

Species-Specific Restriction of Apobec3-Mediated Hypermutation[∇]

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Apobec proteins are a family of cellular cytidine deaminases, among which several members have been shown to have potent antiviral properties. This antiviral activity is associated with the ability to cause hypermutation of retroviral cDNA. However, recent research has indicated that Apobec proteins are also able to inhibit retroviruses by other mechanisms that are independent of their deaminase activity. We have compared the antiviral activities of human and murine Apobec3 (A3) proteins, and we have found that, consistent with previous reports, human immunodeficiency virus (HIV) is able to resist human A3G but is sensitive to murine A3, whereas murine leukemia virus (MLV) is relatively resistant to murine A3 (mA3) but sensitive to human A3G. In contrast to previous studies, we observed that mA3 is packaged efficiently into MLV particles. The C-terminal cytidine deaminase domain (CDD2) is required for packaging of mA3 into MLV particles, and packaging did not depend on the MLV viral RNA. However, mA3 packed into MLV particles failed to cause hypermutation of viral DNA, indicating that its deaminase activity is blocked or inhibited. hA3G also caused significantly less hypermutation of MLV than of HIV DNA. Both mA3 and the splice variant mA3Δ5 exhibited some residual antiviral activity against MLV and caused a reduction in the ability of MLV particles to generate reverse transcription products. These results suggest that MLV has evolved specific mechanisms to block the ability of Apobec proteins to mediate deaminase-dependent hypermutation.

Apobec proteins are a family of cytidine deaminase enzymes, among which several have been identified as having antiviral properties, and hence constitute an important part of the innate immune response to retroviruses (5). At least 12 Apobec genes (*AID*, *APOBEC1*, *APOBEC2*, *APOBEC3A-H*, and *APOBEC4*) are encoded in the human genome, whereas the mouse genome contains only five members of this family (*Apobec1* to *-4* and *AID*) (25). *AID* is critical for antibody class switching and affinity maturation in B lymphocytes, while *Apobec1* directs a site-specific deamination in the mRNA for apolipoprotein B. The *in vivo* roles of *Apobec2*, *-3*, and *-4* remain unclear, although it has recently been demonstrated that mice lacking *Apobec3* exhibit increased susceptibility to MMTV infection (23). In 2002, Sheehy et al. showed that human APOBEC3G (hA3G) was a potent inhibitor of human immunodeficiency virus (HIV) replication if the virus was deficient in *Vif* (26). Subsequently, *Vif* was shown to counteract the antiviral effect of hA3G by binding to it and promoting its proteasomal degradation (17, 27, 28, 32). *Vif* is unable to counteract the murine mA3 protein, however, and HIV is highly susceptible to inhibition by mA3 (16). Several other members of the human Apobec family have been shown to have antiviral properties: APOBEC3A potently inhibits endogenous retroelements such as MusD and IAPs (4); APOBEC3F inhibits HIV, simian immunodeficiency virus (SIV), and murine leukemia virus (MLV) (34); and APOBEC3B and APOBEC3C inhibit HIV and SIV (31). hA3G also inhibits

replication of hepatitis B virus and endogenous retroviruses (8).

Although mouse Apobec3 inhibits HIV, it is considerably less effective in blocking replication of the mouse gammaretrovirus MLV (7, 16), even though MLV does not encode a homolog of *Vif*. Thus, both human and murine retroviruses have evolved mechanisms to resist Apobec3 proteins in their respective hosts, but these mechanisms are unable to defend the viruses against Apobec3 proteins from a different species.

Several models have been proposed to explain the ability of MLV to resist mA3. These include exclusion of mA3 from MLV virions (7) and degradation of mA3 by the MLV protease (1). The murine *Apobec3* gene consists of nine exons and is expressed as at least two distinct splice variants: a full-length 430-amino-acid protein and a shorter form that lacks amino acid residues 198 to 230 encoded by exon 5 (mA3Δ5) (16). mA3 and mA3Δ5 both contain two cytidine deaminase domains (CDD1 and CDD2). A distinct role for the mA3Δ5 splice variant has not been reported, although one group found that mA3Δ5 was resistant to proteolysis by the viral protease, thus rendering it a more potent inhibitor of MLV (1).

