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APOBEC3 Proteins Inhibit Human LINE-1 Retrotransposition*[S]

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The human cytidine deaminase family APOBEC3 represents a novel group of proteins in the field of innate defense mechanisms that has been shown to be active against a variety of retroviruses. Here we examined whether members of the APOBEC3 family have an impact on retrotransposition of human long interspersed nuclear elements (LINE-1s or L1s). Using a retrotransposition reporter assay in HeLa cells, we demonstrate that in the presence of transiently transfected APOBEC3A, L1 retrotransposition frequency was reduced by up to 85%. Although APOBEC3G and -3H did not influence L1 retrotransposition notably, expression of APOBEC3B, -3C, and -3F inhibited transposition by ~75%. Although reverse transcription of L1s occurs in the nucleus and APOBEC3 proteins are believed to act via DNA deamination during reverse transcription, activity against L1 retrotransposition was not correlated with nuclear localization of APOBEC3s. We demonstrate that APOBEC3C and APOBEC3B were endogenously expressed in HeLa cells. Accordingly, down-regulation of APOBEC3C by RNA interference enhanced L1 retrotransposition by ~78%. Sequence analyses of *de novo* L1 retrotransposition events that occurred in the presence of overexpressed APOBEC3 proteins as well as the analyses of pre-existing endogenous L1 elements did not reveal an enhanced rate of G-to-A transitions, pointing to a mechanism independent of DNA deamination. This study presents evidence for a role of host-encoded APOBEC3 proteins in the regulation of L1 retrotransposition.

The long interspersed nuclear element 1 (LINE-1, L1)[4] is the only autonomous non-LTR retrotransposon in the human genome. Approximately 520,000 L1 copies compose ~17% of the chromosomal DNA (1), but only ~80–100 L1s are retrotransposition-competent in the average genome (2). As functional L1 proteins are also mobilizing nonautonomous non-LTR retrotransposons (*e.g. Alu*s) in *trans* (3), they are overall responsible for the generation of at least 30% of the human genome. A functional full-length L1 element is ~6 kb long and includes a 5′-untranslated region (UTR) consisting of an internal promoter, two open reading frames (ORFs) separated by a 63-nucleotide intergenic region, and a 3′-UTR terminating in a poly(A) tail (4). ORF1 encodes an RNA-binding protein (ORF1p) that has nucleic acid chaperone activity *in vitro* (5–7). ORF2 is coding for a protein (ORF2p) with endonuclease (8) and reverse transcriptase activities (9, 10) and a 3′-terminal Zn^{2+} finger-like domain (11).

ORF1p and ORF2p demonstrate a profound *cis* preference for their encoding transcript resulting in the formation of a cytoplasmic ribonucleoprotein particle (RNP) (12–15). After RNP formation, the L1 RNA gains access to the nucleus where it is reverse-transcribed into a cDNA copy most likely via target site primed reverse transcription (8, 16, 17). Integration of the resultant L1 cDNA and the completion of retrotransposition were suggested to occur by alternative mechanisms (18). L1s have altered the genome structure in a multitude of ways (3, 19–22). Host gene expression is influenced by polymerase II promoter activity discovered on sense and antisense strands of the L1 5′-UTR (23) and by transcriptional disruption resulting from intronic L1 insertions (24).

Although there is direct evidence for the regulation of L1 retrotransposition on the transcriptional level, very little is known about host/L1 interactions that might be required for cell type-specific L1 expression and retrotransposition. So far, several host-encoded factors influencing L1 retrotransposition on the transcriptional level have been identified (25–30). There is also evidence strongly suggesting that the human L1 element is susceptible to RNA interference (31). However, to this point, regulation of L1 expression and retrotransposition is far from being understood.

As APOBEC3G, a member of the recently discovered human cytidine deaminase family named apolipoprotein B-editing catalytic polypeptide 3 (APOBEC3), deaminates cytosine residues to uracil in the growing minus strand viral DNA during retroviral reverse transcription, we wanted to evaluate whether members of the APOBEC3 family also interfere with L1 retrotransposition, which is also dependent on reverse transcription of template RNA.

The APOBEC3 family of cytidine deaminases is considered to be an important part of anti-retroviral intrinsic immunity

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[4] The abbreviations used are: LINE-1, L1, long interspersed nucleotide element-1; APOBEC3A, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3A; A3A, APOBEC3A; Vif, virion infectivity factor; HIV-1, human immunodeficiency virus 1; HA, hemagglutinin; UTR, untranslated region; ORF, open reading frame; RNP, ribonucleoprotein; CCAA, mutation of Cys-101 and Cys-106 to Ala; E72A, mutation of Glu-72 to Ala; RT, reverse transcription; LTR, long terminal repeat; PBMC, peripheral blood mononuclear cells; SIV, simian immunodeficiency virus; siRNA, small interfering RNAs; IAP, intracisternal A particle.

(for review see Ref. 32). In primates, the eight paralogues *APOBEC3A*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, and *-H* (*A3A–H*) have been described, although rodents carry only one single *Apobec3* gene. Human APOBEC3G (A3G) and APOBEC3F (A3F) have been shown to be active against human immunodeficiency virus-1 (HIV-1), which lacks the virion infectivity factor (Vif) (33–37). Both APOBEC3s induce C-to-U transitions by cytidine deamination on the viral minus strand DNA during reverse transcription, causing genome degradation or hypermutations (38, 39). Studies showed that other lentiviruses, gammaretroviruses, deltaretroviruses, spumaviruses, LTR retrotransposons, orthohepadnaviruses, and avihepadnaviruses are also sensitive to the latter APOBEC3 proteins (38, 40–53). Human A3B, A3C, A3F, and A3G proteins show a strong activity against simian immunodeficiency virus (SIV)Δ*vif*, whereas the effect of A3B and A3C on HIV-1Δ*vif* is moderate (54). The Vif proteins of the various SIVs preferentially neutralize APOBEC3 proteins of their own species, but activity against APOBEC3s from other primates, including humans, is also described (51, 53–55). Only lentiviruses like HIV-1 and spumaviruses evolved viral proteins, Vif and Bet respectively, that prevent incorporation of APOBEC3 into progeny virions (41, 42, 51). The question of how retroviruses that do not encode Vif or Bet proteins protect themselves against the activity of APOBEC3 during natural infections has not been answered yet. A3G has been shown to inhibit hepatitis B virus and human T cell leukemia virus type 1 without causing genomic mutations in the majority of the viral genomes (43, 44, 49, 50, 56–58). This indicates the existence of an additional antiviral mechanism mediated by APOBEC3 proteins, which is independent of cytidine deamination.

L1 elements have been discussed as potential targets for APOBEC3 activity, because their spreading depends on a reverse transcription step. It was shown previously that L1 retrotransposition is not sensitive to A3G (59). We wanted to evaluate the effect of other members of the APOBEC3 family on L1 retrotransposition and therefore applied a well established L1 retrotransposition reporter assay (60, 61), which was already used successfully to identify other host-encoded factors regulating L1 retrotransposition (27, 30).

