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All APOBEC3 family proteins differentially inhibit LINE-1 retrotransposition

Masanobu Kinomoto[1], Takayuki Kanno[1], Mari Shimura[2], Yukihito Ishizaka[2], Asato Kojima[1], Takeshi Kurata[1], Tetsutaro Sata[1] and Kenzo Tokunaga[1,*]

[1]Department of Pathology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan and
[2]Department of Intractable Diseases, International Medical Center of Japan, Tokyo, 162-8655, Japan

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ABSTRACT

Approximately 17% of the human genome is comprised of long interspersed nuclear element 1 (LINE-1, L1) non-LTR retrotransposons. L1 retrotransposition is known to be the cause of several genetic diseases, such as hemophilia A, Duchene muscular dystrophy, and so on. The L1 retroelements are also able to cause colon cancer, suggesting that L1 transposition could occur not only in germ cells, but also in somatic cells if innate immunity would not function appropriately. The mechanisms of L1 transposition restriction in the normal cells, however, are not fully defined. We here show that antiretroviral innate proteins, human APOBEC3 (hA3) family members, from hA3A to hA3H, differentially reduce the level of L1 retrotransposition that does not correlate either with antiviral activity against Vif-deficient HIV-1 and murine leukemia virus, or with patterns of subcellular localization. Importantly, hA3G protein inhibits L1 retrotransposition, in striking contrast to the recent reports. Inhibitory effect of hA3 family members on L1 transposition might not be due to deaminase activity, but due to novel mechanism(s). Thus, we conclude that all hA3 proteins act to differentially suppress uncontrolled transposition of L1 elements.

INTRODUCTION

Human APOBEC3G (hA3G) is known to be a powerful innate antiretroviral factor, which can suppress Vif (virion infectivity factor)-deficient human immunodeficiency virus type 1 (HIV-1) infection by deaminating viral minus-strand DNA during reverse transcription, resulting in G-to-A hypermutation (1–4). This cytidine deaminase targets not only on retroviruses, such as simian immunodeficiency virus (5–8), primate foamy virus (9,10), human T-cell leukemia virus type I (11) and murine leukemia virus (MuLV (2,7,12)), but also on Hepatitis B virus (13,14) which has a reverse transcription step. Recent findings have revealed that hA3G also restricts transposition of murine MusD and intracisternal A-particle long terminal repeat (LTR) retrotransposons (15). hA3G is one of the APOBEC3 (A3) family containing A3A to A3H in human. Vertebrates from fish to birds do not encode any of A3 proteins (16), while mammals before primates encode a single or two to three A3 proteins (17). Primates have acquired seven tandem A3 genes throughout 33 million years of evolution (18), implying the ancient history of battles between primates and retroelements.

In human genome, retrotransposons have accumulated up to ~42%, divided into two classes, which are non-LTR, and LTR retrotransposons (19). Non-LTR types are subdivided into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). The most common human LINE, called LINE-1 (L1), accounts for ~17% of the human genome, corresponding to 500 000 copies, out of which 100 copies are known to be full-length and transposition-competent (19–21). This non-LTR retrotransposon is 6-kb long, and consists of two open reading frames (ORFs) called ORF1 and ORF2 (22). ORF1 encodes an RNA-binding protein ORF1p (23), and ORF2 encodes an enzymatic protein ORF2p like Pol of retroviruses, carrying an endonuclease (24) and a reverse-transcriptase (25,26), both of which are enzymatically active. Importantly, L1 elements can cause several genetic diseases by *de novo* L1 insertion at dispersed positions in the germinal chromosomes, such as the Factor VIII gene in patients with hemophilia A (27,28), the Factor IX gene in patients with hemophilia B (29), the dystrophin gene in patients with Duchene muscular dystrophy (30,31), and with X-linked dilated cardiomyopathy (32), the β-globin gene in patients with β-thalassaemia (33), the CYBB gene in patients with chronic granulomatous disease (34), and the RP2 gene in patients with type-2 retinitis pigmentosa (35). The fact that colon cancer is caused by L1 insertion into the APC genes of somatic chromosomes (36), indicates that, L1 could be

*To whom correspondence should be addressed. Tel: +81 3 5285 1111; Fax:+81 3 5285 1189; Email: tokunaga@nih.go.jp

active not only in germ cells, but also in somatic cells if innate protection system would not work appropriately. The mechanism by which L1 transpositions are restricted in the normal cells, however, remains unknown.

Here we examined whether antiretroviral innate proteins, human A3 (hA3) family members are able to show inhibitory effects on L1 retrotransposon. hA3 family, in particular, hA3A, hA3B, hA3F and hA3G protein expressions strongly decrease the transposition level of L1 elements that does not correlate with either antiretroviral activity or patterns of subcellular localization. Results of DNA sequencing argue that inhibitory effect of hA3 family on L1 transposition might be independent of deaminase activity. We conclude that all hA3 proteins are able to differentially suppress uncontrolled transposition of L1 retroelements.