The precise mechanism by which Apobec members inhibit viruses has been controversial. Apobec proteins can be packaged into budding retroviral particles through mechanisms that are still unclear and can inhibit viral infectivity by causing hypermutation in proviral cDNA. The C-terminal cytidine deaminase domain of hA3G is absolutely required for this hypermutation (20). Other groups have found, however, that hA3G can inhibit some viruses (human T-cell leukemia virus and hepatitis B virus) without causing hypermutation and that a mutant form of hA3G that lacked the ability to cause deamination and hypermutation was still capable of inhibiting HIV (22). The precise mechanism of this deamination-independent retroviral inhibition remains unknown, but some groups have

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noticed that HIV made in the presence of hA3G or hA3F exhibited reduced accumulation of reverse transcription products in the absence of deamination (2, 12, 18, 30). Furthermore, in some nondividing cell types, such as dendritic cells and resting T cells, hA3G is found in a low-molecular-mass complex that can inhibit early steps in HIV infection (6, 24). It has also been proposed that hA3G can inhibit integration of HIV by interacting with the integrase protein (15).

In the present study we investigated the effect of murine Apobec3 against MLV, and found that it can be packaged into MLV particles with an efficiency similar to its incorporation into HIV but that its ability to cause G-to-A hypermutation is restricted. Packaging of mA3 into MLV was equally efficient in the presence or absence of MLV viral RNA and depended on the CDD2 domain of mA3. Also, mA3 and mA3Δ5 do possess some inhibitory activity against MLV, although this is less potent than their inhibition of HIV. mA3 and mA3Δ5 were able to inhibit the ability of MLV particles to generate reverse transcripts in infected cells. These activities may account for the residual ability of mA3 to inhibit MLV replication.

MATERIALS AND METHODS

Cells and viruses. The wild-type MLV clone used in these experiments was pNCS and was provided by Stephen Goff. This is a full-length replication competent ecotropic Moloney MLV clone. For MLV infectivity assays, this clone was cotransfected with pMIGR, a retroviral derived expression plasmid that contains an internal ribosome entry site-green fluorescent protein (GFP) cassette. For the experiments described in Fig. 1, a replication-competent clone of the HXB2 strain of HIV was used. For all other experiments, the HIV clone used was a virus lacking *env*, *nef*, and *vif* derived from NL4-3 with a GFP cassette inserted into the *nef* open reading frame. MLV virus-like particles (VLPs) were generated by using a pCL-10A1 MLV plasmid that lacks the RNA packaging element (21).

Apobec expression plasmids consist of cloned cDNAs with C-terminal hemagglutinin (HA) tags in the pCDNA3 vector (mA3 NCBI accession number NM_030255). The full-length mA3 plasmid was kindly provided by Bryan Cullen. Three cell lines were used: 293T cells, NIH 3T3 cells, and GHOST-X4 (a human osteosarcoma-derived cell line with a GFP expression cassette under the control of the HIV long terminal repeat). These cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and penicillin-streptomycin.

Infectivity assays. Virus particles were produced by transient transfection of 293T cells with plasmid DNA using the calcium phosphate method, except for the data shown Fig. 2C, for which virus particles were produced by the transfection of 3T3 cells using Lipofectamine (Invitrogen). At 48 h posttransfection, viral supernatants were centrifuged for 5 min at low speed to remove cellular debris and then filtered through a 0.45-μm-pore-size filter. Part of the supernatant was centrifuged at 80,000 × g for 90 min to pellet virus particles and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. These pellets were subjected to immunoblotting for p30 (MLV) or p24 (HIV) to quantify the relative amounts of virus in each supernatant. Equivalent amounts of supernatant were then used to infect subconfluent NIH 3T3 cells in the presence of 10 μg of Polybrene/ml. At 48 h postinfection, the 3T3 cells were washed with phosphate-buffered saline (PBS), treated with trypsin, and analyzed for GFP expression by flow cytometry on an LSRII cytometer. Titers of infectious virus were estimated from the fraction of GFP⁺ cells within the population and normalized to the level of virus as estimated by Western blotting.