In this study, we demonstrate that the presence of A3A reduced the L1 retrotransposition frequency in HeLa cells by up to 85%. Expression of A3B, A3C, and A3F inhibited retrotransposition activity by ~75%, whereas A3G and A3H had no influence. Furthermore, we were able to enhance L1 retrotransposition frequency in the absence of cotransfected APOBEC3s by knocking down endogenously expressed A3C via RNA interference. Although mutation of the putative catalytic deamination domain of APOBEC3A completely abolished its activity against L1 retrotransposition, we did not find editing of L1 *de novo* insertions in the presence of APOBEC3 proteins. In addition, sequence analyses of preexisting L1 insertions did not reveal any enhanced frequency of G-to-A transitions. Our data suggest a novel mode of action of APOBEC3 activity against the non-LTR retrotransposon LINE-1.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were maintained in Dulbecco's high glucose modified Eagle's medium supplemented with 10% fetal bovine serum, 0.29 mg/ml L-glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen; Dulbecco's modified Eagle's medium complete). Human A3.01 T cells (National Institute for Biological Standards and Control, UK) were grown in complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.29 mg/ml L-glutamine, and 100 units/ml penicillin/streptomycin. Human keratinocytes were kindly provided by Dr. August Bernd (Zentrum der Dermatologie und Venerologie, Johann-Wolfgang Goethe-Universität Frankfurt/Main, Germany) and cultivated in Hanks' medium supplemented with 5% fetal bovine serum and 100 units/ml penicillin/streptomycin. Cells were incubated at 37 °C with 100% humidity in 5–7% CO_2 and passaged using standard cell culture techniques. PBMCs were isolated from EDTA-treated whole blood of healthy donors by Histopaque-1077 (Sigma) gradient centrifugation.

Plasmids—L1 retrotransposition reporter construct pJM101/$L1_{RP}$ (62) was a gift from Haig H. Kazazian, Jr. pLLrpII.6 is a plasmid carrying a *de novo* L1 integrant with a spliced *neo*^{r} cassette that is localized on a plasmid backbone derived from the L1 rescue plasmid pCEP4/L1.3mneoI400/ColEI (20). The hemagglutinin (HA)-tagged human APOBEC3 proteins were expressed from the pcDNA3.1/Zeo(+) vector (Invitrogen). The original APOBEC3G-HA and APOBEC3A-HA expression constructs were generously provided by Nathaniel R. Landau (51) and Bryan R. Cullen (35), respectively. Plasmids expressing A3B, A3C, and A3F were generated by performing RT-PCR on RNA of PHA/IL2-activated human PBMCs. To isolate A3B (pcA3B-HA), forward primer CEM15-CM13 (5′-TAAGCGGAATTCTATCTAAGAGGCTGAACATG-3′) and reverse primer CEM15-HA-C (5′-TAGAAGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGATAGTTTTCCTGATTCTGGAG-3′) were used. The amplicon was cloned into pCR4Blunt-TOPO (Invitrogen), and the NotI- and XbaI-restricted fragment was transferred into the NotI and SpeI sites of pcDNA3.1(+) (Invitrogen). To clone A3C (pcA3C-HA), forward primer CEM15-CM13 (see above) and reverse primer CEM15-CM28 (5′-AGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGATACTGGAGACTCTCCCGTAGCCTT-3′) were applied. The amplicon was cloned into pCR4Blunt-TOPO and inserted into the EcoRI and XhoI restriction sites of pcDNA3.1(+). To clone A3F (pcA3F-HA), forward primer CEM15-CM12B (5′-TAAGCGAAGCTTCTTAGTCGGGACTAGCCGGC-3′) and reverse primer CEM15-CM29 (5′-AGTCTAGATCAAGCGTAATCTGGAACATCGTATGGATACTCGAGAATCTCCTGCAGCTTGCTGTC-3′) were used, and the amplicon was cloned into pCR4Blunt-TOPO. After HindIII and SpeI digestion, the resulting fragment was transferred into the HindIII and XbaI sites of pcDNA3.1(+).To clone A3H (pcA3H-HA), the coding region was amplified by PCR using the forward primer CEM15-CM42 (5′-CAGGCGAATTCCTGCTAAGGAAGCTGTGGCC-3′) and the reverse primer CEM15-CM43 (5′-TTCAGCTCGAGTCAAGCGTAATCTGGAACATCGTA-

TGGATAGGACTTTATCCTCTCAAGCCG-3′) with the plasmid IRALp962F0354Q (obtained from the German Resource Center for Genome Research, Berlin) as template. The PCR product was transferred into the EcoRI and XhoI sites of pcDNA3.1(+). APOBEC3 expression constructs used in the retrotransposition reporter assays were generated by subcloning the respective APOBEC3-HA sequence from the above-described pcDNA3.1(+) vectors into pcDNA3.1/Zeo(+) using the following restriction sites: A3A, KpnI and XbaI (pcA3A-HA.ZEO); A3B, HindIII and XhoI (pcA3B-HA.ZEO); A3C, HindIII and XhoI (pcA3C-HA.ZEO); A3F, PmeI and PmeI (pcA3F-HA.ZEO); A3G, EcoRI and XhoI (pcA3G-HA.ZEO); and A3H, EcoRI and XhoI (pcA3H-HA.ZEO). The APOBEC3A mutants pcA3A.E72A and pcA3A.CCAA were generated by overlapping PCR using pcA3A-HA.ZEO as template. 5′ and 3′ fragments were amplified with primer sets specific for the overlap region, including the mutations and the external primers (hu3A1, 5′-GCTTGGTACCACCATGGAAGCCAGCCCAGCATCCG-3′; hu3A4, 5′-CATGCTCGAGTCAAGCGTAATCTGGAACGTC-3′). Subsequently, the mixture of both PCR products was amplified with the two external primers. The resulting fragment was cleaved with KpnI and XhoI and cloned into pcDNA3.1(+)Zeo. Sequence analysis revealed an additional mutation (M153I) in pcA3A-CCAA. The identity of the described APOBEC3 expression plasmids was confirmed by sequence analyses.

Western Blot Analysis—For analysis of APOBEC3 protein expression, 2×10^5 HeLa cells were seeded and transfected with 2 μg of plasmid DNA the next day using FuGENE 6 transfection reagent (Roche Applied Science). Two days after transfection, cells were lysed using RIPA lysis buffer (25 mM Tris, pH 8.0, 137 mM NaCl, 1% glycerol, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2 mM EDTA, pH 8, 0.1% SDS, and protease inhibitors), and lysates were cleared by centrifugation. Samples were boiled in Laemmli buffer and subjected to SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane. HA-APOBEC3 proteins were detected using an anti-HA antibody (1:6,000 dilution, MMS-101P; Covance) and anti-mouse horseradish peroxidase (Amersham Biosciences). For the detection of α-tubulin, an anti-tubulin antibody (1:10,000 dilution, B5-1-2, Sigma) was applied. Signals were visualized by ECL (Amersham Biosciences).

L1 Retrotransposition Reporter Assay—Retrotransposition rates were determined by applying the rapid and quantitative transient L1 retrotransposition assay described previously (61). Briefly, for each transfection reaction, HeLa cells were seeded in 6-well tissue culture dishes at 2×10^5 cells/well. The following day, each well was cotransfected with 0.5 μg of reporter plasmid pJM101/$L1_{RP}$ (62) and 0.5 μg of the respective APOBEC3 expression plasmid using 3 μl of FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. 72 h post-transfection, cells were selected for L1 retrotransposition events in 1,000 μg/ml G418 (Invitrogen). After 11–12 days of selection, $G418^r$ colonies were fixed and stained with Giemsa (Merck) as described previously (60). To control for transfection, HeLa cells seeded in parallel were cotransfected with 0.5 μg of plasmid phMGFP (Promega) expressing the green fluorescent protein and 0.5 μg of reporter plasmid. Two days later, transfection rates of 60–70% were determined by flow cytometry. For titration of APOBEC3 expression against L1 retrotransposition, the retrotransposition reporter assay was done as described above using various amounts of the respective APOBEC3 plasmid (0.5 to 0.002 μg). The total amount of transfected plasmid DNA was held constant by adding empty expression vector pcDNA3.1/Zeo(+).

Toxicity Assay—2×10^5/well HeLa cells were seeded in a 6-well dish. The following day, cells were cotransfected with 0.5 μg of the respective APOBEC3 expression plasmid or empty vector pcDNA3.1/Zeo(+) with 0.5 μg of pcDNA3.1(+) expressing the neomycin resistance gene using FuGENE 6 transfection reagent (Roche Applied Science). Two days post-transfection, the G418 selection (1,000 μg/ml) was initiated and continued for 10–12 days. $G418^r$ colonies were stained with Giemsa and counted.

