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APOBEC3B and APOBEC3F Inhibit L1 Retrotransposition by a DNA Deamination-independent Mechanism*[S]

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The most common transposable genetic element in humans, long interspersed element 1 (L1), constitutes about 20% of the genome. The activity of L1 and related transposons such as Alu elements causes disease and contributes to speciation. Little is known about the cellular mechanisms that control their spread. We show that expression of human APOBEC3B or APOBEC3F decreased the rate of L1 retrotransposition by 5–10-fold. Expression of two related proteins, APOBEC3D or APOBEC3G, had little effect. The mechanism of L1 inhibition did not correlate with an obvious subcellular protein distribution as APOBEC3B appeared predominantly nuclear and APOBEC3F was mostly cytosolic. Two lines of evidence indicated that these APOBEC3 proteins use a deamination-independent mechanism to inhibit L1. First, a catalytically inactive APOBEC3B mutant maintained L1 inhibition activity. Second, cDNA strand-specific C → T hypermutations were not detected among L1 elements that had replicated in the presence of APOBEC3B or APOBEC3F. In addition, lower levels of retrotransposed L1 DNA accumulated in the presence of APOBEC3B and APOBEC3F. Together, these data combined to suggest a model in which APOBEC3B or APOBEC3F provide a preintegration barrier to L1 retrotransposition. A particularly high level of APOBEC3F protein in human testes and an inverse correlation between L1 activity and APOBEC3 gene number suggest the relevance of this mechanism to mammals.

Several human APOBEC3 (A3)[3] proteins have been shown to be capable of inhibiting the replication of a diverse and growing number of retroelements (reviewed recently in Ref. 1). All of these genetic elements replicate by reverse transcribing their genomic RNA into a double-strand DNA. This process requires that the ends of the retroelement be composed of long terminal repeats (LTRs). For LTR-dependent endogenous and exogenous retroelements (retrotransposons and retroviruses, respectively), reverse transcription predominantly occurs in the cytoplasm of the host cell. It is in this subcellular compartment that the human A3 proteins are thought to engage the retroelement as it assembles its core components into a ribonucleoprotein complex required for replication. Much of our current mechanistic understanding has been derived from studying APOBEC3G (A3G), which accesses assembling virus (*e.g.* human immunodeficiency virus-1 (HIV-1)) and virus-like (*e.g.* musD) particles through the Gag protein of the retroelement (2, 3). Considerable evidence indicates that during retroelement reverse transcription, A3G exerts its restrictive potential by deaminating nascent cDNA cytosines to uracils (4–6). The uracils template the incorporation of second cDNA strand adenines and, ultimately, this mutagenic process results in a lethal level of strand-specific retroelement C/G → T/A transition mutations (hypermutations). Additional evidence indicates that several of the other human A3 proteins can also inhibit a variety of retrotransposons and retroviruses by a similar cDNA cytosine deamination mechanism (*e.g.* Refs. 5–15). However, partial inhibition of HIV-1 (16–19), hepatitis B virus (20, 21), human T cell lymphotropic virus (22), and yeast Ty1 (23) was still observed with A3G catalytic mutants, indicating that deaminase activity may be only one part of the overall mechanism.

LTR retrotransposons such as the human endogenous retrovirus make up about 8% of the human genome, and most (if not all) of these are thought to be inactive (24). In contrast, about 75% of the repetitive DNA in the human genome (34% of the total) is composed of retroelements that do not require LTRs for replication. The most prominent examples include long interspersed element-1 (LINE-1 or L1), which replicates autonomously, and Alu elements, the replication of which requires L1 protein activities (25, 26). These elements make up 17 and 11% of the genome, respectively (24). The mechanism of L1 replication is fundamentally different from that of LTR-dependent retroelements. Most importantly, L1 elements reverse-transcribe their RNA genome upon integration into the host cell nuclear DNA by a process called target-primed reverse transcription (26). Although most of the estimated 500,000 L1 elements are incapable of mobilizing, about 100 are active, and they can compromise genomic integrity by disrupting genes, interfering with transcriptional programs, mobilizing other elements, and precipitating recombination (25, 26). One of every 10–250 infants is estimated to contain a new L1 insertion (26). L1 elements can thereby cause disease and, over many generations, contribute to speciation. The cellular mechanisms that limit L1 activity are poorly understood.