Protein analysis. Cellular protein was harvested by lysis of cells with radioimmunoprecipitation assay buffer (50 mM Tris [pH 8], 5 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1% SDS, 1% NP-40, 1% deoxy-cholate, 1% Triton X-100). Genomic DNA was removed by centrifugation, and the supernatant was mixed with an equal volume of SDS-PAGE loading buffer (0.5 M Tris [pH 6.8], 30% glycerol, 4% SDS, 0.2% bromophenol blue). To analyze purified virus particle proteins, viral supernatants were centrifuged at low speed to remove cellular debris and then filtered through a 0.45-μm-pore-size filter. Virus particles in the supernatant were then purified by ultracentrifugation at 80,000 × g for 90 min through 20% sucrose and resuspended in SDS-PAGE loading buffer. Protein samples were then analyzed by electrophoresis on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and subjected to Western blotting in

TBS-Tween (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20) with 2% nonfat milk powder. HA-tagged proteins were detected by using a polyclonal anti-HA antiserum (Zymed). MLV p30 was detected by using polyclonal antiserum provided by Rob Gorelick (NCI). HIV p24 was detected by using a monoclonal antibody (National Institutes of Health).

Virus fractionation. Virus particles were generated by transfection of 293 cells and purified as described above. Viral pellets were resuspended in PBS, and Triton X-100 was added to a final concentration of 1%. After 2 min of incubation, the suspensions were centrifuged at 8000 × g for 10 min. Pellet and supernatant samples were separated and then mixed with SDS-PAGE loading buffer.

Quantitative PCR. Cellular DNA was extracted from infected cells by washing with PBS, trypsin treatment, and resuspension in lysis buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 10 mM NaCl, 0.5% SDS, 1 mg of proteinase K/ml). After incubation for several hours at 37°C, DNA was precipitated by adding 2 volumes of ethanol. Pelleted DNA was then dissolved in distilled H₂O over several hours. Viral DNA was then quantified by using real-time PCR in a Bio-Rad iCycler with SYBR Green Master Mix (Bio-Rad). The primer sets used were as follows. (i) For "early" reverse transcription products (designed against the R-U5 region of MLV), we used R-F (GCGCCAGTCTCCGATTGAC) and U5-R (CGTGACGGGTAGTCAATCACTC). (ii) For "late" reverse transcription products (designed against MLV Gag), we used Gag-F (TAGAGGCTAGAAAGGCGGTGCGG) and Gag-R (CTGGCGATAGTGGACTAGGTGGTTCC). For analysis of MLV viral DNA accumulation (see Fig. 5A), primer sets designed against the eGFP cassette encoded in the virus genomes were used: GFP-F (ATGGTGAGCAAGGGCGAGG) and GFP-R (ATGGGGGTGTTCTGCTGGTAG). Cellular DNA was also quantified by using a primer set designed against the mouse *Apobec3* locus: mA3F (GTTTGATCTTCGGCAGCACAGG) and mA3R (TC TGC CTC CCA TAG GTT AGA TGG). When we quantified viral DNA, we normalized real-time PCR signals for viral DNA to the cellular DNA control and to the amount of virus in the supernatant used for infection (as determined by αCA Western blotting).

Hypermutation analysis. Infected cells were harvested, lysed, and DNA precipitated as described above. The GFP cassette in the viral genome was then amplified by using GFP-specific primers GFP-F and GFP-R (described above). The PCR products (566 nucleotides) were TA cloned into the TOPO-2.1 vector (Invitrogen), and inserts were sequenced. Fifteen to twenty inserts, amounting to 8 to 12 kb, were sequenced for each virus-Apobec combination.

Site-directed mutagenesis. Site-directed mutagenesis was performed by overlapping PCR. For mA3Δ5-CCAA1 and mA3Δ5-CCAA2, the two conserved cysteine residues at the active sites were converted to alanines. All clones were verified by sequencing.

