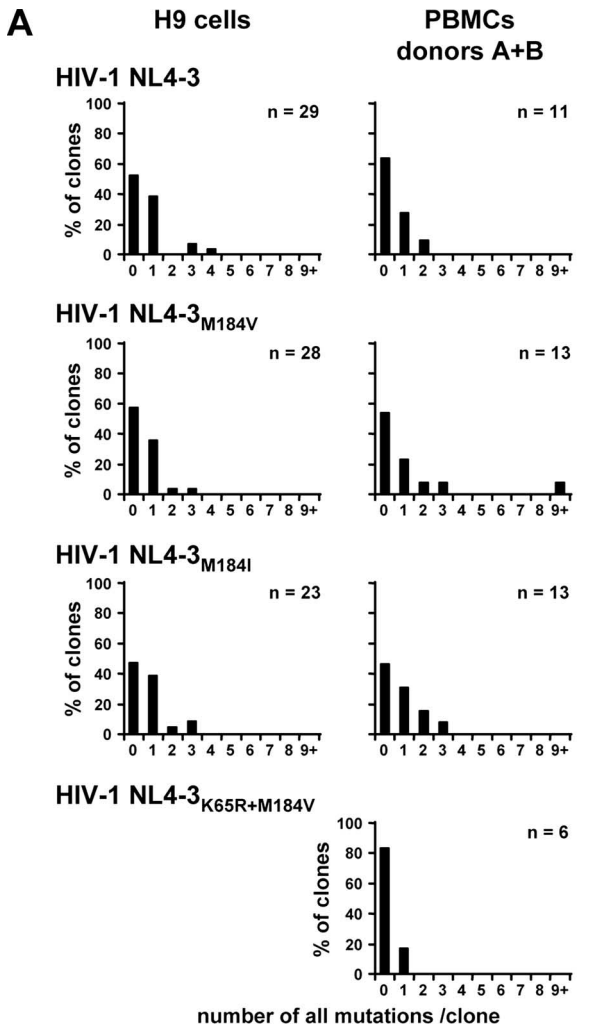

FIG. 2. Total mutation frequencies of viral DNA do not differ in H9 cells and PBMCs infected with HIV-1 NL4-3 harboring different RT variants. (A) H9 cells and PBMCs were infected with HIV-1 NL4-3 containing *vif* and the different RT variants, wild-type RT, M184V, M184I, and K65R+M184V. After the second round of infection, viral DNA was isolated and 466 nucleotides of the *env* gene were amplified, cloned, and sequenced. The percentages of HIV-1 *env* clones carrying the indicated numbers of mutations are shown. The numbers of clones analyzed are given. Two independent experiments using H9 cells were performed, and PBMCs from two different donors, A and B, were used. The infection with HIV-1 NL4-$3_{K65R+M184V}$ was only performed in PBMCs from donor B. (B) Preferences of nucleotide substitutions in HIV-1 *env* clones in H9 cells and PBMCs infected with HIV-1 NL4-3 harboring the different RT variants. The total numbers of mutations are indicated.

APOBEC3G (6, 18, 37, 68, 69) (Fig. 2A). As this result is very unusual in the presence of Vif, all DNA samples isolated after the second round of infection were controlled by real-time PCRs specific for RT mutant alleles and PCRs specific for wild-type *vif* and *vif*-deficient DNA, as well as pNL4-3 vector-specific PCRs. No contamination was observed (data not shown). This suggests that in rare events, APOBEC3-induced G-to-A hypermutation can occur despite the presence of Vif.

Increase of G-to-A mutation frequency of viral DNA in H9 cells infected with HIV-1 NL4-3Δvif$_{M184I}$. To determine the impact of each RT variant on the G-to-A mutation frequency induced by APOBEC3G/F, we infected H9 cells with HIV-1 NL4-3Δvif and its variants HIV-1 NL4-3Δvif$_{M184V}$ and HIV-1 NL4-3Δvif$_{M184I}$ in two independent experiments. A total of 99 clones were analyzed (Fig. 3; Table 1). Three clones harbored no G-to-A mutations. Two clones from the remaining 96 clones harboring G-to-A mutations were identical. Thus, one of those two clones was excluded from the final analysis to avoid any unwanted bias due to possible clonal amplification.