Immunofluorescence Microscopy—Immunofluorescence studies were performed in HeLa cells 2 days after transfection applying the FuGENE technology (Roche Applied Science). Cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice in PBS, permeabilized in 0.1% Triton-X-100 in PBS for 15 min, washed twice in PBS, and blocked with Image-iT (Invitrogen) for 30 min. For APOBEC3 staining, cells were incubated overnight at 4 °C with an anti-HA antibody (MMS-101P; Covance) in a 1:1,000 dilution in blocking solution (Candor). Anti-mouse Alexa Fluor 488 (Invitrogen) was used as secondary antibody in a 1:300 dilution in blocking solution (Candor) for 1 h. Subsequently, 4,6-diamidino-2-phenylindole staining (Chemicon, 1:1,000) was performed for 5 min. Finally, cells were incubated in ProLong Gold (Invitrogen) and analyzed by laser scan microscopy (LSM 510 Meta Zeiss).

Sequence Analyses of L1 Insertions—Genomic DNA was extracted from $G418^r$ HeLa clones using the DNeasy kit (Qiagen) and used as template for PCR with the primers GS260 forward (5′-CAGGTGCTGGAGAGGATGCGGAG-3′) and Neo-B reverse (5′-CGGTGCCCTGAATGAGCTTCAG-3′), which specifically recognizes the spliced *neo*^{r} cassette. PCRs were performed with Expand High Fidelity PCR system (Roche Applied Science) using the following cycle conditions: one cycle at 94 °C for 3 min; 35 cycles at 94 °C for 45 s, 65 °C for 45 s, and 72 °C for 60 s; and one cycle at 72 °C for 10 min. PCR products were gel-purified (Qiagen) and sequenced (MWG) using the primers GS260 and Neo-B. To optimize the annealing temperature, PCR was performed with varying annealing temperatures using pJM101/$L1_{RP}$ or pLLrpII.6 as templates.

To analyze pre-existing endogenous L1 insertions for DNA editing events, 30 full-length L1 sequences were randomly picked from a previously published L1 data base available from the author (18).

Gene Silencing by RNA Interference—siRNA-mediated knockdown of human APOBEC3s in HeLa cells was performed by transfection of predicted, chemically synthesized RNA duplexes (Qiagen). For targeting of A3B and A3C, we used a mixture of two different siRNAs. The A3B-specific siRNAs (SI00298487 and SI00298494; Qiagen) target the noncoding regions 5′-CUCCAUAUUUAGACUAAUAA-3′ and 5′-AAGCAAUGUGCUCCUGAUCAA-3′ of the mRNA. The target

sequences for the A3C-specific siRNAs (SI00298515 and SI00298522; Qiagen) are the coding sequence 5′-AAGCCAACGAUCGGAACGAAA-3′ and the noncoding sequence 5′-CAGCAUAACCAAAUCUUACUA-3′ (siA3C2). For control, a nonspecific control siRNA (1022076; Qiagen) with the target sequence 5′-AAUUCUCCGAACGUGUCACGT-3′ was used. To test for interference efficiency, 300 ng of siRNA mixture (150 ng each) was transfected into HeLa cells using the HiPerfect fast-forward protocol (Qiagen) for 6-well plates according to the manufacturer's instructions. After 48 h, RT-PCR analysis was performed as described below. For cotransfection of the L1 reporter plasmid pJM101/$L1_{RP}$ and the siRNAs, the following protocol was used: 5×10^5 HeLa cells were seeded in 6-well plates. The next day 0.5 μg of pJM1001/$L1_{RP}$ and 300 ng of total siRNA were diluted in 50 μl of HBS buffer (50 nM Hepes, 150 nM NaCl, pH 7.4), and 12 μl of HiPerfect transfection reagent were diluted in 50 μl of HBS buffer. Both solutions were incubated for 5 min at room temperature and mixed. After 15 min of incubation, the transfection solution was added dropwise to the cells. 72 h post-transfection cells were selected for L1 retrotransposition events as described above.

Detection of APOBEC3 RNA by RT-PCR—RNA was prepared from human keratinocytes, freshly isolated human PBMC, HeLa cells, and A3.01 T cells using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. 2 μg of total RNA was used to generate cDNA using SuperScript III reverse transcriptase (Invitrogen). PCR was performed using the following primer pairs: A3A, forward 5′hA3A (5′-ATGGAAGCCAGCCCAGCATC-3′) and reverse 3′hA3A (5′-TCAGTTTCCCTGATTCTGGAG-3′); A3B, forward CEM15-CM30 (5′-CGAAGCTTAGAGCGGGACAGGGACAAGCG-3′) and reverse CEM15-CM6 (5′-TAGAAGCTCGAGGTTTTCCTGATTCTGGAGAATGGC-3′); A3C, forward CEM15-CM13 (5′-TAAGCGGAATTCTATCTAAGAGGCTGAACATG-3′) and reverse CEM15-CM27 (5′-AGCTCGAGTCACTGGAGACTCTCCCGTAGCCTT-3′); A3F, forward CEM15-CM12 (5′-TAAGCGGAATTCCTTAGTCGGGACTAGCCGGC-3′) and reverse CEM15-CM32 (5′-AGTCTAGATCACTCGAGAATCTCCTGCAGCTTGCTGTC-3′); A3G, forward CEM15-CM12 (see above) and reverse CEM15-CM6 (see above); and A3H, forward CEM15-CM41 (5′-TGATAATGAGTGGGATACGGGTCC-3′) and reverse CEM15-CM42 (5′-CAGGCGAATTCCTGCTAAGGAAGCTGTGGCC-3′). The cDNA synthesis was controlled by RT-PCR detection of the constitutive expression of the housekeeping gene β_2-microglobulin using the primers forward β_2-microglobulin-A (5′-CTCGCTCCGTGGCCTTAGCTGTGCTCGCGC-3′) and reverse β_2-microglobulin-B (5′-TAACTTATGCACGCTTAACTATC-3′). The cDNAs were used for 30 cycles of PCR amplification using Phusion polymerase (New England Biolabs) under the following conditions: denaturation for 10 s at 98 °C, primer annealing for 30 s at the temperature mentioned below, and primer extension for 1 min at 72 °C. Annealing temperatures were as follows: 58 °C for APOBEC3A, APOBEC3G, and β_2-microglobulin; 50 °C for APOBEC3B and APOBEC3H; and 60 °C for APOBEC3C and APOBEC3F.

RESULTS

APOBEC3 Family Members A3A, A3B, A3C, and A3F Inhibit L1 Retrotransposition—To analyze the influence of APOBEC3 proteins on L1 retrotransposition, we employed an established cell culture retrotransposition reporter assay (60, 61). This assay is based on the active $L1_{RP}$ element (62) containing an antisense copy of the *neo*^{r} indicator gene disrupted by intron 2 of the γ-globin gene in sense orientation. Splicing and reverse transcription of RNAs from the marked $L1_{RP}$ reporter allow expression of the *neo*^{r} gene and results in G418 resistance after integration of the cDNA into chromosomal DNA (Fig. 1). To measure the effects of the APOBEC3 proteins on L1 retrotransposition, we cotransfected the L1 reporter plasmid pJM101/$L1_{RP}$ with plasmids expressing A3A, A3B, A3C, A3F, A3G, or A3H into HeLa cells. Transfected cells were cultivated for 12 days under G418 selection, and G418^r HeLa colonies were visualized by Giemsa staining. Expression levels of the different HA-tagged APOBEC3 proteins in HeLa cells were confirmed by Western blot analysis (Fig. 2*A*). By determining the number of G418^r colonies, we found differential activity of the single APOBEC3s against L1 retrotransposition. The presence of A3A drastically reduced L1 retrotransposition frequency to ~14.5% of the $L1_{RP}$ control activity, whereas in the presence of A3B, A3C, or A3F, retrotransposition rates were decreased to about 22.8, 22.6, and 25.9%, respectively (Fig. 2*C*). In contrast, A3G and A3H had no significant impact on L1 retrotransposition (Fig. 2*C*). To titrate APOBEC3 proteins against L1 retrotransposition, we decreased the amounts of transfected APOBEC3 expression plasmid from 0.5 to 0.002 μg (Fig. 2*E*). A dose-dependent inhibitory effect of A3A, A3B, A3C, and A3F was observed.