RESULTS

Species-specific activities of human and mouse Apobec3 proteins. Previous studies have indicated that MLV is relatively resistant to the antiviral effects of the murine Apobec3 protein but sensitive to inhibition by hA3G (3, 7, 13, 16). We wanted to compare the potency of human A3G and mouse A3 in terms of their ability to inhibit the replication of MLV or HIV. Consistent with the earlier studies, we found that cotransfecting increasing amounts of mA3 with MLV only weakly inhibited the infectivity of MLV particles that were produced, whereas hA3G potentially restricted MLV infectivity (Fig. 1, left panel). mA3Δ5 was a slightly more potent inhibitor of MLV, despite being expressed at a slightly lower level. In contrast, HIV replication was highly sensitive to inhibition by mA3 and mA3Δ5 but relatively resistant to hA3G, likely due to the action of Vif (Fig. 1, right panel). These results confirm that both HIV and MLV have evolved mechanisms to resist their host species Apobec3 proteins.

mA3 is packaged efficiently into MLV particles. Other investigators have reported that mA3 is excluded from MLV and that this accounts for the relative resistance of MLV to mA3 (7, 13). To investigate this hypothesis, MLV or HIV (lacking *vif* [*vif*−]) were produced in the presence of HA-tagged mA3Δ5,

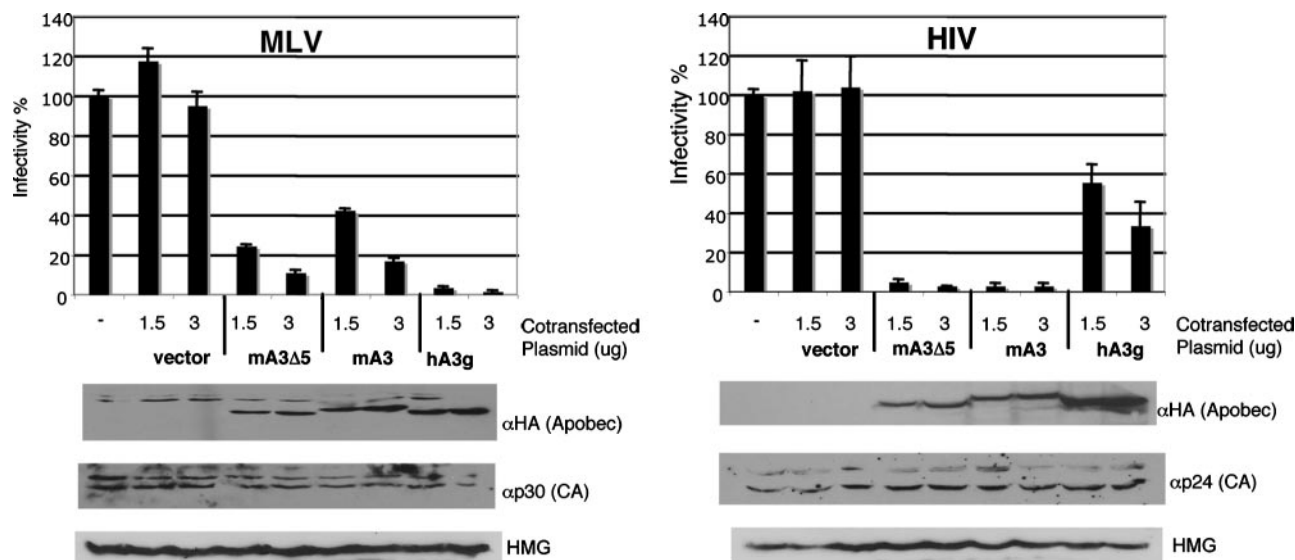


FIG. 1. Species-specific activities of human and mouse Apobec3 proteins. 293T cells were transfected with 10 μ g of MLV or 10 μ g of HIV plasmid DNA, as well as various amounts of HA-tagged Apobec plasmids (0, 1.5, or 3 μ g) or control vector. At 48 h posttransfection, viral supernatants were used to infect either NIH 3T3 cells (for MLV) or CD4- and CXCR4-expressing HOS cells with a GFP reporter under the control of the HIV long terminal repeat promoter (for HIV). At 24 h postinfection, the level of infectious virus in the supernatant was measured by flow cytometry of the infected cells. Infectivity is displayed as a percentage of that for virus alone. The data shown are the average of two independent experiments. Cellular protein from transfected cells was Western blotted for the presence of Apobec proteins (HA), the cellular loading control HMG, and p30 (MLV) or p24 (HIV).

