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Dual inhibitory effects of APOBEC family proteins on retrotransposition of mammalian endogenous retroviruses

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ABSTRACT

We demonstrated previously that the cytosine deaminase APOBEC3G inhibits retrotransposition of two active murine endogenous retroviruses, namely intracisternal A-particles (IAP) and MusD, in an *ex vivo* assay where retrotransposition was monitored by selection of *neo*-marked elements. Sequencing of the transposed copies further disclosed extensive editing, resulting in a high load of G-to-A mutations. Here, we asked whether this G-to-A editing was associated with an impact of APOBEC3G on viral cDNA yields. To this end, we used a specially designed quantitative PCR method to selectively measure the copy number of transposed retroelements, in the absence of G418 selection. We show that human APOBEC3G severely reduces the number of MusD and IAP transposed cDNA copies, with no effect on the level of the intermediate RNA transcripts. The magnitude of the decrease closely parallels that observed when transposed copies are assayed by selection of G418-resistant cells. Moreover, sequencing of transposed elements recovered by PCR without prior selection of the cells reveals high-level editing. Using this direct method with a series of cytosine deaminases, we further demonstrate a similar dual effect of African green monkey APOBE3G, human APOBEC3F and murine APOBEC3 on MusD retrotransposition, with a distinct extent and site specificity for each editing activity. Altogether the data demonstrate that cytosine deaminases have a protective effect against endogenous retroviruses both by reducing viral cDNA levels and by introducing mutations in the transposed copies, thus inactivating them for subsequent rounds of retrotransposition. This dual, two-step effect likely participates in the efficient defense of the cell genome against invading endogenous retroelements.

INTRODUCTION

Since they entered living species millions of years ago, mobile genetic elements have profoundly shaped the genomes of all living organisms, via insertional mutagenesis and subsequent DNA rearrangements (1–7). In mammals, most mobile elements are retrotransposons, which account for >45% of the human and murine genomic DNA (8,9). They can be grouped along two main classes: the well-characterized long interspersed nuclear elements (LINE), which account for ~30% of the genome, and the long terminal repeat (LTR) retrotransposons (or endogenous retroviruses), which are closely related to infectious retroviruses. Most of these endogenous retroviruses, accounting for nearly 10% of the murine or human genome, have lost a replication capacity due to the accumulation of inactivating mutations, but several, including some murine intracisternal A-particles (IAP) and MusD sequences, are still mobile (10,11). These elements move by an intracellular copy and paste process involving an RNA intermediate, its reverse transcription and the integration of the resulting proviral DNA by the retrotransposon-encoded RNA-dependent DNA polymerase and integrase, respectively (Figure 1A).

In fact, the requirement for genome stability has led eukaryotes to adopt several strategies to restrict the proliferation of transposable elements. These include transcriptional silencing through DNA methylation and/or chromatin structure modifications (12–15), post-transcriptional silencing via RNA interference (16–21) and, as shown recently, mutational inactivation of the elements, in the course of their retrotransposition cycle, by cellular cytosine deaminases (22–24).

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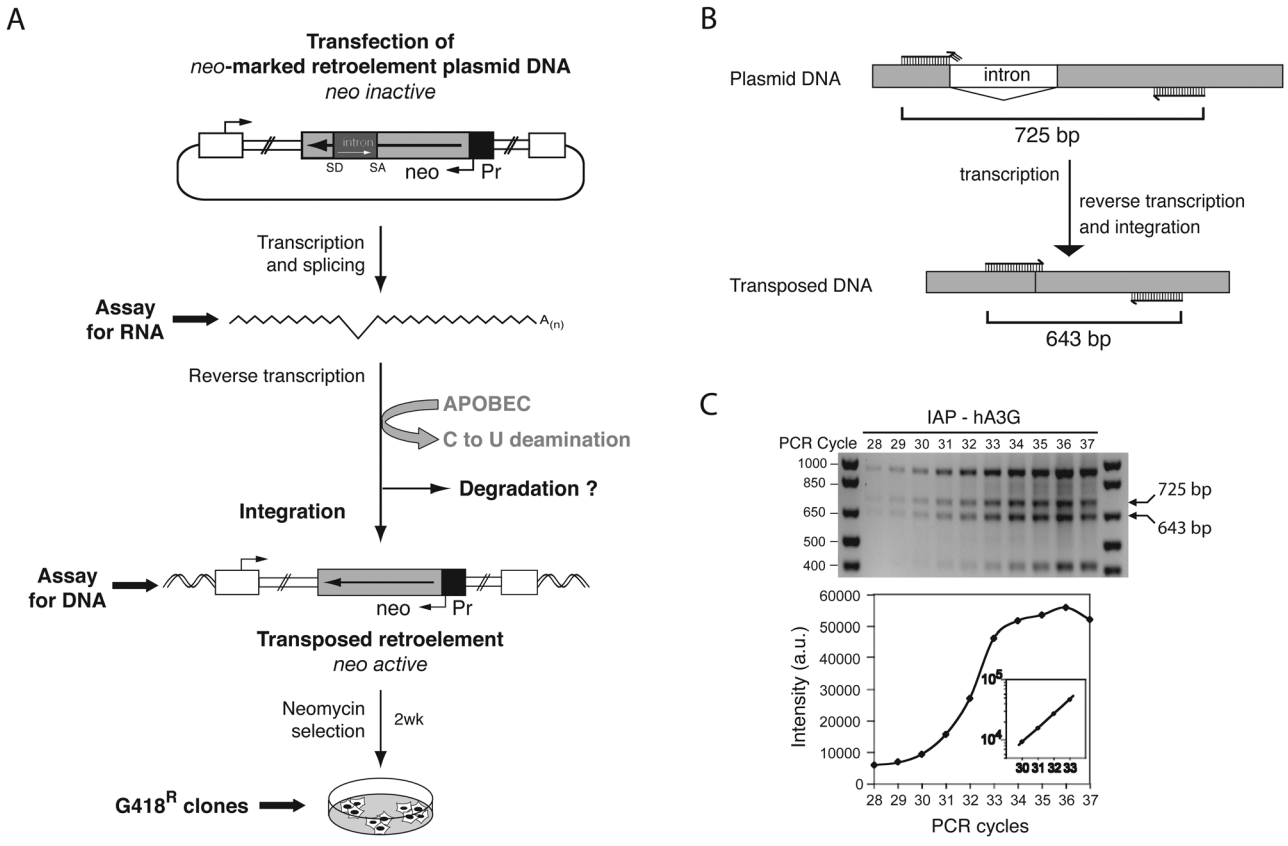