To evaluate whether any general cytotoxic effect of APOBEC proteins could have an impact on L1 retrotransposition rates, we performed toxicity assays. For that purpose, we cotransfected each APOBEC3 expression plasmid with pcDNA3.1 conferring neomycin resistance, instead of the L1 reporter pJM101/$L1_{RP}$, and counted resulting G418^r cell colonies after 12 days of G418 selection. Although we observed a minor toxic effect caused by overexpression of A3A, A3B, A3C, and A3F (Fig. 2*B*), the vast majority of the effect observed in the transposition assays was clearly a result of specific inhibition of L1 retrotransposition.

Mutating the Putative Catalytic Domain of A3A Fully Restores L1 Retrotransposition Activity—All human APOBEC3 family members have a structure in common that represents a putative cytidine deaminase domain. To answer the question whether the presumed catalytic active site of A3A ($HXEX_{27}PCX_4C$) is involved in the inhibition of L1 retrotransposition, we introduced mutations that were described to destroy the editing activity in the context of A3G (63). Mutating the Zn^{2+}-coordinating Cys residues at position 101 and 106 to Ala (CCAA) or mutating Glu72 to Ala (E72A) fully restored the retrotransposition activity of the L1 reporter (Fig. 2*D*), suggesting that the putative catalytic domain of A3A is involved in the inhibition of L1 retrotransposition. In the presence of the A3A mutant proteins, retrotransposition frequency was even

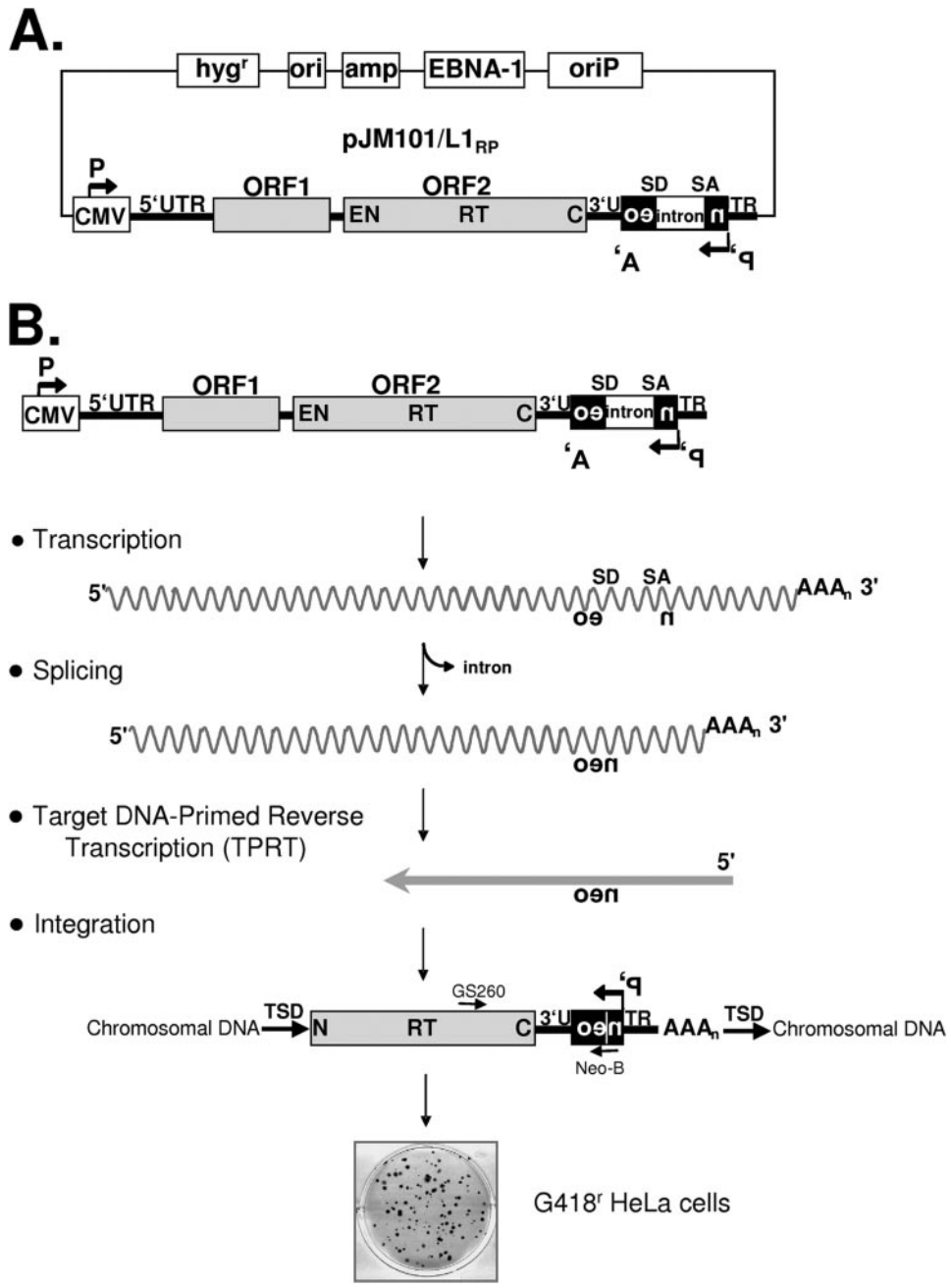
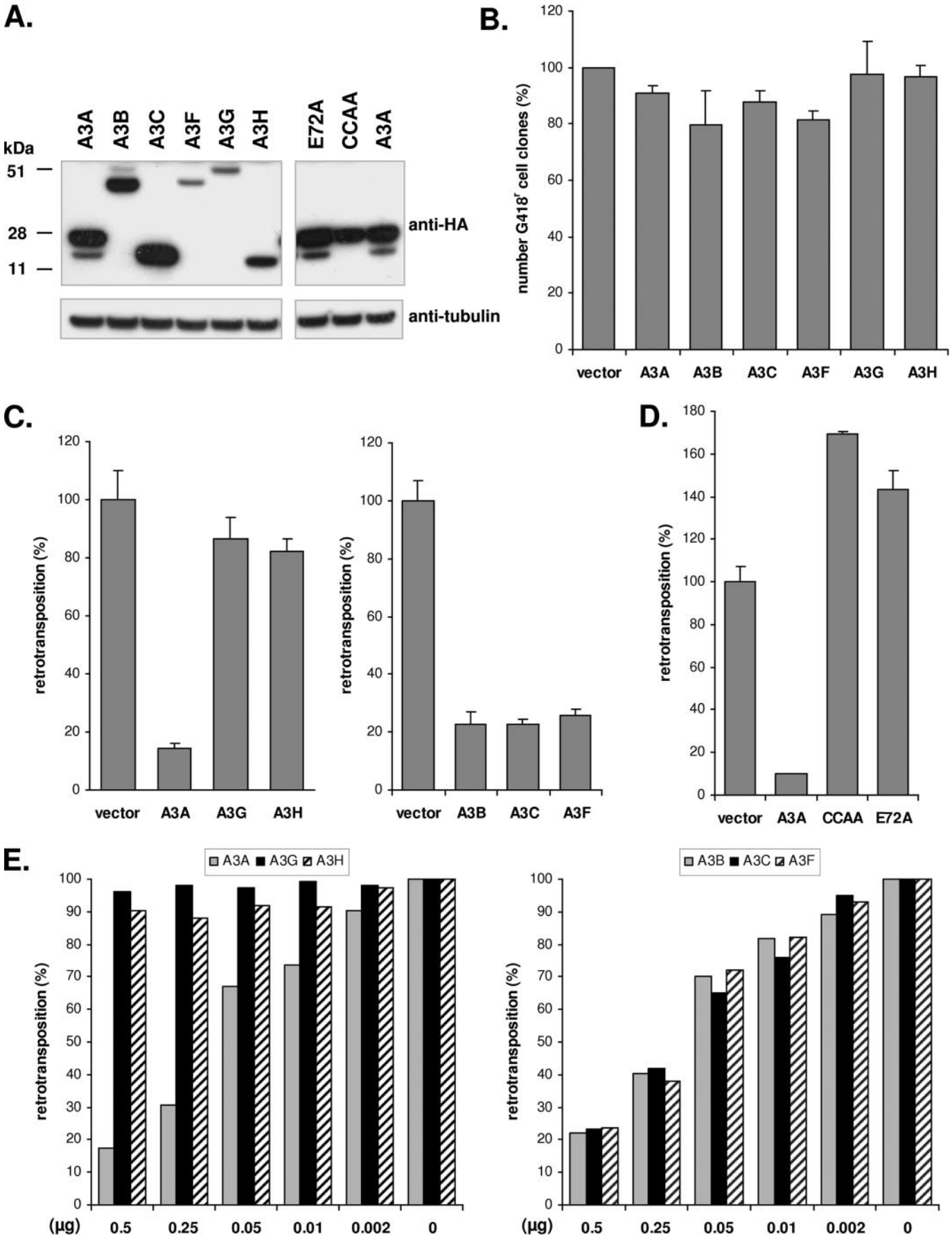