MATERIALS AND METHODS

DNA constructs

The Vif-deficient HIV-1 proviral indicator construct pNL-Luc-F(-)E(-), and the L1 indicator constructs $pL1_{RP}$-EGFP (kindly provided by E.T. Luning Prak) and pCEP4/L1mneoI/ColE1 (kindly provided by N. Gilbert) have previously been described elsewhere (37–39). Total RNA was isolated from H9 cells by using RNAqueous Kit (Ambion), and was subjected to reverse transcription, followed by amplification with oligonucleotides 5′-GGG GTA CCA TGA ATC CAC AGA TCA GAA ATC CG-3′/5′-ATT CTC GAG CTG GAG AAT CTC CCG TAG CCT TC-3′, 5′-GGG GTA CCA TGA AGC CTC ACT TCA GAA AC-3′/5′-CCG CTC GAG AAT CTC CTG CAG CTT GC-3′, 5′-GGG GTA CCA TGA AGC CTC ACT TCA GAA ACA-3′/5′-CCG CTC GAG GTT TTC CTG ATT CTG GAG AAT-3′ and 5′-GGG GTA CCA TGG CTC TGT TAA CAG CCG AAA CAT TCC G-3′/5′-CCG CTC GAG GGA CTG CTT TAT CCT CTC AAG-3′, producing hA3DE, hA3F, hA3G or hA3H fragments, respectively. Total RNA isolated from HeLa cells was subjected to RT-PCR amplification of hA3A, hA3B or hA3C genes using oligonucleotides 5′-ATG GTA CCA TGG AAG CCA GCC CAG CAT C-3′/5′-CAT CTC GAG GTT TCC CTG ATT CTG GAG AAT GG-3′, 5′-GGG GTA CCA TGA ATC CAC AGA TCA GAA ATC CG-3′/5′-CCG CTC GAG GTT TCC CTG ATT CTG GAG AAT GG-3′, 5′-GGG GTA CCA TGA ATC CAC AGA TCA GAA AC-3′/5′-ATT CTC GAG CTG GAG ACT CTC CCG TAG CCT TC-3′, respectively. Amplified hA3 fragments were KpnI/XhoI-digested and cloned into a modified mammalian expression plasmid pCAGGS carrying carboxy-terminal hemaggulutinin (HA)-tag. To generate an indicator retrovector construct pMSCVneo-Luc, a luciferase gene from pGL4.10[luc2] vector (Promega) was PCR-amplified by using oligonucleotides 5′-GGA ATT CGC CAT GGA AGA TGC CAA AAA CAT-3′/5′-AAT CTC GAG TTA CAC GGC GAT CTT GCC GCC C-3′, digested with EcoRI and XhoI restriction enzymes, and inserted into pMSCVneo (Clontech). All constructs were verified by DNA sequencing.

Cell maintenance, transfections and protein analyses

HeLa cells, 293T cells and HT1080 cells were maintained as described elsewhere (40,41). The human osteocarcinoma 143BTK- cells were kindly provided by J. Hayashi and cultured as previously described (42). 293T cells were transfected with HA-tagged hA3 plasmids by using FuGENE 6 transfection reagent (Roche) to confirm hA3 protein expressions. Cell extracts from transfected cells are subjected to western analysis using an anti-HA mouse monoclonal antibody (Sigma). HeLa cells were transfected with the same constructs by using Lipofectamine with Plus reagents (Invitrogen) to determine subcellular localization of hA3 proteins. Cells were fixed, permeabilized and incubated with an anti-HA monoclonal antibody and Alexa 488 conjugating anti-mouse IgG (Invitrogen) used for the first and second antibodies, respectively. Microscopic observation was performed by using FV-1000 confocal microscopy (Olympus).

Viral infectivity assay

To prepare MuLV or HIV-1 supernatants, 7×10^5 293T cells were cotransfected with 0.1 μg of hA3 expression plasmids and 0.1 μg of VSV-G expression plasmid pHIT/G (43), together with either 0.9 μg of pMSCVneo-Luc and 0.9 μg of retroviral packaging vector pVPack-GP (Stratagene), or 1 μg of pNL-Luc-F(-)E(-) by using FuGENE 6 and 0.8 μg of an empty vector. Sixteen hours later, cells were washed with phosphate-buffered saline, and then 2 ml of fresh complete medium was added. After 24 h, supernatants were harvested and treated with 37.5 U/ml DNase I (Roche) for 37°C for 30 min. HIV-1 supernatants were subjected to measure p24 antigen by HIV-1 p24-antigen capture enzyme linked immunosorbent assay (RETRO-TEK). To normalize MuLV supernatants, transfected cells were lysed in 100 μl of passive lysis buffer (Promega), and firefly luciferase activities which indicate transfection efficiencies were determined by Centro LB960 (Berthold). To determine the infectivity, 1×10^4 293T cells were incubated with 0.1 ng of p24 antigen of each HIV-1 or 100 μl of normalized MuLV supernatants. After 48 h, cells were lysed in 100 μl of lysis buffer, and firefly luciferase activities were determined as described above.

L1 retrotransposition assay

L1 retrotransposition assay was performed by cotransfection of 3.5×10^5 293T cells with 0.1 μg of the respective hA3 expression plasmids, 0.5 μg of EGFP-based L1 reporter vector $pL1_{RP}$-EGFP and 0.4 μg of an empty vector. Transfected cells were maintained by puromycin treatment (0.5 μg/ml) for 10 days. EGFP expression resulting from retrotransposition was verified by flow cytometry. Another L1 retrotransposition assay was performed by cotransfection of HeLa cells with equal amounts of the respective hA3 expression plasmids and neomycin-resistant (*neo*^{r})-based L1 reporter vector pCEP4/L1mneoI/ColE1, as previously described (15). After 72 h, cells were trypsinized, re-seeded onto 100 mm dishes for G418 (1 mg/ml) selection and maintained. Fourteen days after selection, resultant G418-resistant

($G418^R$) colonies were fixed, stained with crystal violet (Merck), and counted.

Confirmation of *de novo* L1 copy number

Presumed copy number of retrotransposed L1 elements was determined by real-time PCR targeting the EGFP gene. Ten days after transfection as described above, total cellular DNA was extracted from 293T cells by using DNeasy kit (Qiagen) which is able to recover both chromosomal and episomal DNA (according to the manufacture's manual). One hundred nanograms of each DNA was subjected to real-time PCR detection using oligonucleotides 5′-GAA GAA CGG CAT CAA GGT GAA C-3′/5′-GGT GCT CAG GTA GTG GTT GTC-3′ and a probe 6-carboxyfluorescein (FAM)-5′-AGC GTG CAG CTC GCC GAC CA-3′- black-hole quencher 1 (BHQ1). Real-time PCR was performed by using ABI 7900HT (ABI). L1 DNA levels are presented as copies per 10^4 cells.