All 31 individual *env* sequences derived from HIV-1 NL4-3Δvif infected H9 cells harbored G-to-A mutations (Fig. 3A). Eighteen of 31 clones (58.1%) carried one to eight and 13 of 31 clones (31.9%) nine and more G-to-A mutations. A maximum of 18 G-to-A mutations was found in one clone, i.e., 19.8% of all guanine residues were mutated in this sequence. A similar pattern was observed in the 34 *env* sequences derived from H9 cells infected with HIV-1 NL4-3Δvif$_{M184V}$. Eight of 34 clones (23.5%) harbored nine and more G-to-A mutations, with a maximum of 19 G-to-A mutations in one clone; 24 of 34 clones (70.6%) carried one to eight G-to-A mutations, and no G-to-A changes were observed in two clones (Fig. 3A). Taken together, we observed no significant differences between those two viruses (Fig. 3C).

In contrast, the infection of H9 cells with HIV-1 NL4-3Δvif$_{M184I}$ led to an increased frequency of G-to-A mutations. In one clone, no G-to-A mutations were observed, and 15 of 33 clones (45.5%) harbored one to eight G-to-A mutations. Seventeen of 33 *env* sequences (51.5%) carried nine and more G-to-A mutations (Fig. 3A). In four of those clones, 22, 23, 24, and 40 of 91 guanine residues (24.2 to 44.0%), respectively, were mutated to adenine. These results differ significantly from the G-to-A mutation frequency observed in H9 cells infected with HIV-1 NL4-3Δvif$_{M184V}$ ($P = 0.04$) (Fig. 3C). Although the infection of H9 cells with HIV-1 NL4-3Δvif$_{M184I}$ led to more G-to-A mutations than infection with HIV-1 NL4-3Δvif, those differences were not significant ($P = 0.24$) (Fig. 3C).

As already seen in the results for H9 cells infected with *vif*-positive HIV-1 NL4-3 variants, the most-frequently observed non-G-to-A mutations were A-to-G and T-to-C mutations (Fig. 3B). No differences in the non-G-to-A mutation frequencies or patterns in regard to the different HIV-1 NL4-3 variants were observed.

Inverse correlation between processivities of RTs and G-to-A mutation frequencies of viral DNA in PBMCs infected with HIV-1 NL4-3Δvif variants. It has been shown that the replication capacities of HIV-1 variants differing in the 184th amino acid position of the RT are very similar in T-cell lines, suggesting that the differences in the processivities of those RT variants may not delay substantially the process of reverse transcription in HIV-1-infected T-cell lines (3). However, a

TABLE 1. Summary of total and G-to-A mutation frequencies of analyzed HIV-1 *env* clones[a]

Item analyzed	Cell type infected	No. (%) of clones, base pairs, or mutations for:							
		HIV-1 NL4-3				HIV-1 NL4-3Δvif			
		WT RT	M184V	M184I	K65R+ M184V	WT RT	M184V	M184I	K65R+ M184V
Total no. of clones sequenced	H9	29	28	23	–	31	34	33	–
	PBMC	11	13	13	6	78	105	90	68
Total no. of base pairs sequenced	H9	13,514	13,048	10,718	–	14,446	15,844	15,378	–
	PBMC	5,126	6,058	6,058	2,796	36,348	48,930	41,940	31,688
Total no. of mutations	H9	21	15	17	–	257	232	354	–
	PBMC	5	19	11	1	221	312	416	287
Total no. of G-to-A mutations	H9	4	1	2	–	246	210	334	–
	PBMC	3	12	4	0	190	270	371	254
No. of clones without G-to-A mutations	H9	25 (86.2)	27 (96.4)	21 (91.3)	–	0	2 (5.9)	1 (3.0)	–
	PBMC	9 (81.8)	11 (84.7)	11 (84.7)	6 (100)	29 (37.2)	40 (38.1)	33 (36.7)	36 (52.9)
Avg no. of mutations/100 bp	H9	0.16	0.11	0.16	–	1.78	1.47	2.30	–
	PBMC	0.10	0.31	0.18	0.04	0.61	0.64	0.99	0.91
Avg no. of G-to-A mutations/ 100 bp	H9	0.03	0.01	0.02	–	1.70	1.33	2.17	–
	PBMC	0.06	0.20	0.07	0.00	0.52	0.55	0.88	0.80
Avg no. of G-to-A mutations/ 100 bp (clones without G-to-A mutations excluded)	H9	NA	NA	NA	–	1.70	1.41	2.24	–
	PBMC	NA	NA	NA	NA	0.83	0.89	1.40	1.70