mA3, hA3g, or empty vector. When we examined purified virus particles, we found that similar levels of all Apobec3s were packaged into MLV (Fig. 2A). HIV, by comparison, was more efficient at packaging hA3G than any of the other Apobec3s. Curiously, mA3Δ5 caused an increase in the level of unprocessed Gag in MLV particles. Neither hA3G nor mA3 had any effect on the level of unprocessed Gag in MLV particles, and hA3G and mA3 had little effect on the processing of HIV Gag. Thus, the effect of mA3Δ5 on Gag processing was specific to MLV.

It has previously been reported that mA3 is cleaved by the viral protease inside MLV particles. Although we observed some minor proteolysis of mA3, as well as mA3Δ5 and hA3G, in MLV, the level was insufficient to account for the differential sensitivity of MLV to mA3 and hA3G (Fig. 2A).

Although we observed incorporation of mA3 into MLV (Fig. 2A), we wanted to directly compare the level of mA3 incorporated into equivalent numbers of HIV and MLV particles. First, we purified MLV and HIV particles made in the presence of mA3Δ5 and used SDS-PAGE and Coomassie blue staining to quantify the amount of virus particles in each stock. We then performed Western blotting on equivalent amounts of HIV and MLV particles to determine the efficiency with which mA3Δ5 was incorporated. Surprisingly, HIV and MLV were found to incorporate similar levels of mA3Δ5 (Fig. 2B). Similar results were obtained when the experiment was repeated using mA3, and HIV and MLV were found to incorporate equivalent levels of mA3 over a wide range of mA3 expression levels, indicating that incorporation of mA3 into MLV was not an artifact of overexpression (not shown). This indicates that virion exclusion of mA3 or mA3Δ5 does not

explain the relative resistance of MLV to these proteins compared to HIV.

Next, we wanted to confirm that mA3 is incorporated into MLV particles using a mouse cell line. We cotransfected MLV DNA into NIH 3T3 cells with mA3Δ5 or mA3. At 48 h posttransfection, virus particles were purified from the cellular supernatant and Western blotted for p30 and the presence of Apobec proteins. We found that both mA3Δ5 and mA3 were present in MLV produced in 3T3 cells (Fig. 2C). Interestingly, the increased level of unprocessed Gag in MLV preparations caused by mA3Δ5 in 293 cells was not observed in 3T3 cells, indicating that this effect is specific to 293 cells.

To determine the location of the Apobec proteins in the virus particles, we produced MLV or HIV particles in the presence of mA3Δ5, mA3, and hA3g. Purified virus particles were treated with Triton X-100 to remove the viral envelope, and viral cores were pelleted by centrifugation. mA3Δ5, mA3, and hA3G were all located predominantly in the pellet for MLV and HIV, indicating that they were incorporated into the viral core (Fig. 2D). A subset, however, partitioned into the supernatant. This result indicates that exclusion from viral cores does not explain the relative resistance of MLV to mA3 and mA3Δ5.

MLV packaging of mA3 requires domain CDD2 but not the viral RNA. Apobec proteins are able to bind RNA, and it is likely that they can be recruited into viral particles by an interaction with the viral genomic RNA. It has been reported that MLV VLPs incorporate mA3 at a higher level in the absence of viral RNA (vRNA) than in its presence (1), indicating that the MLV vRNA may play a role in limiting the amount of mA3 that is packaged into MLV virions. We inves-