During A3 protein localization studies, we discovered that A3B is predominantly nuclear, in contrast to A3G, which is mostly cytoplasmic (5, 27). This observation prompted us to test whether A3B could inhibit L1 retrotransposition. Using a GFP-based L1 retrotransposition reporter assay, we found that expression of A3B or A3F strongly inhibits L1 retrotransposition. Expression of the related human proteins A3D or, as shown previously (28), A3G did not significantly affect the rate of L1 retrotransposition. Interestingly, the mechanism of inhibition appeared to be deaminase-independent as hypermutations were not apparent in retrotransposed L1 DNA and inhibition was still observed

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[3] The abbreviations used are: A3, APOBEC3, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like protein 3; A3B, APOBEC3B; A3D, APOBEC3D-E; A3F, APOBEC3F; A3G, APOBEC3G; GFP, green fluorescent protein; HIV, human immunodeficiency virus; L1, long interspersed element-1; LTR, long terminal repeats; HA, hemagglutinin.

with a catalytically inactive A3B protein. Such a mechanism was further supported by PCR experiments showing that less integrated L1 DNA accumulated in the presence of A3B or A3F. These data combined to suggest a model in which these A3 proteins interfere with L1 transposition prior to retroelement integration.

EXPERIMENTAL PROCEDURES

DNA Constructs—The L1 construct, pL1-ac002980-EGFP (29), and the functionally active A3B construct, pcDNA3.1-A3B-HA (13), have been described. The deaminase activity-deficient A3B construct has two amino acid substitutions, W228L and D316N, and it was isolated during the course of PCR using oligonucleotides 5′-GAG CTC GGT ACC ACC ATG AAT CCA CAG ATC AGA AAT-3′ and 5′-GTC GAC CAT CCT TCC GTT TCC CTG ATT CTG GAG-3′ and IMAGE clones 3915193 and 4707934 as amplification templates. We have found that similar substitutions in A3G are important for catalytic activity.[4] A3D, A3F, and A3G were amplified from existing laboratory constructs (6, 12, 30) using oligonucleotides 5′-GAG CTC GGT ACC ACC ATG AAT CCA CAG ATC AGA AAT-3′/5′-GTC GAC TCC CTG GAG AAT CTC CCG TAG C-3′, 5′-GAG CTC AGG TAC CAC CAT GAA GCC TCA CTT CAG AAA C-3′/5′-GTC GAC TCC CTC GAG AAT CTC CTG CAG CTT-3′, and 5′-GAG CTC AGG TAC CAC CAT GAA GCC TCA CTT CAG AAA C-3′/5′-GTC GAC TCC GTT TTC CTG ATT CTG GAG AAT-3′, respectively. The A3B, A3D, A3F, and A3G PCR products were digested with SacI and SalI and cloned into a similarly digested pEGFP-N3 (Clontech). They were also digested with KpnI and SalI and cloned into a modified version of pcDNA3.1(+) (Invitrogen) containing a carboxyl-terminal 3× influenza hemagglutinin (HA) tag. This modified pcDNA3.1 vector was created by cloning a triple HA tag between the XhoI and XbaI sites in the polylinker. The *Escherichia coli* expression plasmids, pTrc99A and pTrc99A-A3G, were reported previously (18, 30). The A3B-expressing derivatives of pTrc99A were constructed by PCR using oligonucleotides 5′-GGT ACC ACC ATG AAT CCA CAG ATC AGA AAT-3′ and 5′-GTC GAC CCC ATC CTT CAG TTT CCC TGA TTC TGG AG-3′, KpnI and SalI restriction, and ligation into similarly digested vector. All constructs were verified by DNA sequencing. The cDNA sequences for A3F and A3G were identical to NCBI reference sequences. The cDNA sequence for A3D is also called A3D-E, and it is represented by GenBank™ cDNA BE888971. Active A3B differed in eight positions from GenBank NP_004891 (K62E, L80P, F107L, T146K, M193V, D205G, T337A, and R372K). The limited number of A3B sequences in GenBank prevented us from determining whether these variations are naturally occurring.