[a] WT, wild-type; –, not performed; NA, not applicable.

correlation between the processivities of those RT variants and their replication abilities has been observed in PBMCs (3). This underlines the role of this effect in vivo, and thus, we expanded our experiments and investigated the impact of different RT variants on the G-to-A mutation frequencies in PBMCs (the physiological target cells of HIV-1) from four different HIV-1-negative donors. In addition, we included HIV-1 NL4-3Δvif$_{K65R+M184V}$, which exhibits a lower processivity than the M184V RT variant (13, 67), to broaden the spectrum of RT variants with reduced processivities.

In total, 363 HIV-1 *env* clones were sequenced and analyzed. Two hundred three clones showed at least one G-to-A mutation. In order to avoid any unwanted bias due to possible clonal amplification, 22 of those 203 clones were excluded from the final analysis, because identical clones derived from the same cell cultures were detected. Thus, 341 clones were included in the final analysis (Fig. 3; Table 1). Forty-nine of 78 clones derived from PBMCs infected with HIV-1 NL4-3Δvif showed G-to-A mutations. Nearly all of those HIV-1 *env* sequences harbored 1 to 7 G-to-A mutations, only one clone showed 9 mutations, and two clones showed 11 G-to-A mutations (Fig. 3A). A similar pattern was observed in HIV-1 *env* clones derived from PBMCs infected with HIV-1 NL4-3Δvif$_{M184V}$. Sixty-five of 105 HIV-1 *env* sequences carried G-to-A mutations. The majority of those clones showed one to seven G-to-A changes (81.8 to 100%). Eight, 9, 12, and 13 G-to-A mutations were observed once, and 10 or 11 G-to-A mutations twice. HIV-1 *env* sequences derived from PBMCs infected with HIV-1 NL4-3Δvif$_{M184I}$ showed higher G-to-A mutation frequencies than those infected with clones of HIV-1 NL4-3Δvif and HIV-1 NL4-3Δvif$_{M184V}$; however, this increase was not significant ($P = 0.06$ and $P = 0.09$, respectively). Here, 57 of 90 clones revealed G-to-A mutations, with 33 of 57 clones (57.9%) harboring one to seven and 20 clones ≥nine G-to-A mutations. In contrast to the other two previously described HIV-1 RT variants, we detected eight clones with 14 to 18 G-to-A mutations; one clone even carried 38 G-to-A mutations, i.e., 41.8% of all guanine residues were mutated to adenine. Such a highly mutated sequence was also observed once in H9 cells infected with the same HIV-1 variant, NL4-3Δvif$_{M184I}$. The HIV-1 variant NL4-3Δvif$_{K65R+M184V}$ was used to infect PBMCs from donors B, C, and D (Fig. 3A). In 68 clones analyzed, we detected 32 HIV-1 *env* sequences with G-to-A mutations. Here, only 15 of 32 clones (46.9%) carried one to seven G-to-A mutations. Seventeen of 32 clones (53.1%) harbored 9 to 16 G-to-A mutations (Fig. 3; Table 1). Thus, HIV-1 NL4-3Δvif$_{K65R+M184V}$ showed the highest G-to-A mutation frequency, which differed significantly from the mutation frequencies of HIV-1 NL4-3Δvif and HIV-1 NL4-3Δvif$_{M184V}$ ($P < 0.001$) (Fig. 3C).

In regard to non-G-to-A mutations, we observed frequencies and distributions that were similar to those of HIV-1 *env* sequences derived from H9 cells infected with *vif*-negative viruses (Fig. 3B), as well as to HIV-1 *env* clones obtained after infection with *vif*-positive HIV-1 variants (Fig. 2B). Of the non-G-to-A mutations, 41.7% were A-to-G changes, followed by T-to-C mutations (27.2%) and C-to-T mutations (19.2%) (Fig. 3B).