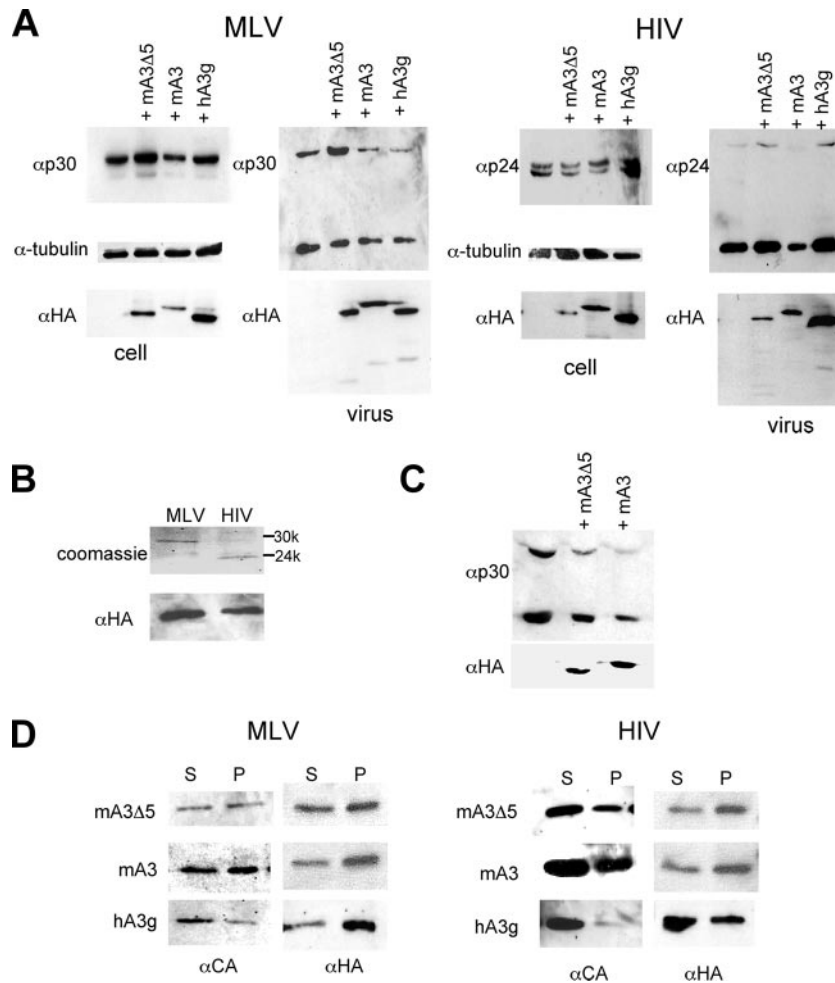


FIG. 2. mA3 is packaged efficiently into MLV particles. (A) MLV or HIV particles were produced by transfecting 293T cells with 10 μ g of MLV or HIV (*vif*⁻) DNA with 3 μ g of mA3, mA3 Δ 5, hA3g, or empty vector. At 48 h posttransfection, transfected cellular protein and purified virus particles were isolated and Western blotted for CA, tubulin, or Apobec proteins (HA). (B) The efficiency of mA3 Δ 5 incorporation into HIV and MLV was compared by transfecting 293T cells with 10 μ g of viral DNA plus 3 μ g of mA3 Δ 5 DNA. At 48 h posttransfection, virus was purified from the supernatant. Purified virus was then analyzed by SDS-PAGE and Coomassie blue stain to compare the relative levels of HIV and MLV and Western blotted for mA3 Δ 5 (HA) to assess incorporation. (C) MLV particles were produced by transfecting 3T3 cells with 10 μ g of MLV alone or with 3 μ g of mA3 Δ 5 or mA3. At 48 h posttransfection, virus was purified from supernatant and then analyzed by Western blotting for CA (p30) or mA3 Δ 5 and mA3 (HA). (D) MLV or HIV particles were produced by transfecting 293T cells with 10 μ g of MLV or HIV DNA with 3 μ g of mA3, mA3 Δ 5, or hA3g. At 48 h posttransfection, purified virus particles were isolated, resuspended, and mixed with Triton X-100. After centrifugation, the detergent-soluble supernatant (S) and the insoluble pellet (P) were Western blotted for CA (p30 or p24) or Apobec proteins (HA).

tigated this hypothesis by producing MLV particles with mA3 or mA3 Δ 5 in the presence or absence of vRNA. We found that the presence of vRNA did not affect the level of mA3 Δ 5 incorporation into MLV and that mA3 was incorporated at a slightly lower efficiency in the presence of vRNA (Fig. 3A). Since Apobec proteins are recruited into virus particles by an RNA interaction (33), it is likely that either viral or cellular RNAs can mediate incorporation of mA3 into MLV.