FIGURE 1. *A,* structure of the L1 retrotransposition reporter plasmid pJM101/$L1_{RP}$. The $L1_{RP}$ retrotransposition reporter cassette was inserted into the episomal pCEP4 vector. *B*, schematic depiction of the L1 retrotransposition assay. The active $L1_{RP}$ element was tagged with the indicator gene (*black box*) containing an antisense copy of the *neo*^{r} gene disrupted by intron 2 of the γ-globin gene in sense orientation (*oe-intron-n*). The splice donor (*SD*) and splice acceptor (*SA*) sites of the intron are indicated. The *neo*^{r} gene is also flanked by a heterologous promoter (′*P*) and a polyadenylation signal (′*A*). Transcripts originating from the cytomegalovirus (*CMV*) promoter drive $L1_{RP}$ expression and can splice the intron but contain an antisense copy of the *neo*^{r} gene. G418-resistant (G418^r) colonies arise only when this transcript is reverse-transcribed, integrated into chromosomal DNA, and expressed from its own promoter P′. Annealing sites of oligonucleotide primers GS260 and Neo-B used to amplify sequences of *de novo* L1 integrants are indicated. The abbreviations used are as follows: *C*, cysteine-histidine-rich domain; *TSD*, target site duplications; AAA_n, poly(A) tail; *EN*, endonuclease.

increased by 70% (CCAA) and 40% (E72A) as compared with the empty expression vector (Fig. 2*D*).

Subcellular Localization of APOBEC3 Proteins Is Not Correlated with Activity against L1 Retrotransposition—APOBEC3 proteins can inhibit retrovirus replication by deamination of the viral minus strand DNA during reverse transcription (38, 39). Because L1 reverse transcription occurs in the nucleus, we analyzed whether the subcellular localization of APOBEC3 offers an explanation for the different impact of the various APOBEC3 gene products on L1 retrotransposition. Using anti-HA antibodies directed against the HA-tagged APOBEC3 proteins, immunofluorescence studies in transiently transfected HeLa cells showed that A3A was mainly localized to the nucleus, but significant amounts were also detected in the cytoplasm (Fig. 3). A3B was found in the nucleus; A3C was equally distributed in both compartments, and A3F and A3G were exclusively localized to the cytoplasm (Fig. 3). In the case of A3H, localization of the protein differed among cells. In some transfected cells, A3H localized predominantly to the nucleus (Fig. 3, *red arrowhead*), and in others it was observed only in the cytoplasm (Fig. 3, *white arrowhead*). The immunofluorescence experiment did not show a general correlation between the subcellular localization of the different APOBEC3 proteins and their activity against L1.

Lack of G-to-A Transitions in L1 Insertions Argues against L1 Inhibition by DNA Deamination—A3G and A3F are cytidine deaminases that induce C-to-U transitions leading to G-to-A substitutions on the positive strand of the retroviral DNA. To examine whether cytidine deamination is also the mechanism that accounts for the inhibition of L1 retrotransposition, we sequenced *de novo* L1 retrotransposition events that occurred in the presence of overexpressed APOBEC3 members. To exclude PCR amplification of endogenous L1 sequences or L1 reporter plasmid DNA, the Neo-B primer was designed to bind specifically to the spliced *neo*^{r} cassette, which is only generated after successful retrotransposition (Fig. 1 and supplemental Fig. 1). PCR conditions were optimized for the specific annealing of the Neo-B primer to the spliced *neo*^{r} cassette (supplemental Fig. 1). For analysis of *de novo* L1 integrants, HeLa cells were cotransfected with pJM101/$L1_{RP}$ and each of the APOBEC3 expression plasmids. Single G418^r HeLa colonies were expanded separately for 3–6 weeks under G418 selection. Genomic DNA was isolated from each clone and used


A.
A3A
A3B
A3C
A3F
A3G
A3H
E72A
CCAA
A3A
kDa
51
28
11
anti-HA
anti-tubulin
B.
number G418r cell clones (%)
vector
A3A
A3B
A3C
A3F
A3G
A3H
C.
retrotransposition (%)
vector
A3A
A3G
A3H
vector
A3B
A3C
A3F
D.
retrotransposition (%)
vector
A3A
CCAA
E72A
E.
A3A
A3G
A3H
A3B
A3C
A3F
retrotransposition (%)
(µg)
0.5
0.25
0.05
0.01
0.002
0

as PCR template in order to sequence *de novo* L1 insertions. We analyzed 7–17 *de novo* L1 insertions resulting from each cotransfection experiment with pJM101/$L1_{RP}$ and A3A, A3B, A3C, or A3G for mutations in a region covering the $L1_{RP}$ sequence from position 5373 to 6000 (628 nucleotides) and 677 bp of the adjacent *neo*^{r} cassette. In total, we analyzed 38,742 nucleotides and found only one point mutation that was not a G-to-A transition (Table 1). These data argue against editing of *de novo* L1 insertions in the presence of APOBEC3 proteins.

In addition, we analyzed pre-existing, endogenous L1 sequences for G-to-A mutations. DNA sequences of 30 full-length L1 insertions obtained from a previously published data base (18) were aligned with the sequence of the active $L1_{RP}$ element (62), and the number and type of identified point mutations were specified. We observed that the frequency of C-to-T as well as G-to-A transitions was higher (1252 and 1242 substitutions) than the frequency of other occurring transitions or transversions (Fig. 4). However, these two transitions are relatively frequent events that can be the consequence of cellular mechanisms such as methyl-CpG deamination, for example (64, 65). These cellular mechanisms affect both DNA strands and therefore increase the number of both G-to-A and C-to-T transitions. In contrast, cytidine deamination of the minus strand DNA, as expected for APOBEC3 activity, would only enhance the frequency of G-to-A transitions on the plus strand. Because our analysis of endogenous L1 sequences showed a comparable number of substituted guanines and cytosines (Fig. 4), we can conclude that a preference for G-to-A substitutions that could be ascribed to APOBEC3 was not detectable.