Cell Lines—Human embryonic kidney 293 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 25 units/ml penicillin and 25 μg/ml streptomycin at 37 °C and 5% CO_2. Transfections were performed using FuGENE 6 according to the manufacturer's protocol (Roche Applied Science). Stable, APOBEC3-expressing 293 cell lines were established by transfecting with linearized APOBEC3-HA expression constructs followed by a selection with growth medium containing 880 μg/ml G418 (Roche Applied Science). Clones expressing HA-tagged APOBEC3 proteins were identified and quantified by anti-HA (Covance) immunoblotting. Maintaining all clones in growth medium supplemented with 220 μg/ml G418 ensured stable APOBEC3 protein expression.

Microscopy—Cells were visualized on a Zeiss Axiovert 200 microscope at ×400 total magnification. 15,000 HeLa or 50,000 293T cells were seeded on LabTek chambered cover glasses (Nunc). After 24 h of incubation, these cells were transfected with 250 ng of the pEGFP-N3-based DNA constructs. After an additional 24 h of incubation, images of the live cells were collected. 293T cells transfected with the APOBEC3-GFP constructs were fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100, stained with 100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI), and imaged, as above. The HA-tagged APOBEC3 proteins were imaged similarly (data not shown). The $A3B_{W228L,\ D316N}$ construct was used to acquire the images presented in Fig. 1. Additional experiments using other A3B constructs have also shown a predominantly nuclear localization (data not shown).

L1 Retrotransposition Assays—L1 assays were performed as originally described with minor modifications (31). For each condition, 3–5 independent APOBEC3 or control 293 clones were plated at a density of 3×10^5 cells per well in 6-well plates. After 24 h of incubation, the cells were transfected with 500 ng of pL1-ac002980-EGFP. After an additional 24 h of incubation, L1 plasmid-containing cells were selected with 0.67 μg/ml puromycin (United States Biological). Maintenance of the L1 substrate was ensured by the continual presence of puromycin (0.15 μg/ml) through the duration of the experiment. At 72-h intervals, the cells were passaged, and at least 25,000 cells were examined by flow cytometry. A GFP-positive region was established such that less than 0.01% of negative-control 293 cells profiled within this gate.

Immunoblotting—Total cellular protein extracts were prepared using a serial freeze/thaw procedure. The extracted proteins were quantified by Bradford assays (Bio-Rad), and equal amounts were fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with monoclonal anti-HA (Covance), monoclonal anti-α-tubulin (Bio-Rad), or polyclonal anti-APOBEC3G (19). Primary antibodies were detected by incubation with horseradish peroxidase-coupled anti-mouse IgG (Bio-Rad) or anti-rabbit IgG (Bio-Rad) followed by chemiluminescence (Roche Applied Science).

L1 DNA Analyses—To ensure the exclusive amplification and detection of L1 retrotransposition events, we designed a PCR-based assay that would specifically amplify intronless GFP DNA. Most importantly, a splice-specific oligonucleotide, 5′-CAG CGT GCA GCT GGC C-3′, was designed to span the intron by annealing to exonic sequences 10 bp immediately upstream and 6 bp downstream. The *GFP* gene-specific annealing of this oligonucleotide would only occur if the intron were precisely spliced out, reverse-transcribed, and integrated. This primer was used in combination with 5′-CGA TCC CCT CAG AAG AAC TCG-3′ or 5′-CGA TCC CCT CAG AAG AAC TCG-3′ to generate a larger 360-bp or a smaller (semi-nested) 240-bp PCR product.

E. coli Mutation Assays—The intrinsic DNA cytosine deaminase activity of several APOBEC3 proteins including APOBEC3G has been assayed by quantifying the accumulation of rifampicin resistance mutations in *E. coli* expressing these proteins (e.g. Refs. 18 and 30).

RESULTS AND DISCUSSION

The cytoplasmic localization of the human A3G protein (5, 27) correlates with its ability to inhibit the replication of LTR-dependent retroelements (reviewed in Ref. 1). Experiments designed to visualize the subcellular compartmentalization of human A3-GFP fusion proteins in HeLa cells confirmed the predominantly cytoplasmic localization of A3G, and they also showed that A3D and A3F locate similarly (Fig. 1*A*). In contrast, GFP alone distributed throughout the cell, and human A3B localized predominantly to nuclei (Fig. 1*A*). Similar results were obtained in human 293T cells with A3-GFP and A3-HA constructs, indicating that localization was not cell-, tag-, or fixation-dependent (Fig. 1*B* and data not shown).