Determination of endogenous levels of L1 and hA3 mRNA expressions in cell lines

Real-time RT-PCR was performed to quantify the endogenous level of L1 and hA3 mRNA expression (and of glyceraldehyde-3-phosphate-dehydrogenase [GAPDH] mRNA expression as an internal control) in various cell types. Total RNA was extracted from peripheral blood lymphocytes (PBL; from two different donors), 293T, HeLa, HT1080 and 143BTK- cells, by using RNAqueous Kit, and then, treated with TURBO DNA-*free* (Ambion) according to the manufacturer's protocols. Specific oligonucleotides (o) and probes (p) used are as follows: L1, (o) 5′-GAG AAC AAA GAC ACC ACA TAC C-3′/5′-GGC ATT TAG TGC TAT AAA TTT CCC -3′ (p) FAM-5′-TCT CTG GGA CGC ATT CAA AGC AGT-3′-BHQ1; hA3A, (o) 5′-CAC ACA TAT TCA CTT CCA ACT TTA AC-3′/5′-GTC CAG GCG CTC CAC TTC-3′ (p) FAM-5′-CAT TGG AAG GCA TAA GAC CTA CCT GTG C-3′-BHQ1; hA3B, (o) 5′-CGC TAA AGG AGA TTC TCA GAT ACC-3′/5′-CAG GAC CCA GGT GCC ATT G-3′ (p) FAM-5′-CCA GGC GCT CCA CCT CAT AGC ACA-3′-BHQ1; hA3C, (o) 5′-CTG TGC TTC ACC GTG GAA GG-3′/5′-TGA CAA TGG GTC TCA GAA TCC AC-3′ (p) FAM-5′-AGC GCC GCT CAG TTG TCT CCT GGA-3′-BHQ1; hA3DE, (o) 5′-ACC GCA CGC TAA AGG AGA TTC-3′/5′-CGA CCA CAG GCT TTC AGT AGG-3′ (p) FAM-5′-ACC CGA TGG AGG CAA TGT ACC CAC AC-3′-BHQ1; hA3F, (o) 5′-TGC CTT GGT ACA AAT TCG ATG AC-3′/5′-AGT GGA AGT AGA ATA TGT GTG GAT AC-3′ (p) FAM-5′-ATT CCT GCA CCG CAC GCT AAA GGA GA-3′-BHQ1; hA3G, (o) 5′-GGC CGA GGA CCC GAA GG-3′/5′-TTC TGA CAC AGG CTG CGA AG-3′ (p) FAM-5′-CCC TGA CCA TCT TCG TTG CCC GCC-3′-BHQ1; hA3H, (o) 5′-GGC TCA CGA CCA TCT GAA CC-3′/5′-AGT CAG CAA ACT CTG GGA AGC-3′ (p) FAM-5′-CGC CTC CCG CCT GTA CTA CCA CTG G-3′-BHQ1; GAPDH (o) 5′-GAT GCT GGC GCT GAG TAC G-3′/5′-GCA GAG ATG ATG ACC CTT TTG G-3′ (p) FAM-5′-TGG AGT CCA CTG GCG TCT TCA CCA CC-3′-BHQ1. Oligonucleotide specificity for each hA3 sequence was confirmed by simultaneously using all hA3 plasmids which gave no non-specific signals. L1 and hA3 mRNA levels were normalized with GAPDH mRNA levels.

Sequencing of *de novo* L1 genes

Total cellular DNAs were isolated by using DNeasy from the cells at 2 and 6 days after cotransfection of $pL1_{RP}$-EGFP with either an empty vector or hA3 constructs. Intronless EGFP DNAs were nested-PCR-amplified by using PfxUltima (Invitrogen) and cloned into pCR-Blunt vector using Zero Blunt® PCR Cloning Kit (Invitrogen). Sequencing was performed by using ABI3130 (ABI).

RESULTS AND DISCUSSION

We first examined if L1 is endogenously expressed in primary and established cells. To do this, we performed real-time RT-PCR targeting ORF1 gene, using total RNA derived from two primary lymphocytes from different donors and from four different established cell lines. As shown in Figure 1, although the expression levels vary among the cells tested, we found that all the cells express L1 mRNA at the level of 10–100 copies/cell. The results strongly emphasize that, even though L1 is expressed in normal cells, its reverse transcription and integration should be blocked by some innate protection system. We therefore hypothesized that antiretroviral innate proteins, hA3 family, might be effective on L1 retrotransposition as well as on that of mouse retroelements (15).

To test this hypothesis, we created HA-tagged hA3 expression plasmids, using cDNA reverse-transcribed from total RNA of H9 cells, or HeLa cells. Protein expressions in the cells transfected with each plasmid were confirmed by immunoblotting using anti-HA antibodies (Figure 2A). Using these plasmids, subcellular localization

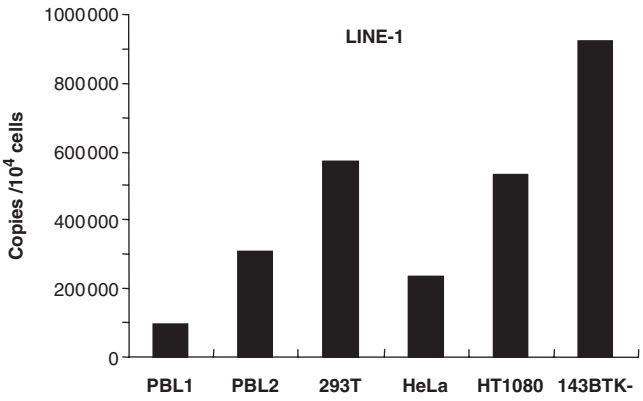

Figure 1. Endogenous expression of LINE-1 in primary and established cells. LINE-1 mRNA copy numbers in PBL (two different donors), 293T, HeLa, HT1080 and 143BTK- cells were determined by real-time RT-PCR using total RNAs isolated from those cells. L1 mRNA levels were normalized with GAPDH mRNA levels, and are presented as copies per 10^4 cells.