Figure 1. Rationale of the retrotransposition assay. (**A**) Schematic representation of the retrotransposition assay and experimental procedure for detection of retrotransposition. A retroelement marked with the *neo* reporter gene driven by its own promoter (Pr) and placed in backward orientation (gray boxes) is introduced by transfection into HeLa cells, together with or without an APOBEC expression vector. The *neo* gene is rendered inactive by the presence of a forward intron, which is spliced out of the RNA intermediate, resulting in a functional gene after reverse transcription and integration, allowing detection of retrotransposition. Cell RNA and DNA are extracted before G418 selection, as indicated. (**B**) Structure of the *neo* gene in the marked elements before and after retrotransposition, with the specific primers used for quantitative PCR analysis and the size of the expected PCR fragments indicated. (**C**) Quantitative PCR analysis of retrotransposed marked elements: (upper) ethidium bromide-stained agarose gel of the PCR products obtained using the primers shown in (B) and cellular DNA from Hela cells transfected with a marked IAP, with the 643 bp fragment specific for the retrotranscribed spliced copies and the 725 bp fragment for the non-transposed parental copies indicated; (lower) plot of the intensity of the 643 bp fragment against PCR cycle number; inset, linear fit using a semi-log scale, yielding an increment value per cycle of 1.75. The 725 bp band, as well as other spurious bands (e.g. ~0.4 and ~0.9 kb), were of variable intensity depending on the experiments, sometimes being hardly detectable (see text and Figure 2). The presence of such parasite bands clearly illustrates why standard real-time qPCR could not be used.

The cytosine deaminase family comprises numerous members including, in humans, AID, APOBEC1, APOBEC2, the APOBEC3A-G group and the recently discovered APOBEC4 (25–27). These enzymes can deaminate cytosine to uracil within DNA and/or RNA molecules, depending on the family member (28), and some of them have known physiological functions [reviewed in Refs. (29–31)]. APOBEC1, the first discovered APOBEC protein, is the catalytic component of the complex that edits the apolipoprotein B (APOB) mRNA by deaminating C^{6666} to U, thereby creating a premature stop codon and leading to the production of a truncated form of the apolipoprotein B lipid-transport protein in gastrointestinal tissues (32–34). AID (activation-induced deaminase) plays a role in the adaptative humoral immune system by initiating immunoglobulin gene diversification (somatic hypermutation, gene conversion and class switch recombination), but its precise mechanism of action, i.e. whether it acts through RNA editing or genomic DNA deamination, is still a matter of debate [for reviews see (35,36) and references therein]. APOBEC3G, the most extensively studied member of the APOBEC family, and APOBEC3F restrict retroviral infections: they convert cytosine to uracil in the nascent single-stranded retroviral cDNA during reverse transcription (37,38), resulting in G-to-A hypermutations in the proviral DNA plus strand (39–45). Although APOBEC3G acts during the early phase of the retroviral life cycle, antiviral activity is observed only if APOBEC3G is expressed in the cell in which the virion is produced (29,40,46–49). Primate lentiviruses counteract this anti-retroviral activity by expressing the retroviral 'Vif' protein that prevents APOBEC3G incorporation into virions, primarily by inducing its degradation via the proteasome (50–58). The physiological roles of APOBEC2, of APOBEC4 and of other members of the APOBEC3 group are still unknown.

Previously, we demonstrated that human APOBEC3G (hA3G) and murine APOBEC3 (mA3) restrict retrotransposition of mammalian endogenous retroviruses, with evidence for 'traces' of this mutagenic activity as G-to-A substitutions within the genomic copies of some of these elements (22). This activity most probably corresponds to a primary function

of the enzymes, as an ancestral defense mechanism against retroelements. By using a cell-based *ex vivo* assay, we have shown that APOBEC3G dramatically inhibits retrotransposition of murine IAP and MusD elements, and induces G-to-A hypermutations in their transposed DNA copies. The assay used retroelements marked with a *neo*-containing indicator gene that becomes active only after retrotransposition, and subsequent selection of the resulting G418-resistant cells (10,11,22,59,60). To determine whether the observed decrease in the retrotransposition rate is only due to proviral cDNA editing *per se* acting at the level of the *neo* gene, or whether it is additionally the consequence of a decrease in the number of integrated copies, we presently measured the copy number of transposed MusD, IAP and control L1 elements, using a refined quantitative PCR (qPCR) method, in the absence of any selection. We show that APOBEC3G has a dual effect on endogenous retroviruses, resulting firstly in a decrease of the number of transposed copies and secondarily in extensive editing of the transposed copies. The effects of other members of the cytosine deaminase family on the retrotransposition of murine retroelements are also examined.