[4] N. Martemyanova and R. S. Harris, unpublished data.

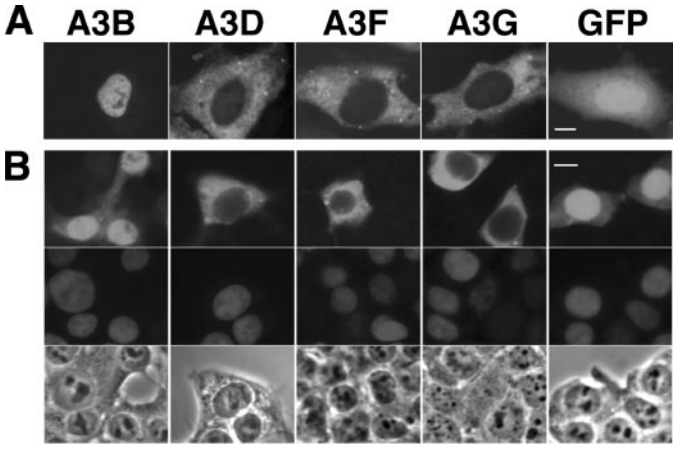

FIGURE 1. **Subcellular distribution of human A3B, A3D, A3F, and A3G.** *A*, representative live HeLa cells showing the subcellular localization of GFP-tagged human A3B, A3D, A3F, and A3G. *B*, representative fixed 293T cells showing the subcellular localization of GFP-tagged human A3B, A3D, A3F, and A3G (*top panel*). The *middle* and *bottom panels* show 4′,6-diamidino-2-phenylindole (DAPI) stained and bright field images of the same fields, respectively. The *scale bars* indicate 10 μm.

The nuclear compartmentalization of A3B suggested that it might function to inhibit replication of L1. To test this possibility, it was necessary to establish human 293 cell clones expressing near equivalent amounts of HA-tagged A3B, A3D, A3F, and A3G (Fig. 2*A*). It is important to note that these A3 constructs were not massively overexpressed as control immunoblots using a polyclonal antibody specific to A3G showed that the A3G-HA levels were comparable with the levels of endogenous A3G in two well studied, HIV-infectable T cell lines (H9 and CEM; *e.g.* Refs. 3 and 14 and data not shown). In most instances, A3F has appeared co-expressed with A3G (12, 14), indicating that the A3F levels observed here are also not overly high. Thus, to a reasonable approximation, the stable cell lines were expressing near physiological levels of A3F, A3G, and likely also the other A3 proteins.

Successful replication of an L1 element requires that it be transcribed, transported to the cytosol, assembled into a ribonucleoprotein complex, returned to the nucleus, and reverse-transcribed upon integration (26). Plasmid-based L1 elements harboring a reporter gene in the antisense orientation, which itself is disrupted by a sense orientation intron, provide a robust system for monitoring retrotransposition in cell lines (Fig. 2*B*) (31, 32). In the GFP-based retrotransposition system, L1 transcription, splicing, reverse transcription, and integration are necessary for GFP expression. This reporter has an advantage over drug-based selection systems because it enables measurements over extended periods of time and therefore the determination of retrotransposition rates.

To assess whether A3B was able to inhibit L1 retrotransposition, the GFP-based L1 reporter construct was transfected into several independent 293 clones stably expressing a control vector, A3B, A3D, A3F, or A3G. These analyses focused on the human double cytosine deaminase domain APOBEC3 proteins because of their overall similarity to A3B and because one of them (A3G) was expected to function as a negative control (28). The steady accumulation of GFP-positive cells (~1% per day) indicated that robust levels of retrotransposition occurred in 293 cells expressing the control vector, A3D and A3G (Fig. 2*C*; Supplemental Fig. 1). In contrast, expression of A3B decreased the rate of L1 retrotransposition by 5–10-fold. Additionally, contrary to expectations based on our A3 protein localization studies, expression of A3F also provided a similarly strong barrier to L1 retrotransposition.