of hA3 proteins was determined by immunofluorescence microscopy. As shown in Figure 2B, results were consistent with the molecular size of each protein, i.e. ~46 kDa proteins, which are larger than nuclear pore exclusion limit (44), hA3DE, hA3F and hA3G proteins, localized into the cytoplasm, while ~23 kDa proteins, which are smaller than the limit, hA3A, hA3C and hA3H, passively diffused into the nucleus. Although a larger protein, hA3B was exceptionally found in the nucleus, this localization is consistent with the recent report that hA3B harbors a nuclear localization signal (45).

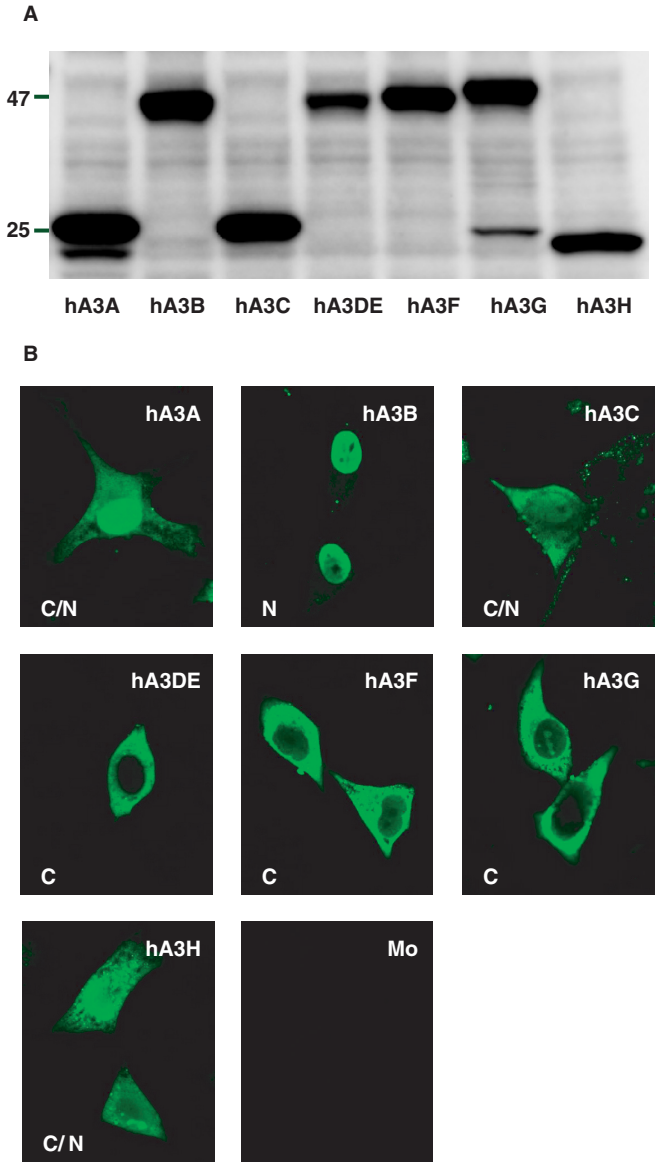

Figure 2. Western analysis and subcellular localization of the hA3 family proteins. (**A**) Western blot analysis was performed by using extracts from 293T cells transfected with HA-tagged hA3 expression plasmids. Antibodies specific for HA were used. (**B**) To analyze subcellular localization of hA3 proteins, immunofluorescence microscopy was performed by using HeLa cells transfected with the HA-tagged hA3 expression plasmids. An anti-HA monoclonal antibody and Alexa 488 conjugating anti-mouse IgG were used for the first antibody and for the second antibody, respectively. C, N or C/N in the pictures indicates cytoplasmic, nuclear or both localizations, respectively.

We next examined inhibitory effects of hA3 family proteins on Vif-deficient HIV-1 and MuLV. Using virions from the cells cotransfected with each virus construct and hA3 expression plasmids, we determined infectivity reduction by hA3 proteins. In accordance with the previous reports (2,3,39,46–51), hA3B and hA3F as well as hA3G revealed robust inhibitory activity on HIV-1, while hA3C and hA3DE showed partial activity (Figure 3A). MuLV infectivity was reduced by hA3B, hA3C and hA3G protein expressions (Figure 3B), as previously reported (2,49,50). Therefore, with respect to the antiretroviral activity, we here confirmed known phenotypes of all hA3 family members as observed in these experiments.

To determine if hA3 family members are able to inhibit L1 retrotransposition, we first employed EGFP-based retrotransposition assay (38). In this assay system, we utilized a L1 clone DNA carrying a reverse-oriented EGFP gene separated by a gamma-globin intron, and also carrying a puromycin resistance gene which allows selection of transfected cells. After transfection of the cells with this construct, followed by puromycin selection, EGFP with L1 is transcribed, spliced, reverse-transcribed and integrated. Then, the EGFP integrated with L1 is driven by CMV promoter and expressed. In other words, EGFP-positive cells are the cells in which L1