MATERIALS AND METHODS

Plasmids

Expression plasmids containing the mouse MusD (pCMVMusD-6neo^{TNF}), mouse IAP (pGL3-IAP92L23-neo^{TNF}) and human L1 (p220.CMV-L1.2Bneo^{TNF}) retrotransposons have been described previously (10,11,61). The hA3G and mA3 expression plasmids used were a gift from A. Hance and N. Landau, respectively. The AID, hA1, hA2, hA3B, hA3C, hA3F and agmA3G expression plasmids were obtained through the NIH AIDS Research and Reference Reagent program (45,57). All the APOBEC open reading frame containing fragments were re-cloned in pcDNA6 expression plasmid (Invitrogen).

Cells, transfection and retrotransposition assay

Human HeLa cells were grown in DMEM containing Glutamax I and sodium pyruvate (Invitrogen) and supplemented with 10% fetal calf serum (Gibco BRL), 100 μg/ml streptomycin and 100 U/ml penicillin. HeLa cells (3×10^5), seeded in 60 mm diameter plates, were transfected with 1.5 μg *neo*^{TNF}-marked retrotransposon and 1.5 μg cytosine deaminase expression vector by using the Lipofectamine Plus kit (Gibco BRL). Two days post-transfection, two-thirds of the cells were recovered for RNA and DNA analyses (see below); the remaining one-third was maintained in culture for 1 week, and cells were then seeded at 3×10^5 cells per 100 mm dish for G418 selection. After 14 day selection, G418^R foci were fixed, stained and counted.

Nucleic acid extractions and quantifications

Cellular DNA from the transfected cells was extracted as described elsewhere (59) and was used as a template for specific PCR amplification of the spliced *neo*^{TNF} domain. PCR amplification was performed using the forward primer 5′-CAG TTC GGC TGG CGC GAG GCC-3′ and the reverse primer 5′-CAG TTC CGC CCA TTC TCC G-3′ in 50 μl containing 0.5 μg of cellular DNA, 1× Buffer II and 1.5 U AccuPrime *Taq* DNA polymerase (Invitrogen), with an initial denaturation step at 94°C (3 min) followed by at least 30 cycles of amplification (50 s at 94°C, 50 s at 60°C and 1 min at 68°C). PCR products were electrophoresed on agarose gels in the presence of ethidium bromide (1 μg/ml) and quantified by densitometry, using the Kodak 1D Image Analysis Software (Kodak). For qPCR (see Results), 5 μl aliquots were removed from the PCR mixture at each of the 10 last cycles, and analyzed as above. A regression equation $y = a \times b^n$ (where y is the intensity of the band, n the cycle number, a the template amount, and b the efficacy of amplification per cycle) was fitted to the data in the linear portion of the amplification reaction curve. In some experiments, the PCR products were electrophoresed on agarose gels, purified with the Nucleospin Extract II kit (Macherey-Nagel) and cloned into pGEM-T easy vector (Promega). After transformation of DH5α cells, 20–25 clones were sequenced (Applied Biosystem sequencing kit) to determine the editing potency of each cytosine deaminase.

Total RNA was extracted from transfected cells with the Nucleospin RNA II kit (Macherey-Nagel). RNA (1 μg) was reverse transcribed in a 20 μl volume reaction using 50 U Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Applied Biosystems), 20 U RiboNuclease inhibitor (Applied Biosystems), 1 mM dA/T/G/C (Amersham-Pharmacia Biotech), 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 10 mM KCl and 2.5 μM random hexamers (Applied Biosystems). After an initial primer-annealing step at room temperature for 10 min., reverse transcription was performed at 42°C for 45 min and ended by heating at 99°C for 7 min. qRT–PCR was then carried out with 1 μl of the RT-treated sample using the same PCR conditions as above.

RESULTS

Rationale of the assay for retrotransposition

We have used the previously described *neo*-marked MusD and IAP retroelements to assay the effects of APOBEC cytosine deaminases on the retrotransposition of these elements. The rationale of the experiment is illustrated in Figure 1A, and includes (i) transfection of the marked retroelement in the presence—or not—of an expression vector for the APOBEC proteins, (ii) extraction of total RNA from a fraction of the cells two days post-transfection to assay for possible effects of the APOBEC proteins on retroelement transcript levels, (iii) plating of the cells for selection in G418-containing medium, to assay for the rate of retrotransposition, via measurement of the number of G418-resistant (G418^R) clones and, in parallel (iv) DNA extraction from a fraction of the cells before selection, to tentatively measure by direct methods (see below) the extent of retroelement transposition in the absence of any selection. For the latter measurement, we took advantage of the presence within the marked retroelements of an intron inserted into the *neo* gene, which is spliced out during the retrotransposition process [also resulting in the activation of the *neo* gene and survival of the cells with retrotransposed elements in G418-containing medium, feature used for assay (iii) above] (22,59,60). Specific primers for the amplification of the spliced *neo* gene in the retrotransposed copies