As an additional control for A3 protein function, we monitored the infectivity of an HIV-based retrovirus produced in the presence of each of the A3-HA constructs or in the presence of an empty control vector

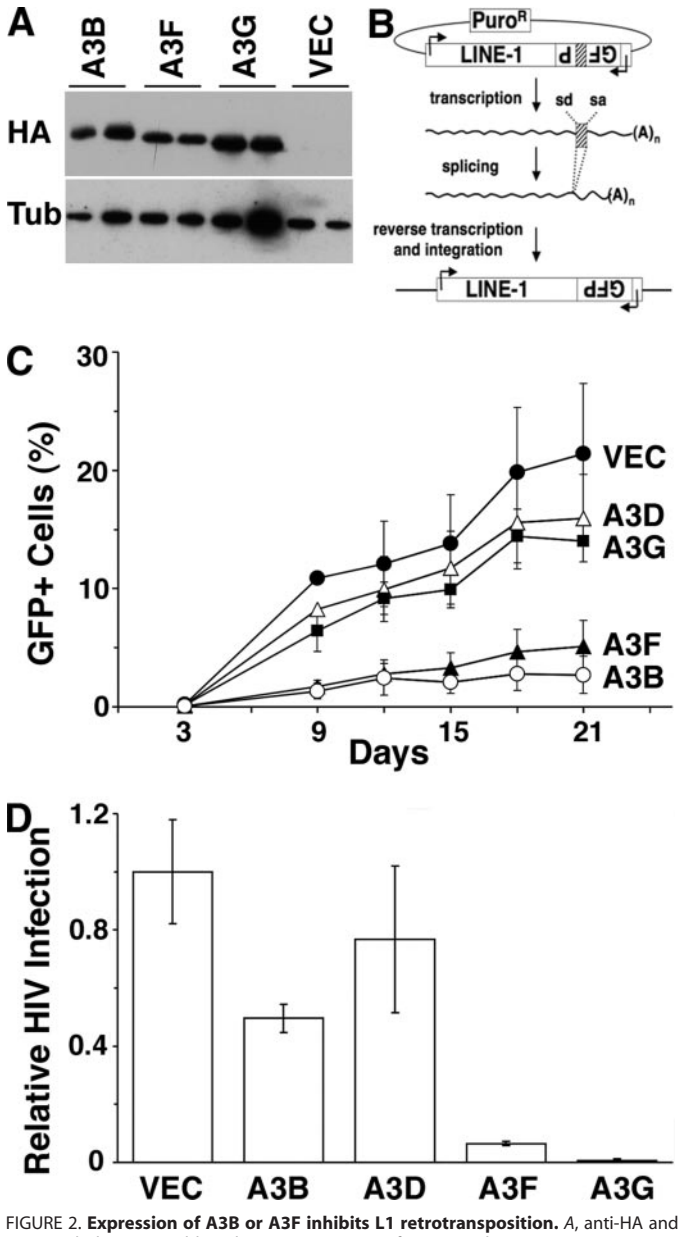

FIGURE 2. **Expression of A3B or A3F inhibits L1 retrotransposition.** *A*, anti-HA and anti-α-tubulin immunoblots showing expression of HA-tagged A3 proteins in representative stable 293 cell lines. The A3B clones shown express the catalytically inactive $A3B_{W228L,\ D316N}$ construct. The active A3B construct contains a single HA epitope (13) and, as expected, its immunoblot intensity was approximately one-third that of the triple HA-tagged proteins (data not shown). *B*, a schematic of the GFP-based L1 retrotransposition system (31). The parental L1 plasmid harbors an antisense, intron-interrupted GFP cassette. L1 transcription, splicing, reverse transcription, and integration are necessary for GFP expression (*sd*, splice donor; *sa*, splice acceptor). GFP fluorescence as monitored by flow cytometry provides a quantitative measure of L1 activity. *C*, A3B and A3F inhibit L1 retrotransposition. The accumulation of GFP-positive cells over time is shown. For each data set, the mean and S.E. of 3–5 independently derived 293 clones, which stably express the control vector (*VEC*) or a human A3 protein, are indicated. *D*, single cycle HIV infectivity assays. HIV-GFP viruses were produced in the presence of the control or the indicated A3-HA constructs and used to infect non-fluorescent 293T target cells, as described previously (12, 18). After 48 h of incubation, target cell GFP fluorescence was monitored by flow cytometry, providing a quantitative measure of a single round of infectivity. Data are presented relative to control, and the mean of three parallel experiments is indicated.