High number of HIV-1 *env* clones without G-to-A mutations in PBMCs infected with HIV-1 NL4-3Δvif variants. Only 3 of 98 HIV-1 *env* sequences (3.1%) derived from H9 cells did not show any G-to-A mutation, and one of those three clones

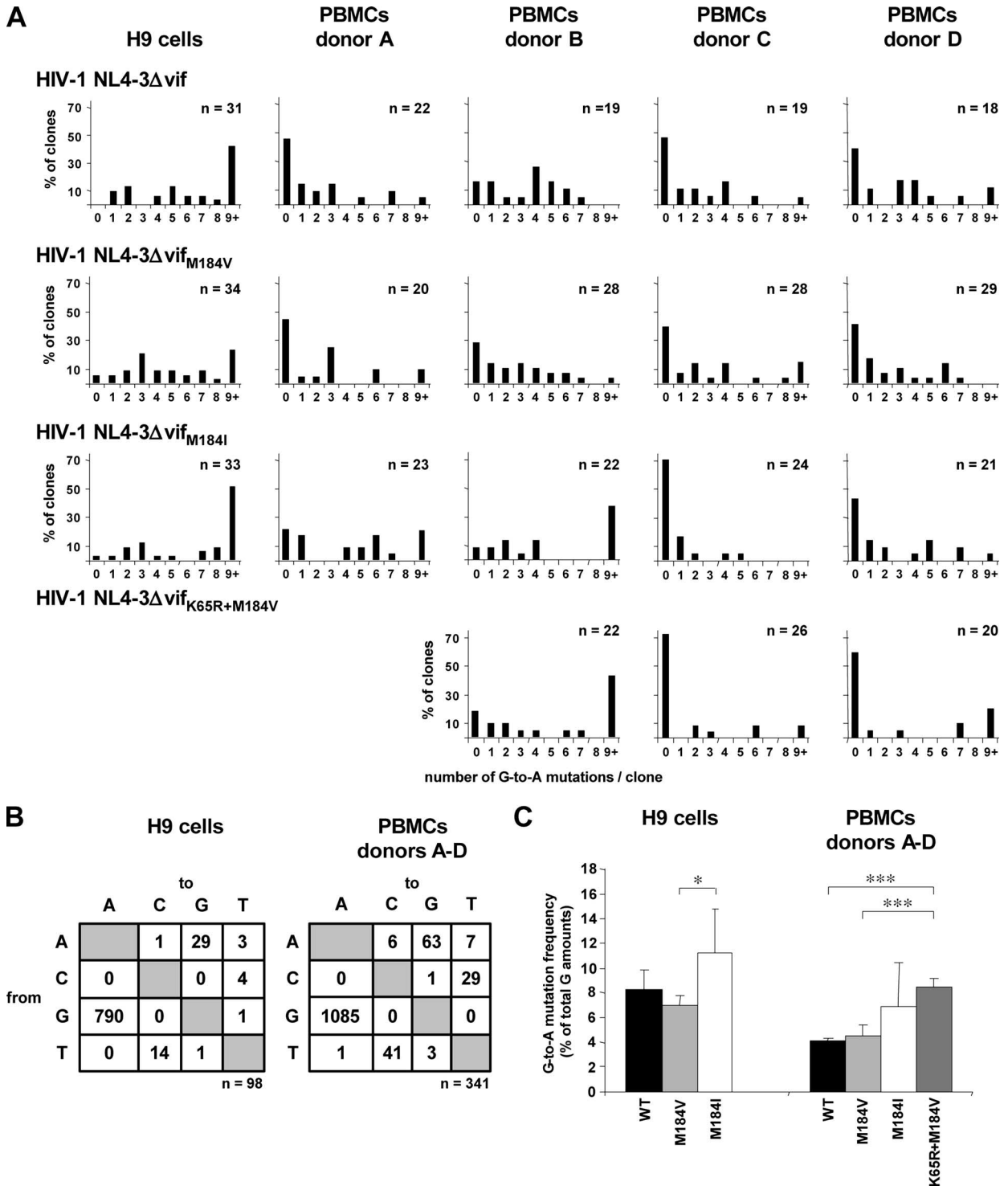

FIG. 3. Impact of impaired processivities of HIV-1 RT variants and the cell type on the G-to-A mutation frequencies induced by APOBEC3 proteins after infection with HIV-1 NL4-3Δvif variants. Shown are summarized results of two independent experiments using H9 cells and of independent experiments using PBMCs from four different donors, A to D. Cells were infected with HIV-1 NL4-3Δvif, HIV-1 NL4-3Δvif$_{M184V}$, and HIV-1 NL4-3Δvif$_{M184I}$. PBMCs from donors B to D were also infected with HIV-1 NL4-3Δvif$_{K65R+M184V}$. Viral DNA was isolated after the second round of infection, and 466 nucleotides of the *env* gene were cloned, sequenced, and analyzed. (A) The percentages of HIV-1 *env* clones carrying the indicated numbers of G-to-A mutations are shown. The numbers of clones analyzed are given. (B) Preferences of nucleotide substitutions in HIV-1 *env* clones in H9 cells and PBMCs infected with HIV-1 NL4-3Δvif harboring different RT variants. The total numbers of mutations are indicated. (C) Comparison of G-to-A mutation frequencies in HIV-1 *env* sequences harboring G-to-A mutations and generated from H9 cells and PBMCs after infection with HIV-1 NL4-3Δvif, HIV-1 NL4-3Δvif$_{M184V}$, HIV-1 NL4-3Δvif$_{M184I}$, and HIV-1 NL4-3Δvif$_{K65R+M184V}$. Error bars show standard deviations. WT, wild type. Asterisks represent statistical significance within both groups, i.e., H9 cell- and PBMC-derived viral *env* sequences. *, $P < 0.05$; ***, $P < 0.001$.

carried an A-to-G mutation. In contrast, no G-to-A mutations were found in 138 of 341 HIV-1 *env* clones (40.5%) derived from PBMCs. This effect was slightly donor dependent. PBMCs from donor B harbored the lowest number of HIV-1 *env* clones without G-to-A mutations (9.1 to 28.6%) (Fig. 3A), and PBMCs from donor C the highest quantity (39.3 to 73.1%) (Fig. 3A). Overall, 51 of those 138 clones harbored different non-G-to-A mutations, the number of identical clones harboring G-to-A mutations was low, and the results of all our control experiments did not reveal evidence for clonal amplification or contamination.