were devised, with a forward primer overlapping the exon junction within the *neo* gene (Figure 1B), in order to minimize the 'parasitic' amplification of the vast excess of non-transposed *neo*-marked plasmid copies present in the transfected cells (retrotransposition frequency is at most 10^{-3} event per cell, i.e. DNA copies with a spliced out intron are about 3 logs less numerous than the parental marked copies). However, even with these primers and a *Taq* polymerase enzyme with improved priming accuracy (AccuPrime *Taq*, Invitrogen), several bands were detected in the PCR amplification product of the genomic DNA from cells with transposed elements (see Figure 1C and legend). Sequencing demonstrated that the 643 bp species corresponded to the product expected for spliced, transposed *neo*-marked copies, whereas the 725 bp species corresponded to the background PCR amplification of unspliced, non-transposed copies of the marked retroelements. Other bands were occasionally observed, with a variable intensity depending on the transfection experiment—and not on the presence of the APOBEC expression vectors—that were found to correspond to either alternative splicing events (for the <643 bp bands) or unexpected structures (for the high molecular weight band) due most probably to recombination events between transfected plasmid DNA molecules. This precluded the use of standard real-time qPCR methods. We therefore devised a manual, indirect method in which an aliquot was removed at each cycle of the PCR, the products separated by agarose gel electrophoresis and the intensity of the 643 bp band quantified by densitometry. As illustrated in Figures 1C and 2, a plot of the intensity of the specific band as a function of the PCR cycle number yielded sigmoïd curves, similar to those obtained in real-time qPCRs with an intermediate phase disclosing a sharp increase corresponding to the linear range of the amplification reaction, i.e. where the amount of amplified fragment was proportional to the input amount of target DNA. Based on a fit of this linear portion of the graphs (see logarithmic representation in inset of Figure 1C), we established that the efficacy of amplification was 1.75-fold per PCR cycle, satisfactorily close to the theoretical value of 2 for optimal PCR amplifications (see Materials and Methods).

APOBEC3G reduces the amount of transposed MusD and IAP elements

Using this PCR assay, it can be clearly shown that the presence of hA3G significantly reduced the copy number of transposed MusD and IAP elements (Figure 2). We observed a significant difference in the number of PCR cycles (up to 5 cycles for MusD and 2 cycles for IAP) required to reach similar amplification levels. Quantitatively, this difference corresponds to a 10- to 20-fold and a 2- to 3-fold decrease in MusD and IAP cDNA levels, respectively (Figure 2A). This effect actually closely correlates with the results obtained in the standard retrotransposition assay using G418 selection, with a 50-fold and a 4-fold decrease in the number of $G418^R$ clones for each of these two retrotransposons, respectively [Figure 3A and see Ref. (22)]. The transposed copy number of a control non-LTR LINE retroelement (L1) was not affected by the presence of hA3G (Figure 2A), in agreement with the previous observations that L1 elements are insensitive to hA3G activity (22,62). To rule out a possible effect of hA3G on the efficiency of the PCR assay *per se* due to, for instance, decreased matching of the primers used for the PCR as a result of the editing activity of hA3G on the DNA template, we performed a series of controls, using other sets of primers and/or primers with degenerate nucleotides at the positions prone to the editing effect (Supplementary Figure S1). The results obtained with such primers disclosed an identical level of reduction in the amount of retrotransposed copies generated in the presence of hA3G. Altogether, the present results clearly indicate that APOBEC3G has an effect on the number of transposed copies of endogenous retroviruses, independently of any 'selection' procedure. The slightly higher reduction levels observed when measuring the $G418^R$ clone number could simply be accounted for by a mutagenic inactivation of the *neo* gene in some of the transposed copies, resulting in the loss of the corresponding cells upon G418 selection.

We next asked whether the reduction in the number of MusD and IAP transposed copies by APOBEC3G could be related to a decrease in the level of viral RNA templates. To this end, we applied our quantitative PCR method to cellular RNAs prepared two days after transfection and reverse transcribed *in vitro*. As illustrated in Figure 2B, hA3G did not affect the level of the detected MusD, IAP or L1 transcripts, with the *neo* intron spliced out.

Conclusively, these results demonstrate that hA3G reduces the amount of retrotransposed copies. This inhibitory effect does not take place at the viral RNA level but during or after reverse transcription, most probably via deamination (see below) and subsequent degradation of the nascent viral cDNAs.

'Anti-viral' effects of other members of the APOBEC family

We then investigated the effect of other members of cytosine deaminase family on the retrotransposition of the MusD retroelement. We tested human AID (hAID), APOBEC1 (hA1), APOBEC2 (hA2) and APOBEC3B, APOBEC3C, APOBEC3G and APOBEC3F (hA3B, hA3C, hA3G and hA3F) family members. hA3G is the first cytosine deaminase that was demonstrated to have an antiviral activity, hA3F was then shown to be active against HIV, whereas hA3B only modestly affects HIV and SIV, and hA3C has antiviral activity only against SIV (41,42). The African green monkey APOBEC3G (agmA3G) and murine APOBEC3 (mA3) proteins, which have been demonstrated previously to display a potent anti-retroviral activity (51,57,63), were also included in the assay. HeLa cells were co-transfected with the marked MusD element and an expression vector for each of these proteins. Cells were then analyzed as above, both by G418 selection and by qPCR.

Three groups of APOBEC proteins could be distinguished, according to their anti-MusD activity (Figure 3). The first group corresponds to efficient anti-retroviral proteins (hA3G, hA3F, agmA3G and mA3) which induced a decrease in the number of $G418^R$ clones of 50-, 5-, 6- and 5-fold, respectively. The second group includes hAID, with only a modest—but reproducible—2-fold reduction effect on MusD retrotransposition. The third group corresponds to non-inhibitory proteins (hA1, hA2, hA3B and hA3C). Analysis of the amount of retrotransposed MusD copies by qPCR