(Fig. 2*D*). As reported previously by several laboratories, the infectivity of Vif-deficient HIV was decreased strongly by A3G, moderately by A3F, and weakly by A3B (160-, 16-, and 2-fold, respectively) (*e.g.* Refs. 11–14). Expression of A3D caused little effect. These controls are important because they show that the L1 inhibition data are not simply

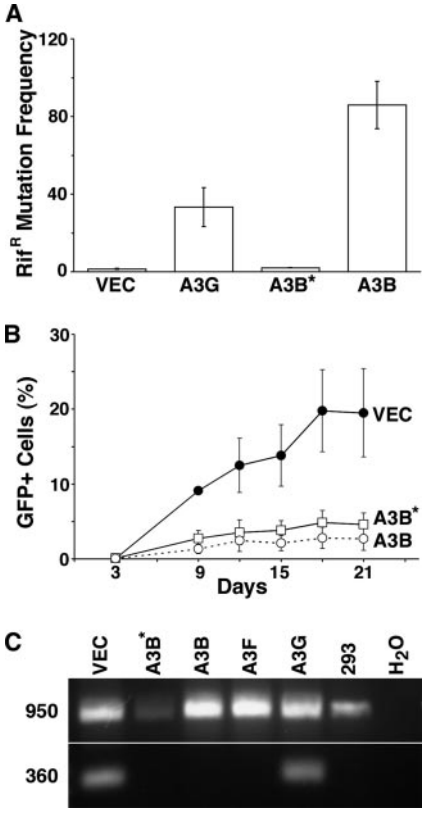

FIGURE 3. **The inhibition of L1 retrotransposition does not require DNA cytosine deaminase activity.** *A*, the intrinsic DNA cytosine deaminase activity of A3B compared with A3G. *VEC*, control vector. *B*, L1 inhibition by catalytically active and inactive A3B proteins. The *asterisk* denotes the $A3B_{W228L, D316N}$ construct. This experiment was performed as described in the legend for Fig. 2 and, for comparison, the A3B data from Fig. 2*B* are shown (*dotted line*). *C*, an ethidium bromide-stained agarose gel showing less integrated L1 DNA in cells expressing A3B or A3F (a 360-bp specific band). A nonspecific higher molecular weight band (~950 bp) indicates that similar amounts of genomic DNA were used in each PCR reaction. See "Experimental Procedures" for additional details.

phenocopies of the HIV restriction activities of the human A3 proteins. Moreover, these data strongly suggest that other trivial explanations for the L1 phenotype (such as nonspecific nucleic acid binding) are not likely. For instance, A3G potently restricts many LTR-dependent retroviruses (Fig. 2*D*) (see also Refs. 1–15), and it binds single-strand nucleic acid (17), but it clearly does little to the rates of L1 retrotransposition (Fig. 2*C*).

To begin to dissect the mechanism of L1 inhibition, we isolated a DNA cytosine deaminase-deficient A3B mutant, $A3B_{W228L, D316N}$. Several previous studies have demonstrated that expression of human A3 proteins in *E. coli* causes a mutator phenotype, thereby providing a sensitive readout of the intrinsic DNA cytosine deaminase activity of these proteins (*e.g.* Refs. 18 and 30). *E. coli* expressing A3B and A3G showed, respectively, 58- and 23-fold increases in the frequency of mutation to rifampicin resistance (Rif^R; Fig. 3*A*). In comparison, control vector- and $A3B_{W228L, D316N}$-transformed cells showed much lower and nearly identical levels of Rif^R mutation, which were attributable to spontaneous mutations that occur during *E. coli* growth. The *E. coli* studies therefore demonstrated that $A3B_{W228L, D316N}$ is not able to catalyze DNA cytosine deamination and that catalytically active A3B is a potent DNA mutator.