Finally, we compared the G-to-A mutation frequencies of each HIV-1 NL4-3Δvif variant in H9 cells and PBMCs. We observed significantly lower frequencies of G-to-A mutations for all HIV-1 NL4-3Δvif variants in PBMCs than in H9 cells (HIV-1 NL4-3Δvif, $P < 0.001$, and HIV-1 NL4-3Δvif$_{M184V}$ and HIV-1 NL4-3Δvif$_{M184I}$, $P = <0.01$) (Fig. 3C).

G-to-A mutations predominantly occur within the GG dinucleotide context in viral DNA in H9 cells and PBMCs infected with HIV-1 NL4-3Δvif variants. The analyzed HIV-1 *env* fragment contains 466 nucleotides, of which 91 are guanines. We analyzed all 290 clones derived from H9 cells and PBMCs infected with HIV-1 NL4-3Δvif variants which contained G-to-A mutations in regard to their GN dinucleotide context. Only 6 of 13 GC and 11 of 22 GT dinucleotides were mutated to AC and AT, respectively, at low frequencies of 0.3 to 1.0%. In total, less than 1.5% of all G-to-A mutations occurred within those two GN dinucleotides. GA dinucleotides, the preferred target site of APOBEC3F (7, 37, 68), were more-frequently mutated to AA, accounting for 13.5% of all G-to-A mutations. Only 1 of 31 GA dinucleotides was not mutated; 27 of 31 GA-to-AA mutations were observed with frequencies of 0.7 to 4.1%. Three GA nucleotides showed mutation frequencies of 6.9, 10.3, and 12.8% (Fig. 4). However, 85.1% of all G-to-A mutations occurred within GG dinucleotides, which is the target site specific for APOBEC3G (6, 18, 37, 68, 69), suggesting that the deamination of viral cDNA occurs predominantly due to APOBEC3G. Twenty-two of the 25 GG dinucleotides were mutated in at least 5% of the HIV-1 *env* clones (Fig. 4). Only three GG dinucleotides were less-frequently mutated, and one of those GG dinucleotides was not mutated to AG in any of the 290 clones. Nine of 25 GG dinucleotides were mutated frequently, showing GG-to-GA mutation frequencies of 26.9 to 71.7% (Fig. 4). No clear patterns in regard to the 5′ and 3′ neighboring nucleotides of GG dinucleotides appeared; however, this might be due to the limited number of GG dinucleotides in our HIV-1 *env* sequence. The G-to-A mutation frequencies, which were significantly higher in viral DNA derived from H9 cells than in PBMCs, were reflected in the GG-to-AG mutation frequencies, which were 1.7 ± 0.6 times higher in viral DNA derived from H9 cells. However, the pattern of frequently versus rarely mutated GG dinucleotides was similar in both cell populations.