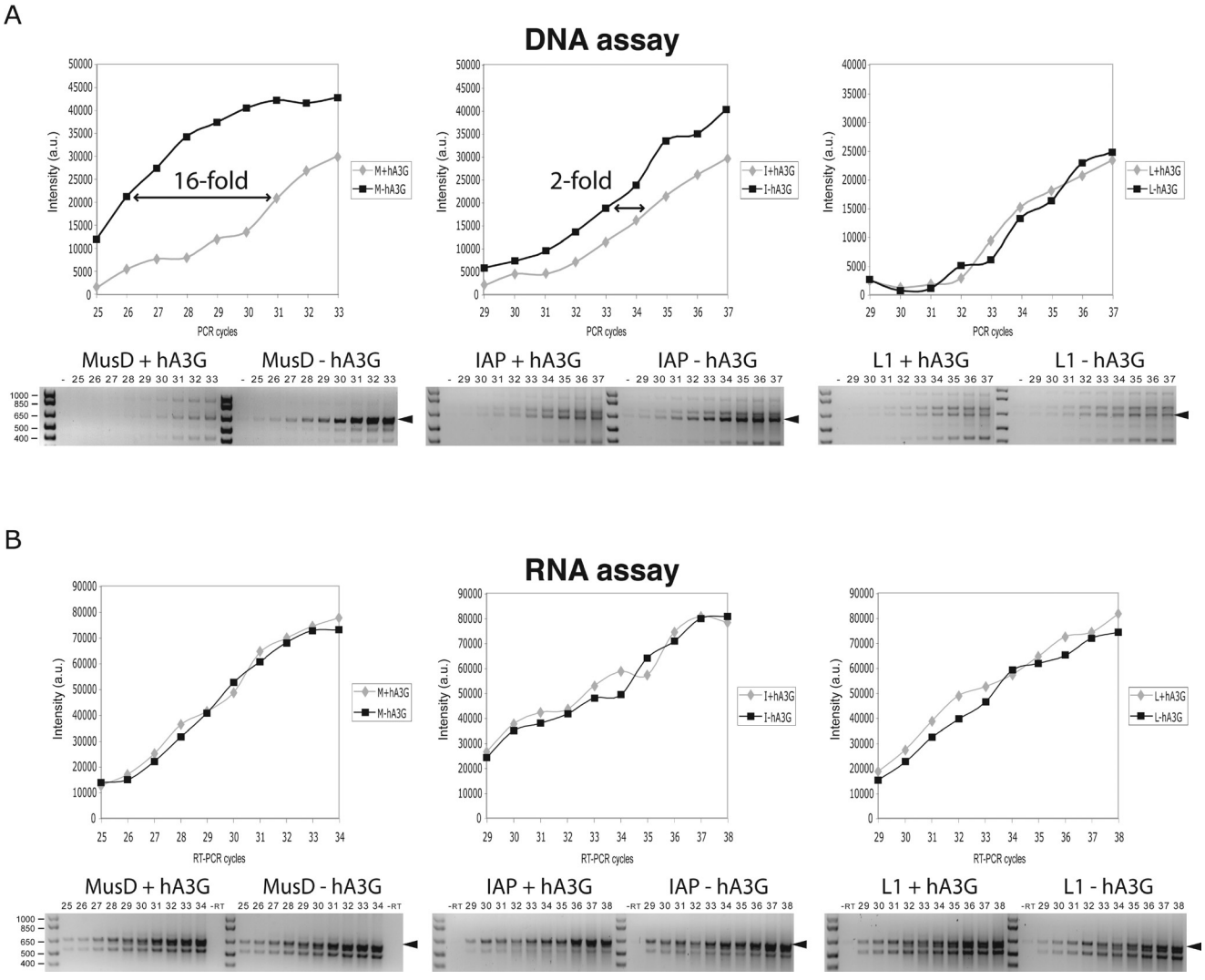

Figure 2. Effects of hA3G on the amount of MusD, IAP and L1 transposed copies and intermediate RNAs. (**A**) Quantitative analysis of the effects of human hA3G on the number of retrotransposed MusD, IAP and L1 *neo*-marked elements. HeLa cells were transfected with 1.5 μg of the indicated marked retrotransposon, in the presence or absence of 1.5 μg of hA3G expression vector. DNA was extracted 2 days post-transfection, and PCR was carried out with 0.5 μg of cellular DNA. The results are represented as in Figure 1C, with the band of the expected size (643 bp) for the retrotransposed copies indicated with black arrowheads. Data points in the intensity/cycle plots are the means of duplicate determinations, and each curve is representative of at least three independent experiments with a mean fold reduction of 13.6 ± 3.4 for MusD ($n = 5$), 3.4 ± 1.5 for IAP ($n = 3$) and no significant difference for L1 ($n = 3$). (**B**) hA3G has no effect on the retroelement transcript levels. RNAs were extracted 2 days post-transfection from the same cell populations as in (A) and reverse transcribed *in vitro*. Quantification of the cDNAs was performed as in (A). Data points in the plots are the means of duplicate determinations and the curves are representative of two independent experiments.

provided consistent results (Figure 3B), with a similar rank order for the effect of the various cytosine deaminases tested and, as already noticed in the previous section for APOBEC3G, a slightly smaller magnitude of the reduction-most probably associated with the editing of the *neo* gene which enhances the inhibitory effect in the G418 selection assay.

Differential editing of the retrotransposed elements by various cytosine deaminases

We then analyzed the editing potency of the various APOBEC proteins on a fragment of the retrotransposed MusD elements recovered by direct PCR, without prior G418 selection of the cells. For each APOBEC protein, 643 bp *neo*-containing PCR fragments were cloned, and at least 20–25 different sequences determined. As illustrated in Figure 4, we observed a high number of mutations in the presence of hA3G, hA3F, agmA3G, mA3 and hAID, i.e. ∼104, 55, 21, 61 and 23 mutations per 10 000 bases analyzed, respectively. Furthermore, a large majority of the mutations were G-to-A transitions (93% for hA3G, 88% for hA3F, 68% for agmA3G, 81% for mA3 and 53.5% for hAID), with the other substitutions randomly distributed (Figure 4A). The distribution of the G-to-A substitutions per individual 643 bp sequence ranged from none to up to 36 (see histograms in Figure 4A), and the overall mutation rate was rather high, reaching a mean of eleven mutations per MusD-derived 643 bp fragment in the case of hA3G, consistent with the results obtained previously with the same enzyme, but following prior selection of $G418^R$