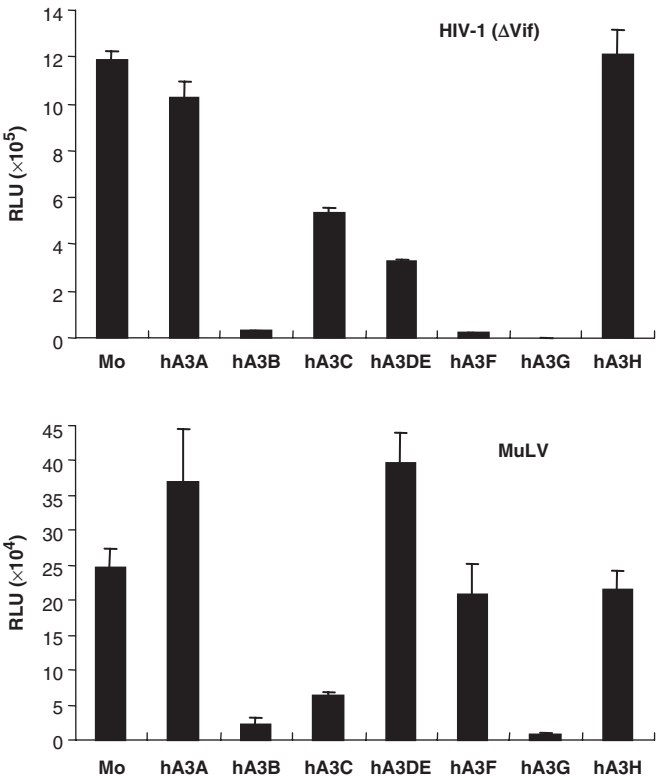

Figure 3. Inhibitory effect of hA3 family proteins on retroviruses. 293T cells were cotransfected with VSV-G and hA3 expression plasmids, together with a luciferase-based HIV-1 or MuLV construct. After 48 h, each viral supernatant was harvested. Normalized supernatants were incubated with 293T cells for additional 48 h. Cells were then lysed and subjected to luciferase assay. Data shown are mean ± SD. RLU: relative light units.

retrotransposition successfully occurred. EGFP expression in cells cotransfected with each hA3 plasmid and the L1-EGFP vector were visualized by microscopy (Figure 4A), and quantitatively analyzed by flow cytometry (Figure 4B). Without coexpressions of hA3 proteins, L1 transposition normally occurred at the level shown in the upper left panel of Figure 4. In contrast, coexpressions of any of hA3 proteins differentially inhibited L1 retrotransposition, and in particular, hA3A, hA3B, hA3F and hA3G expressions strongly decreased the transposition level of L1 elements to 0.9, 14.0, 15.6 and 10.2%, respectively. These activities against L1 did not correlate with their patterns of subcellular localization (Figure 2B) and inhibitory activities on retroviruses (Figure 3A and B). In this assay, we observed no cytotoxic effect of A3 proteins (data not shown) as previously reported (52,53).

To assess reproducibility of this experiment, we next performed a different type of retrotransposition assay, using L1 vector which carries *neo*^{r} gene (37) instead of the EGFP gene. In this assay, after neomycin selection following transfection, we were able to quantify the retrotransposition level, simply by counting G418^r colonies. As shown in Figure 5A and B, this assay gave results equivalent to that obtained with EGFP-based assay. Thus, on the basis of the data presented here, we conclude that all hA3 proteins act to differentially suppress uncontrolled L1 retrotransposition. Importantly, in striking contrast to the recent reports (45,53–56), we observed the inhibitory effect of hA3G proteins on retrotransposition in both assays (Figures 4 and 5).

To confirm that hA3G is indeed effective on retrotransposition inhibition, we next measured presumed copy number of retrotransposed L1 elements by performing real-time PCR using total DNA which was isolated from the cells used in Figure 4. To exclude detection of genomic DNA, we targeted intronless EGFP DNA which is detectable only in spliced and reverse-transcribed L1 elements carrying the EGFP gene. As shown in Figure 6, results obtained with this assay were fully consistent with those observed in flow cytometry analysis, and in neo-resistance assay. We therefore conclude that hA3G is indeed able to inhibit L1 retrotransposition as well as hA3A, hA3B and hA3F. Importantly, the fact that results shown in Figure 6 were obtained by detecting not only integrants, but also reverse transcripts, and are

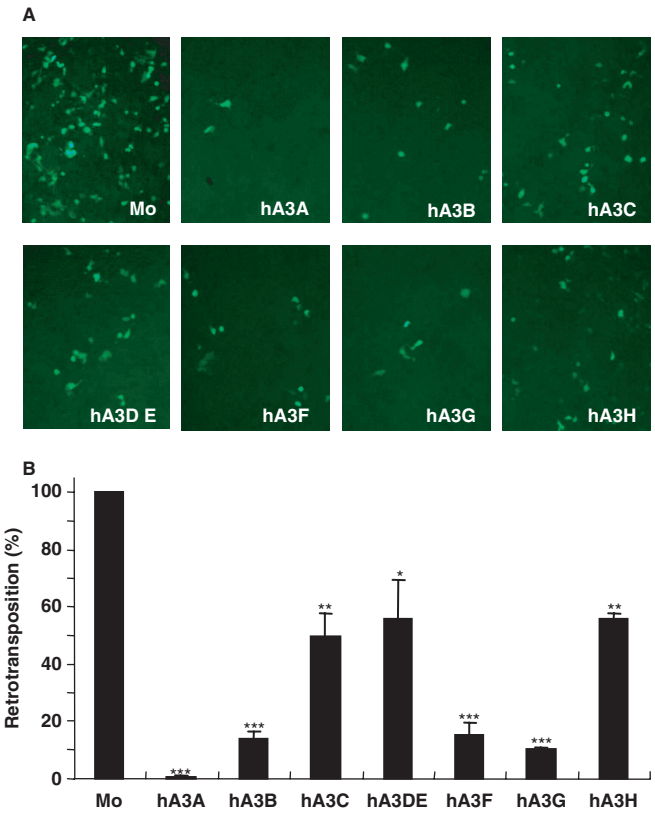

Figure 4. EGFP-based retrotransposition assay. 293T cells were cotransfected with the EGFP-based L1 retrotransposon indicator construct pL1$_{RP}$-EGFP and hA3 expression plasmids. After 48 h, cells were subjected to puromycin (0.5 μg/ml) selection. After 8 days of puromycin selection, cells were observed by fluorescence microscopy (**A**) and subjected to flow cytometry analysis (**B**). Retrotransposition level in the absence of hA3 proteins was set as 100%. Data shown are mean ± SD; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *t*-test.