To address whether deaminase activity is required for L1 inhibition, retrotransposition assays were done using 293 clones stably expressing a vector control or $A3B_{W228L, D316N}$. Interestingly, the catalytically inactive A3B protein also caused 5–10-fold reductions in the rate of L1 retrotransposition (Fig. 3*B*). These data indicated that the L1 inhibition activity of A3B is independent of deaminase activity. In further support of such a mechanism, retrotransposed L1 DNA showed no signs of the hallmark, strand-specific C/G to T/A transition mutations that are commonly found in A3-restricted LTR-type retroelements. Between 7 and 22 retrotransposed L1-GFP substrates were sequenced for each experimental condition, and only three base substitutions were found in 26,640 bp sequenced (see "Experimental Procedures"). In contrast, HIV experiments with A3F and A3G yielded 8 and 21 C → T transitions, respectively, per 1000 bp of viral DNA analyzed (18). Finally, we noticed that the L1 PCR experiments repeatedly produced less intense or barely visible GFP-specific retrotransposition bands from the genomic DNA samples of cells expressing A3B or A3F (Fig. 3*C*). In many instances, an additional semi-nested PCR was required to visualize retrotransposed L1 DNA (data not shown). All of these observations combined to strongly indicate that A3B and A3F interfere with L1 retrotransposition prior to integration by a process that does not require DNA cytosine deamination.

Initially, we were surprised that the L1 inhibition activity failed to correlate with obvious subcellular localization. However, the similar inhibitory potentials of A3B and A3F suggested that this activity might derive from a common domain. Indeed, these proteins are 96% identical between residues 66–190 and 65–189, respectively, whereas the remainder of these polypeptides shares less than 57% identity. It is likely that this region of concentrated identity mediates L1 inhibition. This is particularly interesting because the corresponding region of A3G is less than 50% identical, and it is required for associating with HIV-1 Gag and thereby mediating its specific encapsidation (17). By analogy, this region of A3B and A3F may mediate L1 inhibition by associating with the L1 ORF2 protein, which, like the nucleocapsid region of HIV Gag, binds both zinc and nucleic acid (26). This ORF2 region is conserved among L1 and related elements. Thus, an A3-ORF2 association may also interfere with the mobilization of related autonomous retroelements and with some non-autonomous ones, such as Alu, because they require ORF2 for transposition. Additional studies on the mechanism of L1 inhibition are clearly warranted.

As regards the physiological relevance of the L1 inhibition activity reported here, a key prediction is that A3B or A3F should be expressed in tissues in which L1 retrotransposition occurs, namely in the cells of the human germline (33). A3B antibodies are presently unavailable, and transcripts are relatively rare as GenBank BLAST searching revealed less than six full-length A3B mRNAs and approximately 12 expressed sequence tags (mainly from cancer cells or tissues). The current lack of data and reagents prevents immediate conclusions regarding A3B. A3F transcripts have been detected in a broad number of human tissues, although mRNA from testes and ovaries was not examined (12, 14). However, the Human Protein Atlas consortium has developed a polyclonal A3F-specific antibody, and immunohistochemistry experiments involving nearly 50 human tissues revealed strong levels of A3F protein expression in spleen and testes (34). A subset of these images is shown in the supplemental materials, in which strong A3F expression is clearly apparent in the seminiferus epithelium region of the testes (Fig. S2). Female germ cells were not specifically examined. Nevertheless, this striking expression profile implies that A3F might constitute a male germ cell-specific barrier to L1 retrotransposition.

The primate A3 proteins are under a strong positive selection (35). Taken together with the data presented here, it seems likely that one of their major, evolutionarily conserved functions is to inhibit the mobility of L1 and related nuclear retroelements. The intrinsically high level of retroelement genetic variation would thereby help select for propor-

tionately adaptable A3 proteins, the genes of which expanded from one in rodents to seven in humans. This affords a probable explanation for why L1 retrotransposition levels are much higher in germ cells of mice than in humans (25). Moreover, periodic episodes of uncontrolled mobilization, which have clearly occurred in the past for L1 (36) (and which may be occurring now with HIV), may be explained by the acquisition of new retroelement genetic variations that render it A3-resistant and/or by the cross-species transmission of an element resistant to the A3 protein repertoire of the new host.

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