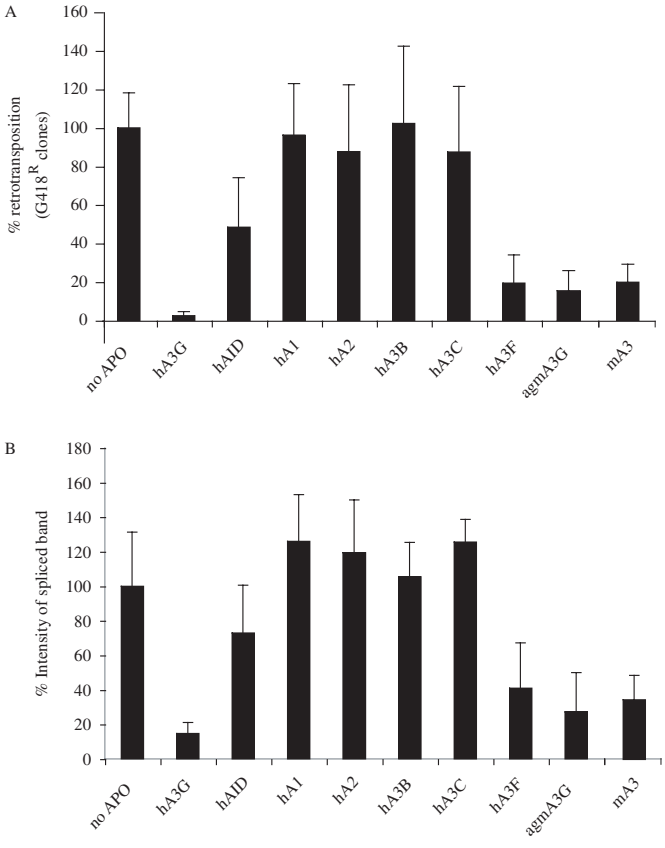

Figure 3. Differential effects of cytosine deaminases on the retrotransposition of MusD. Analysis of the effect of human hA3G, hAID, hA1, hA2, hA3B, hA3C, hA3F, African green monkey agmA3G and murine mA3 on MusD retrotransposition. (**A**) Measurement of the number of $G418^R$ clones after selection in G418-containing medium of the cells co-transfected with the marked MusD and the expression vector for the indicated cytosine deaminase; values are given as the percentages of the corresponding values obtained in the absence of cytosine deaminase expression vector (no APO). Data are the means ± SD of at least three independent experiments. (**B**) Quantitative analysis as in Figure 2 of the number of retrotransposed MusD copies, in the absence of G418 selection. DNA was extracted from cells transfected as in (A). Values of the intensity of the specific 643 bp PCR fragment, measured after 30 cycles of amplification, are expressed as the percentages of the corresponding values in the absence of cytosine deaminase. Data points are the means ± SD of at least three independent experiments.

cells (22). It is noteworthy that, in agreement with previous reports (22,64,65), a large fraction of the G-to-A mutations occurred in the typical GG dinucleotide context (at the underlined nucleotide) for hA3G and agmA3G (up to 71 and 79%, respectively), GA dinucleotide context for hA3F (up to 62%) and GXA trinucleotide context for mA3 (up to 66%) (Figure 4C). For hAID, no clear-cut sequence specificity could be unraveled. For hA1, hA2, hA3B and hA3C, as expected from the retrotransposition assay, only few mutations were observed, which further appeared to be random (Figure 4A and B).

DISCUSSION

Despite profound similarities between infectious retroviruses and endogenous LTR retroelements, which relate to their common ancestral history, the 'physiological' impact of both classes of elements are different. Indeed, the primary 'goal' of infectious retroviruses is to replicate in the host, i.e. remain functional as an invading element, whereas endogenous retroelements are already present within the host genome as multicopy 'genes' most of which are inactive. Although the difference is tenuous, it may explain why the recently discovered effects of the ancestral cellular cytosine deaminases on infectious retroviruses have mainly concerned their editing potency, which renders them defective for further replicative cycles and propagation. This editing potency also applies in the case of endogenous elements. Indeed, it has been recently demonstrated that several LTR retroelements are subjected to editing by the APOBEC3G enzyme, as illustrated both by using retrotransposition assays [for Ty1 see Refs. (23,24) and for mammalian retrotransposons Ref. (22)] and by performing an *in silico* search for traces of the expected mutations within the numerous copies of these elements that have been generated in the course of genome evolution and retroelement expansion (22). Yet, the effect of cytosine deaminases on the amount of proviral copies—edited or not—that are generated by functional endogenous retrotransposons remains an essential parameter for their mobility and their resulting potential for insertional mutagenesis.

The present study, therefore, is an important step in the characterization of the effects of cytosine deaminases and of their impact as defense enzymes against these elements. By using an appropriate PCR-based quantification method, we demonstrate that the human APOBEC3G actually reduces the number of retrotransposed copies of the MusD and IAP retrotransposons, by 10- to 20-fold and 2- to 3-fold, respectively. We show that this reduction is not due to an effect of the cytosine deaminases on the transcript levels of the marked retroelements. Sequencing of the rescued retrotransposed copies further demonstrates editing of the proviral DNA, a process which most probably takes place during reverse transcription of the IAP and MusD transcripts. Although one cannot formally exclude that cytosine deaminases inhibit the reverse transcription process *per se*, it is likely that the observed decrease in the amount of proviral DNA is due to a degradation of the deaminated reverse transcripts, prior to integration [for HIV-1 see also Refs. (38,57) and for Ty1 (23)]. Cellular DNA repair enzymes such as uracil-DNA glycosylases could be responsible for this degradation (66) Along this line, it is noteworthy that preliminary experiments that we carried out using mutants in the catalytic domain of hA3G [i.e. the M2 (23) and the E259Q (67) mutants] disclosed a reversion of the inhibitory effect on MusD retrotransposition. Altogether, the present results show that APOBEC enzymes have a dual effect on endogenous retroviruses (Figure 5): they reduce the number of proviral copies that are generated by the IAP and MusD marked elements, and they have an editing activity which results in the introduction of mutations into the transposed copies. The latter effect is important since mutations can indeed inactivate the newly integrated copies, render them defective for autonomous retrotransposition and cut short additional retrotransposition cycles, but most probably the primary 'line of defense' against retroelements is mediated by the former effect, which reduces the number of transposed copies whose integration can directly mediate insertional mutagenesis.