Down-regulation of Endogenous A3C Expression by RNA Interference Increases L1 Retrotransposition Rates—The presented data demonstrate that expression of A3A, A3B, A3C, and A3F has inhibitory effects on L1 retrotransposition. We next wanted to evaluate whether in HeLa cells or other cell types *APOBEC3* genes are expressed that could modulate the level of L1 retrotransposition. Therefore, we performed RT-PCR analyses on total RNA from HeLa cells, A3.01 T cells, unstimulated PBMC, and primary human keratinocytes (Fig. 5*A*). As positive controls, the respective APOBEC3 expression plasmids were used as PCR templates (Fig. 5*A*). APOBEC3 gene products, which we have shown to inhibit L1 retrotransposition, were differentially expressed in all samples. In addition to the findings of a previous report that demonstrated A3A expression in keratinocytes (66), we observed A3A expression in unstimulated PBMC. Interestingly, in HeLa cells, no expression of A3A or A3F was detected, which is consistent with the high retrotransposition rate of tagged L1 reporter elements in these cells. Instead, HeLa cells expressed A3B and A3C, which we also found to be inhibiting in the retrotransposition assay. To investigate whether endogenous A3B and A3C contribute to the inhibition of L1 retrotransposition, we intended to down-regulate the expression of these proteins by RNA interference. siRNAs directed against A3B and A3C or a nonspecific control siRNA were transfected into HeLa cells, and mRNA levels of A3B and A3C were analyzed by RT-PCR. The amount of A3C mRNA was strongly decreased by the A3C-specific siRNA, but not by control siRNA, and only moderately by A3B-specific siRNA (Fig. 5*B*). In contrast, we did not observe efficient down-regulation of A3B in the presence of any tested siRNA. Therefore, we performed our L1 retrotransposition assay only in HeLa cells with silenced A3C expression. Cotransfection of the L1 reporter construct with A3C-specific siRNA resulted in an increase of the L1 retrotransposition frequency by ~78% as compared with cells transfected with nonspecific control siRNA (Fig. 5*C*). This result demonstrates that endogenous A3C plays a role in controlling L1 retrotransposition in HeLa cells.

DISCUSSION

In this study we set out to evaluate whether functional human L1 retrotransposons are regulated by members of the APOBEC3 protein family. By applying a cell culture-based retrotransposition reporter assay, we observed that in the presence of A3A, L1 retrotransposition rate was reduced by ~85%. A3B, A3C, and A3F had a lower but still significant inhibitory activity repressing transposition by ~74–77%, whereas A3G and A3H had no substantial effect.

So far, studies on the function of A3A are rare. It has been shown that A3A does not exhibit any activity against HIV-1, primate foamy virus, or murine leukemia virus (35, 37, 40, 41). In the course of the preparation of this manuscript, a report from Bogerd *et al.* (67) was published showing that A3A and A3B are potent inhibitors of LTR-retrotransposon function in human cells; this was the first time that any function has been ascribed to APOBEC3A. In our study, we present evidence that A3A is also highly active against the human non-LTR retrotransposon LINE-1. A3G and A3F are more thoroughly characterized factors and exhibit strong activity against *vif*-deleted HIV-1, hepatitis B virus, and primate foamy virus (34, 35, 37, 41, 49). As we observed an effect of A3F, but not of A3G, against L1 retrotransposition, our results suggest that these two factors possess different specificity, although they are believed to be

FIGURE 2. **Effect of APOBEC3 proteins on L1 retrotransposition rates.** *A*, Western blot analysis showing protein expression from APOBEC3 expression plasmids. HeLa cells were transfected with 2 μg of the respective APOBEC3 (A3) expression plasmids. Two days later, APOBEC3 protein levels were detected by anti-HA Western blot. Protein levels of tubulin (kDa) were analyzed as loading control. *B*, evaluation of cytotoxic effects of APOBEC3 proteins. HeLa cells were cotransfected with 0.5 μg of each APOBEC3 expression plasmid or parental plasmid pcDNA3.1/Zeo(+) (vector) and 0.5 μg of pcDNA3.1 conferring neomycin resistance. After 12 days of G418 selection, G418^r colonies were stained and counted. The number of G418^r colonies in the absence of APOBEC3 was set as 100%. Data are means ± S.D. for three independent experiments. *C*, relative L1 retrotransposition frequencies in the presence of APOBEC3 proteins. HeLa cells were cotransfected with 0.5 μg of both L1 reporter pJM101/$L1_{RP}$ and APOBEC3 expression plasmids. After 12 days of G418 selection, the number of G418^r colonies was determined. Retrotransposition rate in the presence of the empty expression plasmid (vector) was set as 100%. Data are means ± S.D. for six (A3A, A3G, A3H) or three (A3B, A3C, A3F) independent experiments. *D*, L1 retrotransposition frequencies were analyzed in the presence of APOBEC3A proteins with mutations in the putative deamination catalytic center (CCAA, E72A). Cotransfection and G418 selection were performed as described in *C*. Data are means ± S.D. for three independent experiments. *E*, titration of APOBEC3 proteins against human L1 retrotransposition. HeLa cells were cotransfected with a fixed amount of L1 reporter construct pJM101/$L1_{RP}$ and decreasing amounts of each APOBEC3 expression plasmid. The total amount of transfected plasmid DNA was held constant by adding empty parental APOBEC3 expression vector. After 12 days of G418 selection, the number of G418^r colonies was determined. Retrotransposition rate in the absence of APOBEC3 was set as 100%. One representative experiment out of three is shown.