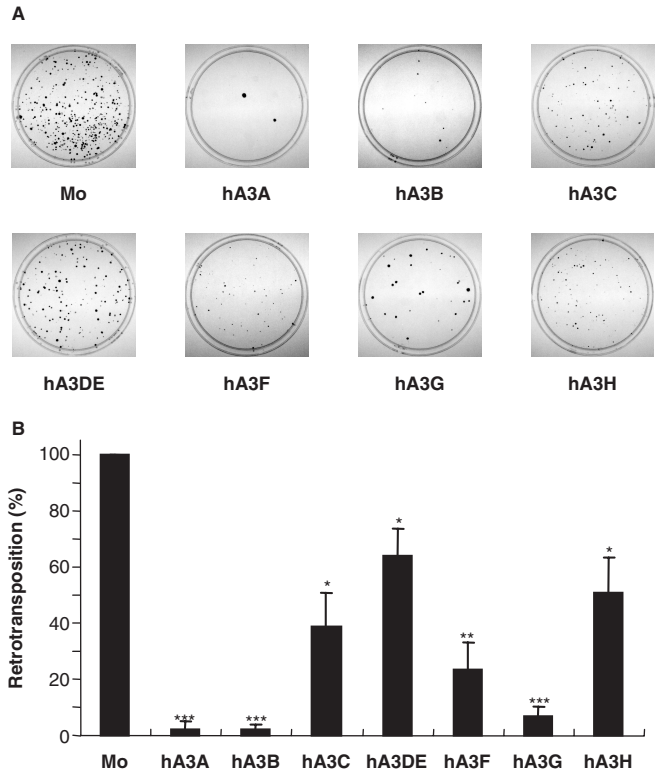

Figure 5. *neo*^{r}-based retrotransposition assay. HeLa cells were cotransfected with pCEP4/L1mneoI/ColE1 and the respective hA3 expression plasmids. Seventy-two hours later, cells were trypsinized, re-seeded onto 100 mm dishes, and subjected to G418 (1 mg/ml) selection. Fourteen days after selection, resultant G418^R colonies fixed, stained with crystal violet (**A**), and counted to determine the level of L1 retrotransposition (**B**). Transposition level in the absence of hA3 proteins was set as 100%. Data shown are mean ± SD; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *t*-test.

similar to those observed in Figure 4 in which levels of EGFP expression represent L1 integrants, suggest that hA3 proteins at least hA3A and hA3G suppress *de novo* L1 DNA synthesis, but not integration.

To examine whether cytidine deamination would explain the mechanism by which the retrotransposition is inhibited, we sequenced L1 elements resulting from retrotransposition which was reduced by hA3G protein. We targeted intronless reverse-oriented EGFP genes for the reason given above. Surprisingly, we observed only one C-to-T mutation (G-to-A mutation on plus-strand DNA) out of 4200 bp nucleotide sequences (Figure 7). As a control experiment, we analyzed deaminase activity of hA3G on Δvif HIV-1 reverse transcripts by sequencing the 3′ end of *env* genes in infected cells. In contrast to Figure 7, G-to-A mutations were found in 367 out of 5900 bp in total (unpublished data). In other hA3 protein expressions, we were not able to find any mutation (0 out of 25 200 bp, data not shown). These results suggest the possibility that inhibitory effect of hA3G on L1 retrotransposition might be independent of its deaminase activity as previously observed in studies of antiviral phenotypes (11,13,57), but this needs to be elucidated with further experiments using mutant hA3G, such as catalytically inactive mutants.

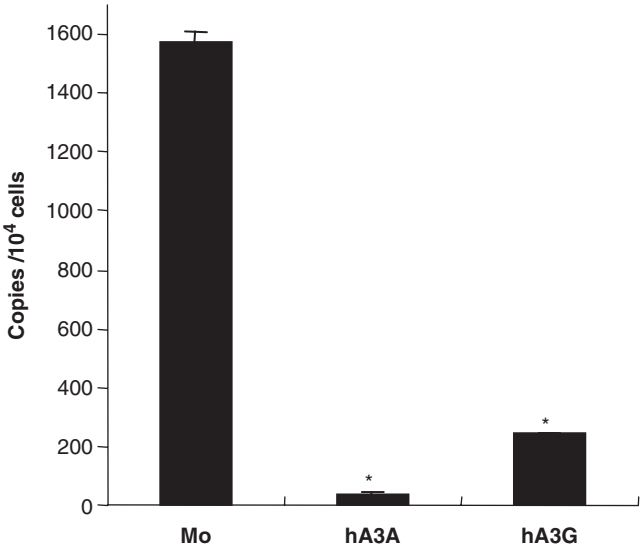

Figure 6. Real-time PCR targeting spliced EGFP genes. Total cellular DNA was extracted from the cells used in Figure 4 and subjected to real-time PCR analysis. Dual labeled probe was designed for the detection of spliced EGFP. Mock, hA3A and hA3G were shown as representatives. Data shown are mean ± SD; $^{*}P < 0.001$, *t*-test.