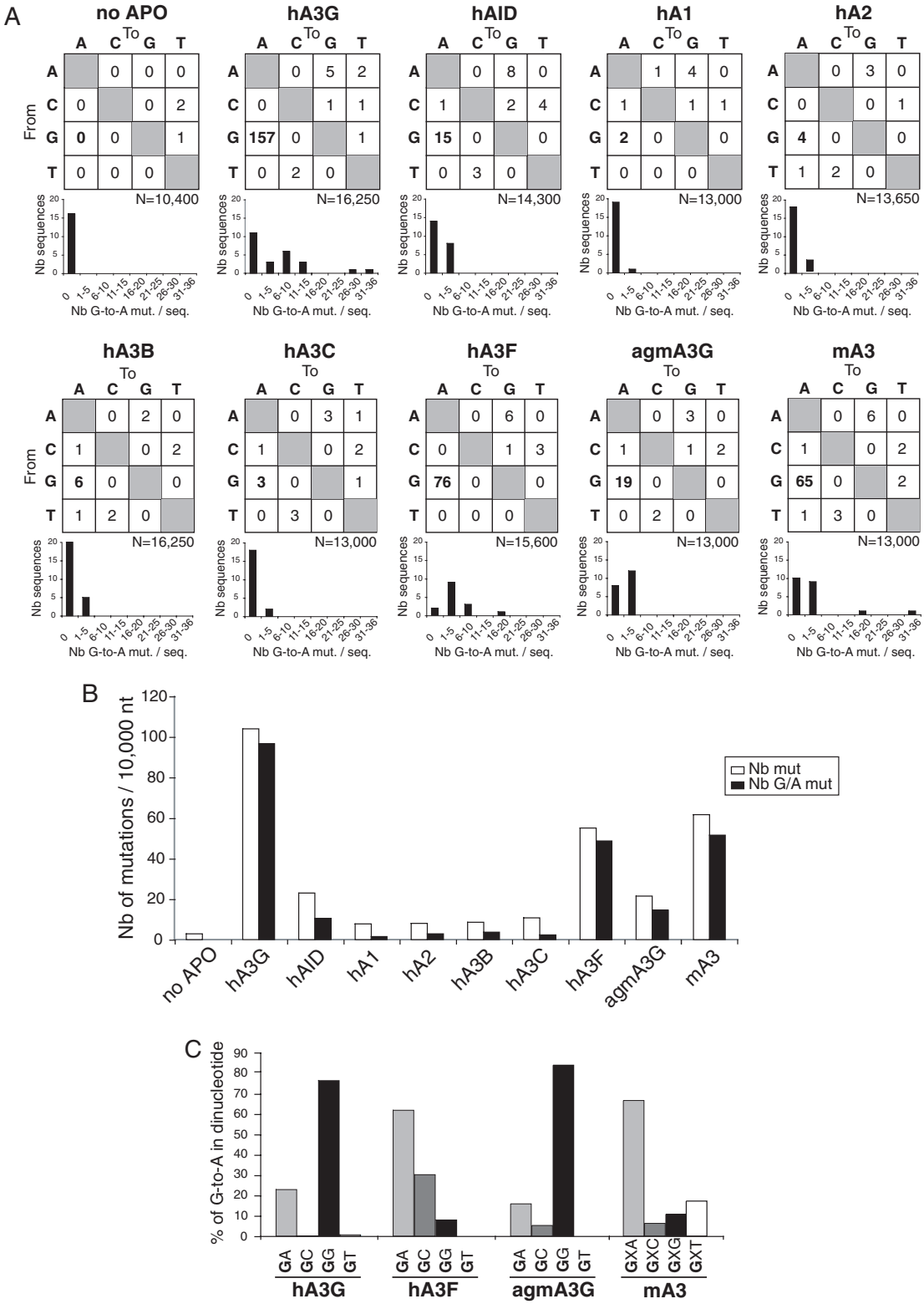

Figure 4. Differential effect of cytosine deaminases on G-to-A hypermutations. (**A**) Upper panels: Two-entry tables showing nucleotide substitution preferences for MusD mutations detected in the absence of cytosine deaminase (no APO) or in the presence of hA3G, hAID, hA1, hA2, hA3B, hA3C, hA3F, agmA3G and mA3. At day 2 post-transfection, retrotransposed MusD copies were amplified by PCR, cloned and sequenced (643 bp *neo* fragment). Clones (20–25) were sequenced under each condition (only 16 for the 'no APO' control). *N*, total number of bases sequenced. Lower panels: Histograms showing the number of G-to-A mutations per individual 643 bp sequence in the retrotransposed MusD elements. (**B**) Number of mutations induced by each cytosine deaminase in the retrotransposed MusD copies, per 10 000 nt. Open bars represent the total number of mutations for 10 000 sequenced nucleotides and black bars the number of G-to-A mutations. (**C**) Target sequence preference of hA3G, hA3F, agmA3G and mA3. The influence of the 3′ neighboring nucleotides for the G-to-A mutations identified in the retrotransposed MusD copies is illustrated.