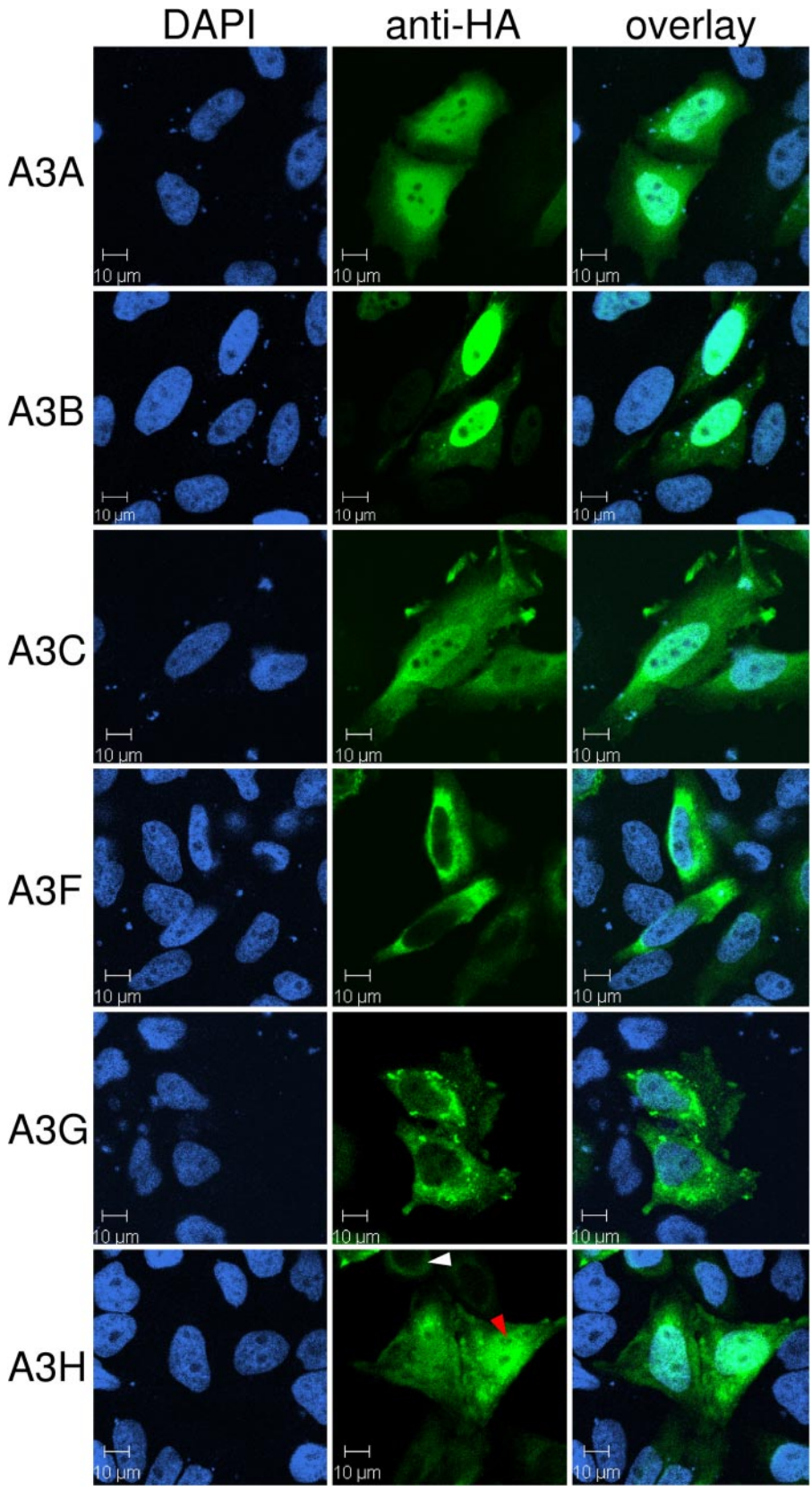


FIGURE 3. **Subcellular localization of the different APOBEC3 proteins.** Human HA-tagged APOBEC3 proteins were expressed in HeLa cells. To detect APOBEC3 proteins, immunofluorescence staining was performed with anti-HA antibodies. Nuclei were visualized by 4,6-diamidino-2-phenylindole (*DAPI*) staining. *White arrowhead,* cytoplasmic localization of A3H; *red arrowhead,* predominant nuclear localization of A3H.

coregulated (35). A3B and A3C, which do not affect HIV-1Δ*vif* but SIVΔ*vif* (54), were inhibiting L1 retrotransposition by ~77%. So far, no function has been identified for A3H, and we did not find any effect of A3H against L1 retrotransposition as well. The molecular structure separates this protein from the other APOBEC3 family members and indicates an exceptional position. Although the zinc coordination domains of all other human APOBEC3s belong to the so-called Z1 clade, APOBEC3H possesses a domain of the Z2 clade that phylogenetically separates this factor from the others (68). Taken together, APOBEC3 family members differ in their specificity, and data suggest that a major function of A3A is combating the replication of retrotransposons.

After A3G and A3F had been shown to deaminate viral DNA during reverse transcription of retroviruses (38, 39), retroelements have also been discussed as potential targets. However, most studies were focusing on A3G, the member of the APOBEC3 family discovered first. When Turelli *et al.* (59) examined the impact of A3G on human L1 retrotransposition, the authors did not observe an influence of A3G on the retrotransposition rate. These results are consistent with our data and were explained by the cytoplasmic localization of the A3G DNA deaminase, which bars the protein from acting on L1 reverse transcription taking place at the genomic target DNA. This hypothesis was supported by a recent publication that describes the inhibition of the retrotransposition of endogenous retroviruses by A3G (48). The latter publications suggest that the cytoplasmic A3G was only capable of inhibiting retroelements if their reverse transcription occurred in the cytoplasm. However, in the light of our localization studies this conclusion cannot be generalized. Whereas A3A, which had the strongest inhibitory effect, was predominantly localized to the nucleus, nuclear localization of the remain-

TABLE 1

Analysis of nucleotide substitutions in *de novo* L1 insertions

HeLa cells were cotransfected with pJM101/$L1_{RP}$ and different APOBEC3 expression plasmids. Single $G418^r$ clones were expanded under G418 selection, and *de novo* L1 insertions were sequenced. Number of *de novo* insertions, total number of analyzed nucleotides, and number and type of the detected substitutions are listed.

Overexpressed APOBEC3s	No. of *de novo* insertions	No. of analyzed nucleotides	No. of point mutations	Substitutions
A3A	17	15,569	1	C→A
A3B	7	6440	0	
A3C	8	7777	0	
A3G	9	8956	0	

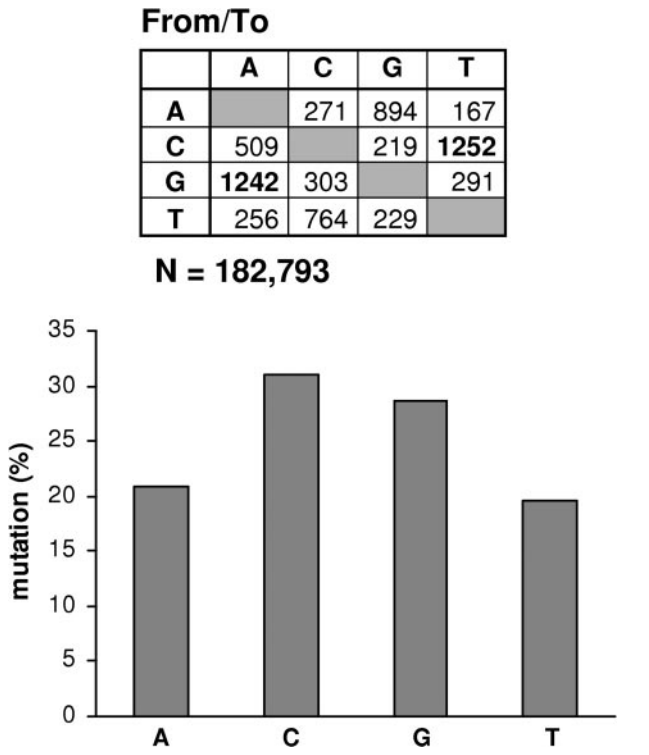


FIGURE 4. **Analysis of endogenous L1 sequences.** *Upper panel,* 30 pre-existing genomic full-length L1 sequences were extracted from a human L1 data base and aligned with the active $L1_{RP}$ element. Numbers of substitutions are depicted. *N,* total number of nucleotides analyzed. *Lower panel,* percentage of substitutions for each type of nucleotide. The total number of substitutions is defined as 100%.

ing APOBEC3 proteins did not always correlate with anti-retrotransposition activity. A3B, A3C, and A3F inhibited L1 retrotransposition to the same degree but exhibited completely different cellular localization patterns. Whereas A3B was localized to the nucleus, A3C was equally distributed, and A3F appeared exclusively in the cytoplasmic compartment. According to our findings, it seems rather unlikely that all APOBEC3 members exhibiting activity against L1 retrotransposition act in the nucleus.

To clarify whether DNA editing occurs during L1 reverse transcription, we sequenced parts of *de novo* L1 insertions, occurring in the presence of the different overexpressed APOBEC3 proteins. Unexpectedly, we found no evidence for editing of *de novo* L1 insertions. In addition, our analysis of endogenous L1 elements also did not reveal any tendency for G-to-A transitions. C-to-T and G-to-A transitions were observed at a