DISCUSSION

In this study, we have shown that the G-to-A mutation frequencies in the viral genome that are induced mainly by APOBEC3G/F are increased in HIV-1 variants expressing RTs which exhibit low processivities. Furthermore, we discovered that the G-to-A mutation frequencies are generally lower in PBMCs than in the T-cell line H9.

Our HIV-1 NL4-3-derived viruses carrying different RT variants do not exhibit different overall mutation frequencies in the context of *vif*-positive viruses, either in the T-cell line H9 or in PBMCs from various donors. This is in contrast to results obtained with purified RT enzymes carrying the M184I, M184V, or M814V+K65R mutations in cell-free nucleotide misincorporation, mismatch extension, and M13-based forward-mutation assays (13, 49, 50, 66). In these biochemical assays, the fidelity of those RT variants was enhanced. In the virus replication assays, the results were contradictory. Lower fidelities of M184I and M184V variants have been observed than for wild-type HIV-1 (14). In contrast, no differences have been shown in regard to the fidelities of those variants and wild-type viruses in cell cultures (24, 26). This suggests that additional viral or cellular factors might also influence the diversity of HIV-1.

In the context of Vif expression, G-to-A, A-to-G, and T-to-C mutations were the most-frequent substitutions observed in H9 cells and PBMCs. This is in accordance with the results of a large survey based on the Los Alamos HIV-1 drug resistance database which has shown that 47% of the resistance-conferring mutations are due to those three substitutions (5). In PBMCs, we found one clone with G-to-A hypermutation, which occurred predominantly in the context of GG dinucleotides and, therefore, is probably caused by APOBEC3G deaminase activity. G-to-A hypermutation has occasionally been detected in cell cultures infected with *vif*-positive HIV-1 (65). Hypermutated proviral DNA has also been detected in patients in up to ~20% of sequences, predominantly in the GG and GA dinucleotide context, without evidence of infection with *vif*-deficient HIV-1 (15, 22, 29). This suggests that APOBEC3G/F can deaminate the viral genome even in the presence of Vif. It is also possible that Vif occasionally became defective due to mutation(s) during reverse transcription in the first round of infection, leading to the incorporation of APOBEC3G/F into virions and subsequently causing G-to-A hypermutation in newly infected cells.

In H9 cells and PBMCs infected with *vif*-deficient viruses, we observed an increase in G-to-A mutation frequencies in the viral DNA derived from HIV-1 NL4-3Δvif$_{M184I}$ compared to the frequencies in DNA from HIV-1 NL4-3Δvif and HIV-1 NL4-3Δvif$_{M184V}$. The G-to-A mutation frequencies were further and significantly enhanced when PBMCs were infected with HIV-1 NL4-3Δvif$_{K65R+M184V}$. These observations indicate that the G-to-A mutation frequencies induced by APOBEC3G/F are inversely correlated to the processivity of the HIV-1 RT. It is unlikely that this is due to direct interactions of the HIV-1 RT with APOBEC3G/F, because it has been reported that the deaminase capacity of APOBEC3G does not require the presence of the HIV-1 RT (62). Thus, it can be assumed that RT enzymes with impaired processivities increase the time period when viral DNA remains single stranded and, therefore, is accessible for deaminases, like APOBEC3G/F. In two elaborative studies, a 5′→3′ G-to-A mutational gradient has been observed (61, 69). Yu and colleagues have found the highest frequencies of G-to-A mutations in the 5′ region of *nef* and the lowest in the 5′ LTR (69). A twin gradient was observed in the other study, with two