Finally, we performed real-time RT-PCR analysis to determine endogenous expression levels of hA3 proteins in various types of cells. As shown in Figure 8, we found that the endogenous levels of hA3 expressions vary depending on the cell types. Especially, some cell types abundantly coexpress hA3B and hA3G proteins which are able to effectively suppress L1 transposition. Therefore, the different results in other studies might be due to the different cells used.

In this study, we first presented evidence showing that primary and established cells endogenously express L1 mRNA at the level of 10–100 copies/cell. Although

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Reference   1 :TGTTCACC GGGGTGGTGCCCATC CTGGTCGAG CTGGACGGCGACGTA AACGGCCAC AAGTTCAGCGTGTCC GGCGAGGGC GAGGGCGATGCCACC TACGGCAAG CTGACCCTGAAGTTCATCTGCACC ACCGGCAAGCTGCC CGTGCCCT 150
A3G-d2-#1   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d2-#2   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d2-#3   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d2-#4   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d2-#5   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d2-#6   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d2-#7   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#1   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#2   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#3   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#4   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#5   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#6   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
A3G-d6-#7   1 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 150
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Reference 151 :GGCCCACC CTCGTGACCACCCTG ACCTACGGC GTGCAGTGCTTCAGC CGCTACCCC GACCACATGAAGCAG CACGACTTC TTCAAGTCCGCCATG CCCGAAGGC TACGTCCAGGAGCGCACCATCTTC TTCAAGGACGACGG CAACTACA 300
A3G-d2-#1 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d2-#2 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d2-#3 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d2-#4 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d2-#5 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d2-#6 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d2-#7 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#1 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#2 151 :.........T. .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#3 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#4 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#5 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#6 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
A3G-d6-#7 151 :.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 300
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Figure 7. Sequence analysis of L1 reverse transcripts. Total DNA was extracted from 293T cells cotransfected with $pL1_{RP}$-EGFP and hA3 expression plasmid, at 2 and 6 days after transfection. Reverse transcribed EGFP genes were PCR-amplified and inserted into the cloning vector. Alignment of partial sequences of EGFP genes in hA3G-cotransfected cells is shown.

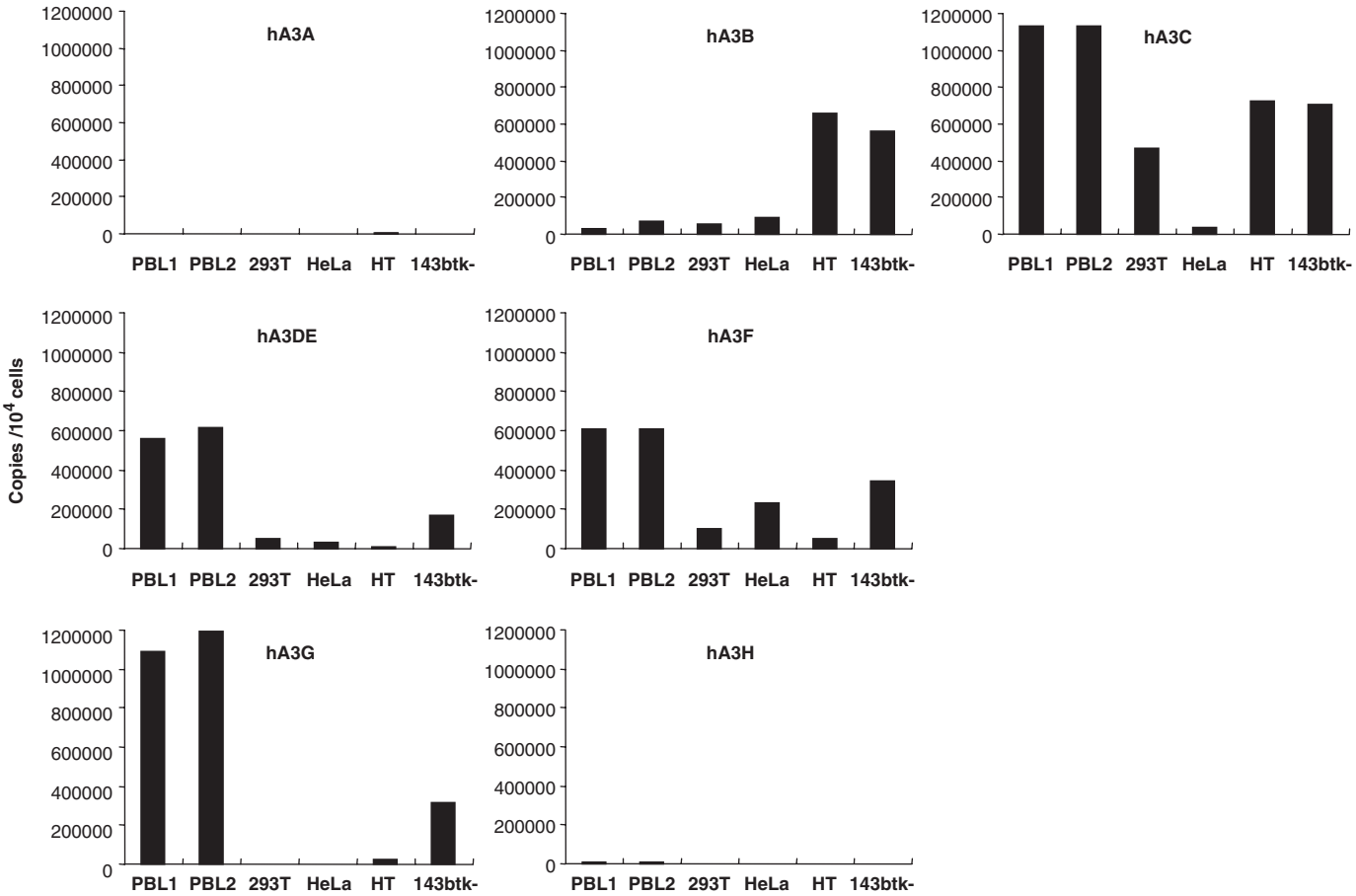

Figure 8. Endogenous expressions of hA3 family members in primary and established cells. Total RNAs were extracted from PBL (from two different donors), 293T, HeLa, HT1080 (HT) and 143BTK- cells, and subjected to real-time RT-PCR to determine the copy numbers of the respective hA3 mRNAs. Levels of hA3 mRNA were normalized with GAPDH mRNA levels, and are presented as copies per 10^4 cells.