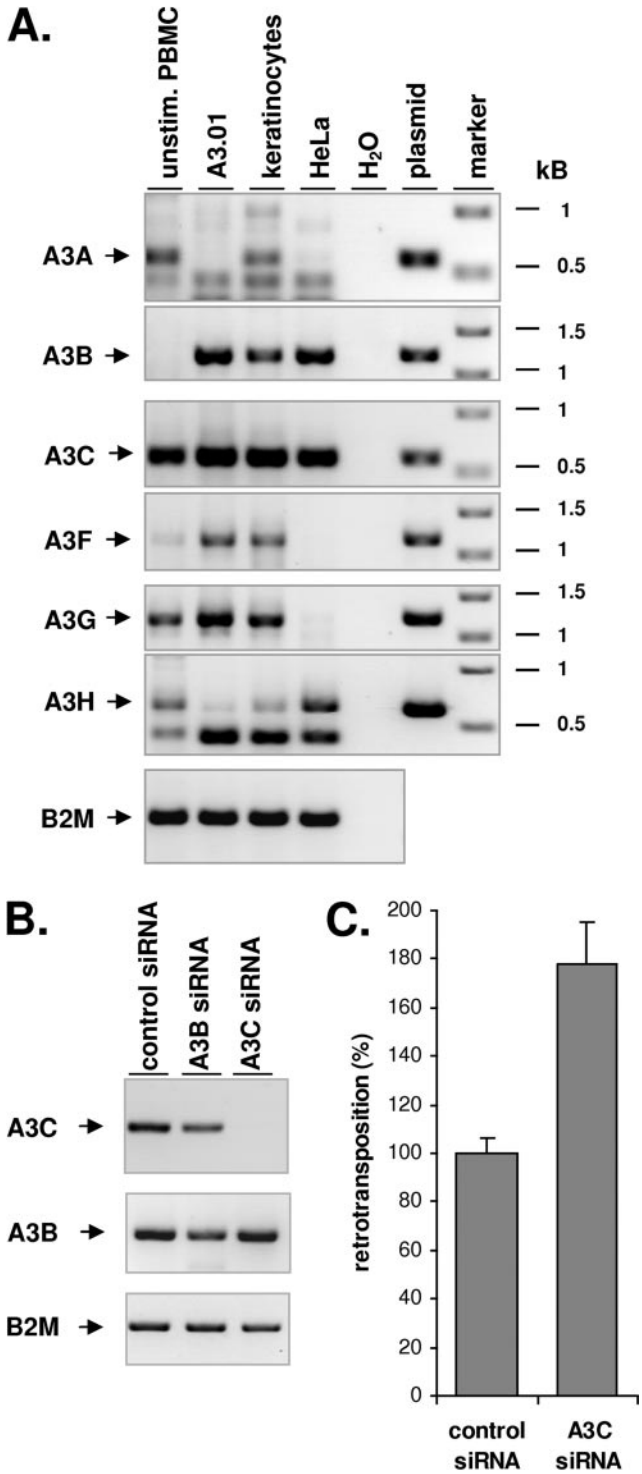


FIGURE 5. **Impact of endogenous APOBEC3s on L1 retrotransposition.** *A,* total RNA of different human cell lines was isolated and reverse-transcribed into cDNA. PCR with specific primers was performed in order to amplify A3A (600 bp), A3B (1203 bp), A3C (610 bp), A3F (1167 bp), A3G (1200 bp), and A3H (668 bp) sequences. As a control, β_2-microglobulin (*B2M*) RNA-specific RT-PCR was performed (704 bp). APOBEC3 expression plasmids were used as positive control templates. *B,* endogenous A3C and A3B RNA levels were analyzed by RT-PCR 2 days after transfection of siRNA directed against A3C or A3B or a nonspecific RNA (control siRNA) into HeLa cells. β_2-Microglobulin-specific RT-PCR served as control. *C,* HeLa cells were cotransfected with pJM101/$L1_{RP}$ and 300 ng of A3C-specific siRNA or control siRNA. After 12 days of G418 selection, cells were stained, and $G418^r$ colonies were counted. The number of $G418^r$ colonies in the presence of control siRNA was set as 100%. Data are means ± S.D. for three independent experiments.

higher frequency than other possible substitutions. The substitution pattern we observed for endogenous human L1 sequences is consistent with the one previously described for endogenous mouse L1 elements (48). As the frequency of G-to-A transitions is not exceeding the C-to-T substitution rate, the increased G-to-A transition frequency can probably be ascribed to cellular mechanisms, for example methyl-CpG deamination (64, 65).

Our observations are reminiscent of earlier reports proposing a second so far elusive antiviral activity for A3G, as the inhibition of hepatitis B virus and human T cell leukemia virus type 1 by A3G rarely results from G-to-A hypermutations (43, 44, 49, 50). Similarly, HIV-1 reverse transcripts formed in resistant resting human $CD4^+$ T cells did not show extensive cytidine deamination. Recently, the HIV-1 resistance in these resting T cells was linked to the expression of a low molecular mass complex of A3G (69), supporting the possibility of an unknown mechanism of A3G interference with HIV-1. Although inhibition of the murine LTR retrotransposon intracisternal A particle (IAP) by A3B and A3G was shown to be the result of G-to-A hypermutations (48, 67), inhibition of IAP by A3A was not associated with edited genomes (67). Therefore, it is still unknown whether A3A possesses a cytidine deaminase activity *in vivo*.

A single copy of the consensus zinc-coordinating region, the cytidine deaminase active site His-*X*-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys, is present in A3A, A3C, and A3H, whereas A3B, A3F and A3G carry two copies (70). Many studies showed the relevance of individual amino acids in the second catalytic domain of A3G for its activity against HIV-1Δ*vif,* which correlated with cytidine deamination activity in all (38, 39, 63, 71) but one study (72). We tested two mutants of A3A (E72A and CC101/106AA), carrying amino acid changes in the presumed catalytic domain. Both A3A mutants completely lost the ability to inhibit L1 retrotransposition, indicating a functional role of the putative catalytic domain. Bogerd *et al.* (67) recently described an inhibitory activity of A3A on IAPs, using a similar detection assay as we did. Interestingly, this study demonstrated no decrease of the inhibitory activity of A3A when disrupting the catalytic domain in the mutant S99A/P100A/C101A. However, both systems have in common that A3A affects the activity of either L1 or IAP without causing G-to-A hypermutations. The different activities of the A3A mutants might point to element-specific interactions or an unexpected functional diversity of the zinc-coordinating region.

Our data suggest that the inhibition of L1 *de novo* retrotransposition by APOBEC3s is mediated by a novel mechanism, not involving cytidine deamination of the L1 genome. Instead, active APOBEC3 proteins could disturb the generation of the cytoplasmic L1 RNP by sequestering either the RNA or proteins of this complex or by competitive binding. Alternatively, APOBEC3 could directly decrease the activity of the L1-encoded enzymes without interfering with RNP formation. Unlikely, but not formally ruled out, APOBEC3s could repress transcription, RNA export, or translation of L1-encoded proteins.

Very little is known about the regulation of L1 retrotransposition and the involvement of host-encoded factors. Because L1 retrotransposition is presumed to occur predominantly in germ cells and none of the currently identified L1 transcription factors is germ cell-specific, it is likely that there are more L1 regulating factors waiting to be discovered. The fact that A3A and A3F expression is suppressed in HeLa cells is consistent with the high retrotransposition rates of tagged L1 reporter elements in these cells. However, moderate repression of L1 retrotransposition in these cells is possibly mediated by the activity of endogenous A3B and A3C gene products, whose expression we detected by RT-PCR. At least for A3C we showed that knocking down expression by RNA interference resulted in an ~78% increase of the L1 retrotransposition rate. It is striking that transfection of the inactive A3A mutants also increased retrotransposition frequencies by ~40–70% as compared with the absence of transiently expressed APOBEC3 proteins. It can be hypothesized that the inactive A3A mutants relieve part of the repression by blocking the binding of endogenous A3C and/or A3B proteins to L1 compounds.

During the revision of this manuscript, two studies were published confirming our data on the inhibitory effects of APOBEC3 proteins on human LINE-1 retrotransposition (73, 74). Chen *et al.* (73) showed in agreement with our data that active site mutants of A3A lost their inhibitory effect against L1 in HeLa cells, and Stenglein and Harris (74) found that a catalytically inactive A3B mutant was still able to inhibit L1 retrotransposition in 293 cells.

Our study demonstrates that in addition to exogenous and endogenous retroviruses and pararetroviruses, the human non-LTR retrotransposon L1 is permissive to the restriction by APOBEC3 proteins. Although most if not all APOBEC3 family members act by cytidine deamination, L1 seems to be inhibited by a different pathway, which might also contribute to the activity of APOBEC3 proteins against other retroelements.

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