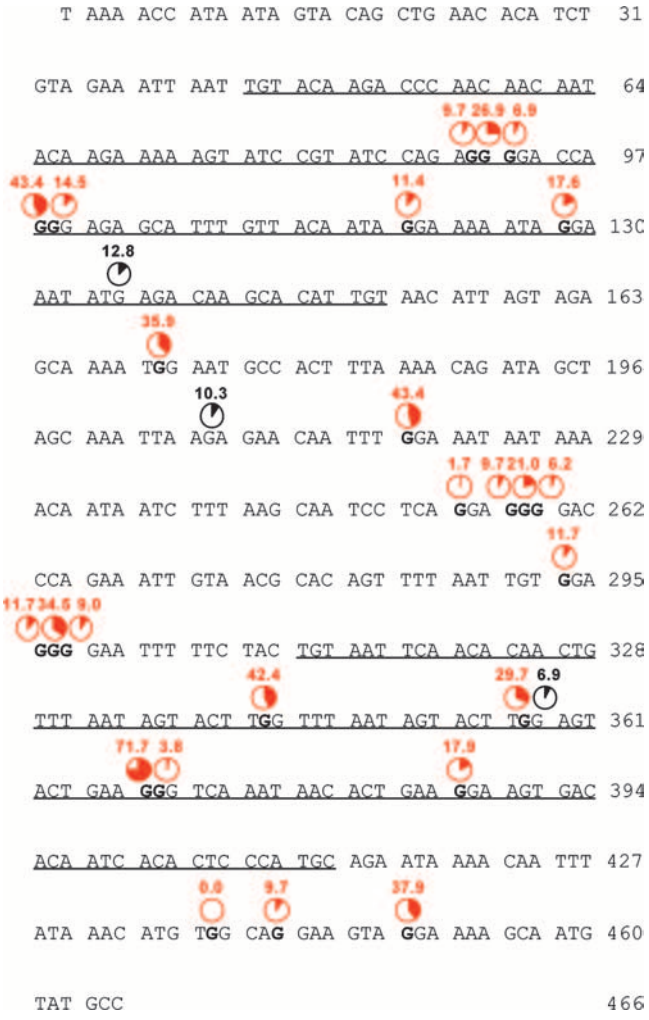

FIG. 4. G-to-A mutation spectrum of viral DNA in H9 cells and PBMCs. Shown are nucleotides 7057 to 7522 of the 466-bp *env* fragment of HIV-1 NL4-3, which was cloned and sequenced. The V3 and V4 loops are underlined, and all first guanine residues of the GG motifs, which are preferentially targeted by APOBEC3G, are depicted in bold. Given in red are the percentages of guanine residues mutated to adenine in a total of 290 HIV-1 *env* sequences obtained by the infection of H9 cells (95 clones) and PBMCs from four different donors (195 clones) harboring at least one G-to-A mutation. Percentages of G-to-A mutations within GA motifs, which are the preferred motifs of APOBEC3F, are depicted in black when more than 5% of those motifs have been mutated.

maxima of G-to-A mutation frequencies, one at the 5′ region of *nef* and the second one just 5′ of the central polypurine tract (61). Those results fit nicely to the model of lentiviral reverse transcription and demonstrate that the deamination process is time dependent.

Our analysis of G-to-A mutation sites in regard to the GN dinucleotide context revealed that approximately 85% of all G-to-A mutations occurred at the first guanine of GG dinucleotides, although GG dinucleotides represented only 27.5% of all four possible GN dinucleotides within the chosen 466-bp fragment of HIV-1 NL4-3 *env*. GT and GC dinucleotides were only rarely mutated to AT and AC, respectively. No differences were observed between H9 cells and PBMCs from four different donors. GG dinucleotides are the specific target for APOBEC3G (6, 18, 37, 68, 69), suggesting that APOBEC3G is mainly responsible for the G-to-A hypermutation seen in the viral DNA of *vif*-deficient viruses in H9 cells and PBMCs.