we were unable to directly demonstrate endogenous expressions of L1 proteins *per se*, many, if not most, of L1 retroelements must be encoded from mRNA detected in our experiments, and should be sensitive to some innate immunity defenses. Otherwise, uncontrolled L1 transposition becomes obviously deleterious to the cells. Based on the hypothesis that hA3 family proteins of human innate antiretroviral factors might be the key to understanding how transposition of L1 retroelements is naturally inhibited in the cells, we preformed two different types of established retrotransposition assays using all hA3 (A, B, C, DE, F, G and H) expression plasmids. Results obtained in these assays revealed differential inhibitory effect of all hA3 family member proteins on L1 retrotransposition. However, we could not observe any correlation between the levels of L1 transposition inhibition by hA3 and either their subcellular localization or antiviral activities against retroviruses.

An intriguing finding in this study was that hA3G was also identified as an intrinsic inhibitor of L1 retrotransposition. This finding was indeed reproducible in two different retrotransposition assays, and a real-time PCR analysis. Sequence analysis, however, revealed no DNA deamination effect of hA3G on *de novo* L1 retrotransposons, strongly suggesting that cytidine deaminase hA3G inhibits L1 transposition by novel mechanism(s). Although we cannot rule out that hA3G might deaminate L1 reverse transcripts which could result in rapid degradation by cellular enzymes such as uracil DNA glycosylase and would be therefore undetectable, hA3G protein has been indeed found to have dual inhibitory effects on murine endogenous retroviruses, which are G-to-A editing and reduction of viral cDNA yields (58). Besides, antiretroviral function of hA3G has been demonstrated to be dissociated from its deaminase activity because deaminase mutants of hA3G still retained antiviral activity (59–61). Taken together, it is likely that hA3G could prevent L1 DNA synthesis *per se*. This still needs to be elucidated.

Cytoplasmic expression of hA3G protein, as observed in Figure 2B, has been thought to cause inaccessibility of this protein to L1 reverse transcripts which are synthesized in the nucleus, leading to the lack of inhibitory activity of hA3G on L1 transposition (45,56). Yet, another cytoplasmic hA3 family protein hA3F is reported to be also active on inhibition of L1 transposition (53,54), consistent with that observed in our study. This discrepancy might be explained by the mechanism of L1 replication as follows: L1 DNA synthesis in the nucleus is based on 'target-primed reverse transcription' in which reverse transcripts

are generated at L1 endonuclease-nicked chromosomal DNA using polyadenylated L1 RNA as a template (62). Importantly, at an early phase of replication before target-primed reverse transcription, L1 forms a ribonucleoprotein complex as a retrotransposition intermediate by associating its own encoding RNA with ORF1p and ORF2p in the cytoplasm (63–66). At this step, cytoplasmic hA3G protein as well as hA3F protein might be able to find a critical, if narrow, window of opportunity for access to the L1 RNA complexed with ORF1p and ORF2p, since hA3G is able to bind RNA nonspecifically (67). If this is the case, hA3G (or hA3F) protein might be able to enter the nucleus with the ribonucleoprotein complex in which ORF2p harbors a putative nuclear localization signal (68,69), and inhibit L1 reverse transcription effectively, by physically blocking the access of the ORF2p to the nicked chromosomal DNA, or by hindering the movement of the reverse transcriptase of ORF2p on a template L1 RNA.

Turelli *et al.* (56) originally reported that hA3G does not affect L1 retrotransposition in the EGFP-based retrotransposition assay which we also utilized in this article. The cells used in their experiments, 143BTK- osteocarcinoma cells, were found in our study to endogenously coexpress high level of hA3B and hA3G (Figure 8) which are able to effectively inhibit LINE-1 transposition. It seems likely that coexpression of hA3B and hA3G proteins might reduce the basal level of L1 retrotransposition. In fact, 143BTK- cells transfected with the $pL1_{RP}$-EGFP construct in our study showed only faint EGFP signals (data not shown) compared with those in 293T cells, although transfection efficiencies were equivalent in both the cells. Besides, reflecting phenotypes of cells expressing hA3B and hA3G proteins, 143BTK- cells transfected with an HIV-1 proviral construct with or without an intact *vif* gene produced ~50-fold less infectious virions, than did 293T cells (unpublished data). These virions showed no effect of Vif protein on infectivity rescue, just as described by Turelli *et al.* (56). This could be due to that hA3B which can be incorporated into virions as well as hA3G (48) might be more effective on inhibition of infection than does hA3G, leading to the lack of Vif-negative phenotypes.

The cell-type preference of hA3 protein expressions, however, might not be able to account for the results in three other articles very recently published, showing inability of hA3G to inhibit L1 transposition by using *neo*^{r}-based L1 retrotransposition assay in HeLa cells (45,53,55), as used in our study. A possible explanation can be drawn from the fact that HeLa cells are highly divergent (70) in which expression pattern of hA3 proteins might differ depending on derivatives of this cell line. Overall, this preferential hA3 expression in a cell type-dependent manner as observed in this study [and also in a tissue-specific manner described in other reports (1,17,46,48,49,51,71)] might explain the difference of results obtained. It will be of interest to further examine whether coexpression of hA3 proteins are able to either additively or cooperatively inhibit L1 retrotransposition.

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