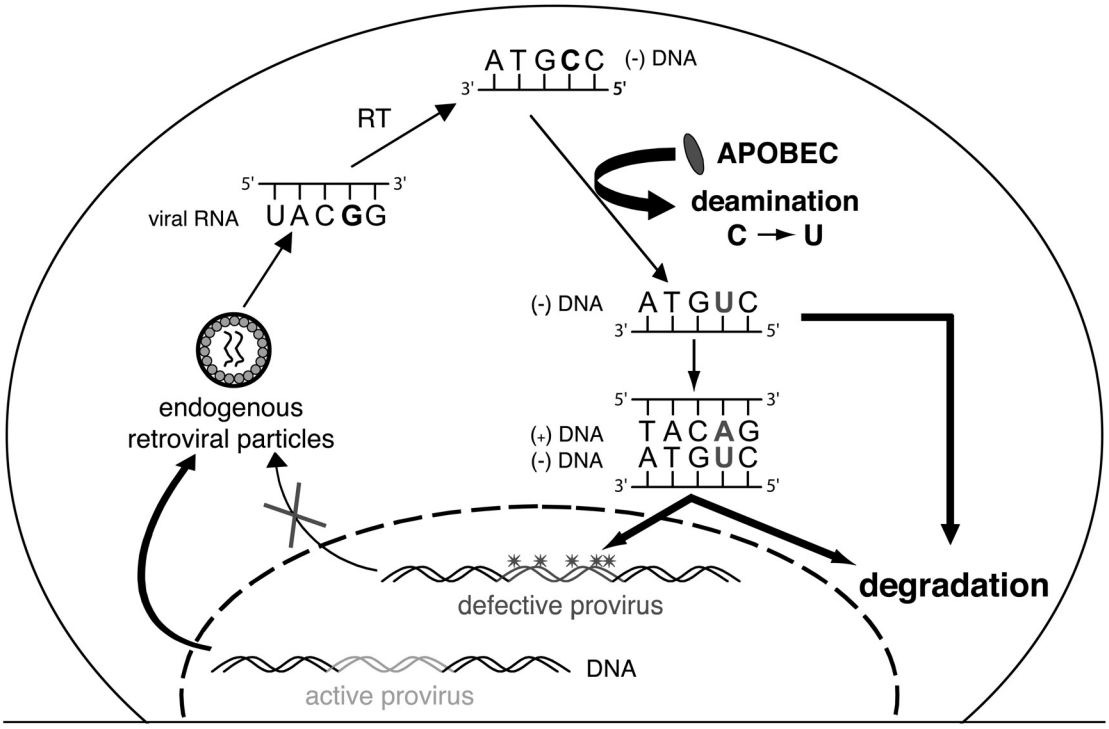

Figure 5. Model for the dual effect of APOBEC activity on retrotransposition. Active endogenous proviruses—such as MusD—produce endogenous retroviral particles, whose RNA is reverse transcribed into single-strand DNA that is sensitive to APOBEC deoxycytidine deaminase activity. Uracil-containing cDNA (either single-strand or double-strand) is then degraded before integration, possibly by cellular UNGs, a process which reduces the number of proviral copies prone to cause insertional mutagenesis (first 'line of defense'). The proviral copies that have escaped degradation can integrate but disclose G-to-A mutations that render them defective for subsequent retrotransposition cycles (second 'line of defense').

Another outcome of the present study is the demonstration that several—but not all—APOBEC proteins have the capacity to restrict retrotransposition of LTR retroelements in cell culture experiments, with a dual effect. Consistently, the human hA3G protein efficiently inhibits retrotransposition of MusD retrotransposons and induces G-to-A hypermutations in their genomes, and the human hA3F, simian agmA3G and murine mA3 proteins are also strongly active. Interestingly, the human and simian APOBEC3G proteins target the same expected consensus site for the G-to-A substitutions, namely the GG dinucleotide, whereas the human hA3F and the murine mA3 proteins disclose G-to-A mutations in the GA dinucleotide and GXA trinucleotide, respectively, in agreement with previous reports (41–44). Interestingly, we show here that the human hAID protein displayed a limited—but reproducible—effect on the rate of MusD retrotransposition, and induced a small number of mutations in the transposed copies. The AID protein is known to edit immunoglobulin gene DNA, and thus to function in the nucleus. However, AID is a nucleocytoplasmic shuttling protein and is also partly localized in the cytoplasm (68–70). This may explain the limited effects of hAID on MusD retrotransposition. Whether this unexpected activity of hAID on endogenous retroviruses is of physiological relevance awaits further analysis. Similarly, differences in subcellular localization and/or intrinsic enzymatic activities might account for the absence of effect that we observed for the hA3B and hA3C proteins, as well as for the two other APOBEC family members tested (hA1 and hA2).

Finally, it is noteworthy that in the case of the most active APOBEC protein, we found that the murine MusD LTR retroelement is sensitive to the human, simian and murine enzymes (hA3G, agmA3G and mA3), without significant species-specific differences. This strong functional conservation is most probably relevant to the primary role of this APOBEC protein as a defense mechanism, against elements that are extremely diverse even within a given species, with for example more than a hundred distinct families of endogenous retroviruses in the human genome. This diversity, resulting from the repetition of 'invasions' during species evolution, has most probably imposed, at the level of the eukaryote genomes, the conservation of a guardian gene with a large spectrum of action, and concomitantly, at the level of the genomes of emerging retroviruses, the invention of refined counteracting strategies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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