Some GG dinucleotides within the analyzed part of *env* were frequently mutated, whereas others were only rarely mutated. Moreover, one GG dinucleotide was not mutated in any clone. Remarkably, the corresponding amino acid, W427, of gp120 is important for the binding of the viral envelope to the CD4 receptor and highly conserved among primate immunodeficiency viruses (33). Here, the GG-to-AG mutation would result in a stop codon. A cytosine is positioned immediately downstream of this GG dinucleotide. It has been shown that G-to-A mutations do not or only rarely occur in the context of GGC (4, 18, 34, 69). This suggests that some protective mechanisms against APOBEC3 protein-mediated deamination might exist.

In all of our experiments performed with different *vif*-deficient viruses, we have observed that the G-to-A mutation frequencies were significantly higher in H9 cells than in PBMCs. This was contrary to the expected results. It has been shown that the replication abilities of HIV-1 variants harboring the M184V or M184I mutation are impaired in PBMCs, which correlates with the processivity of each RT variant. This effect has not been observed in a T-cell line. The authors argued that the dNTP concentrations, which are lower in primary cells than in T-cell lines, caused the impaired replication abilities of those HIV-1 variants in PBMCs (3). It has recently been confirmed that a dNTP utilization defect is one reason for decreased processivities and slower polymerization rates of HIV-1 RT variants carrying M184I or M184A mutations, which is exacerbated by limiting dNTP concentrations (16). Thus, dNTP concentrations being lower in PBMCs than in H9 cells should lead to slower polymerization rates and subsequently to higher frequencies of the G-to-A mutations induced by APOBEC3 proteins. In fact, we have obtained opposite results. Furthermore, on average 40% of viral *env* clones derived from PBMCs did not carry any G-to-A mutation. This effect was not observed to the same extent in H9 cells. Several other factors might explain those different observations in H9 cells and PBMCs. Recently, it has been shown that the deaminase activity of endogenous APOBEC3G in T-cell lines and PBMCs is lower than the deaminase activity of exogenous APOBEC3G derived from APOBEC3G-transfected epithelial cells (64). The authors showed that the deaminase activity of APOBEC3G is suppressed by a yet-unidentified cellular factor(s) which is present in T-cell lines and primary $CD4^+$ cells, but not in epithelial cells. This inhibitory activity might be lower in H9 cells than in PBMCs, and the expression levels of this factor(s) could vary in PBMCs from different donors. In addition, the level of APOBEC3G/F expression might be lower in PBMCs than in H9 cells. Variations in the level of APOB EC3G mRNA expression have been observed in PBMCs from different donors (23).

In regard to the high number of sequences without G-to-A mutations in PBMCs, it is also conceivable that other antiretroviral mechanisms besides the deaminase activity of APOBEC3G/F are responsible for this observation and that those mechanisms are not similarly present and active in T-cell lines and PBMCs from different donors. Several groups have

shown that APOBEC3G exhibits additional antiretroviral activities; for instance, postentry restriction of HIV-1 in resting $CD4^+$ T cells (12), inhibition of the accumulation of reverse transcripts (17, 19, 44), and inhibition of DNA strand transfer during reverse transcription and the integration of proviral DNA (36, 40, 44). Another possibility might be the existence of a population of cells within PBMCs which are susceptible to HIV-1 infection and support virus replication but do not express APOBEC3G/F.

In summary, we have shown that the frequencies of G-to-A mutations induced by APOBEC3G/F are increased when viruses are expressing RT enzymes with impaired processivities. Moreover, the deaminase activity of endogenous APOBEC3G/F is substantially lower, if not partly absent, in PBMCs than in H9 cells. Further experiments are required to explore whether this is caused by antiretroviral mechanisms of APOBEC3G/F other than their deaminase activity, by suppressive cellular factors inhibiting APOBEC3G/F, by primary cell populations not expressing a sufficient quantity of APOBEC3G/F, and/or for other reasons.

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