Previous studies of human A3G have found that the two cytidine deaminase domains of this molecule have distinct functions. The N-terminal domain (CDD1) was found to be important for viral packaging, while the C-terminal domain (CDD2) was required for deamination activity (20). The cytidine deaminase domains of murine A3, in contrast, have a reversed functional organization: CDD1 is required for deamination, whereas CDD2 is required for packaging in HIV (11).

In order to determine the role played by the cytidine deaminase domains of mA3 Δ 5 in its antiviral activities, we constructed mutant versions with the conserved cysteine residues within the zinc finger of either the N-terminal (mA3 Δ 5-CCAA1) or C-terminal (mA3 Δ 5-CCAA2) cytidine deaminase domain converted to alanines. These mutant proteins accumulated to considerably lower levels in transfected cells than wild-type mA3 Δ 5 (Fig. 3B). It is possible that mutations in the cytidine deaminase domains affect the stability of the mA3 Δ 5 proteins, although, notably, it has been shown that such mutations did not affect the stability of full-length mA3 (11). Thus, this phenomenon may be specific to the mA3 Δ 5 variant.

Production of MLV particles in the presence of wild-type or mutant forms of mA3 Δ 5 demonstrated that mA3 Δ 5-CCAA1 was incorporated into MLV particles at a level relative to wild-type that was proportional to its relative cellular expres-

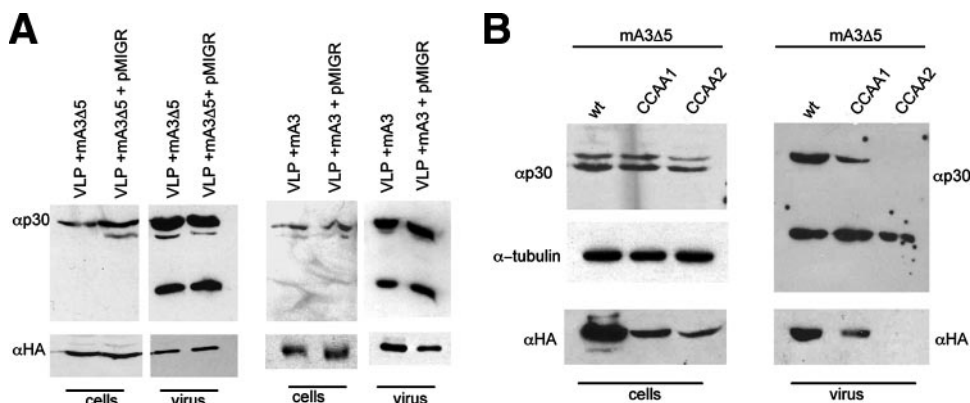


FIG. 3. CDD2 but not viral RNA is required for m3 packaging in MLV. (A) MLV VLPs were generated by transfection of 293T cells with a plasmid that expresses all MLV viral proteins but lacks an RNA packaging element. VLPs were produced in the presence of m3Δ5 (left panels) or m3 (right panels) and in the presence or absence of a viral RNA (pMIGR). At 48 h posttransfection, both transfected cells and purified MLV particles were Western blotted for the presence of m3Δ5 or m3. (B) 293T cells were transfected with 10 μ g of MLV DNA and 3 μ g of m3Δ5, m3Δ5-CCAA1, or m3Δ5-CCAA2. At 48 h posttransfection, transfected cells and virus particles purified from the supernatant were Western blotted for m3Δ5 and p30.

sion level (Fig. 3B). In contrast, m3Δ5-CCAA2 was not incorporated into MLV. This indicates that CDD2 but not CDD1 is required for the viral packaging of m3Δ5 into MLV. We also examined the antiviral activities of the m3Δ5 mutants. Both m3Δ5-CCAA1 and m3Δ5-CCAA2 exhibited reduced ability to inhibit HIV, as well as MLV, relative to wild-type m3Δ5 (not shown), although the relatively poor expression levels of the mutant proteins precludes making a definitive estimate of the role of the CDDs in antiviral activity of m3Δ5.

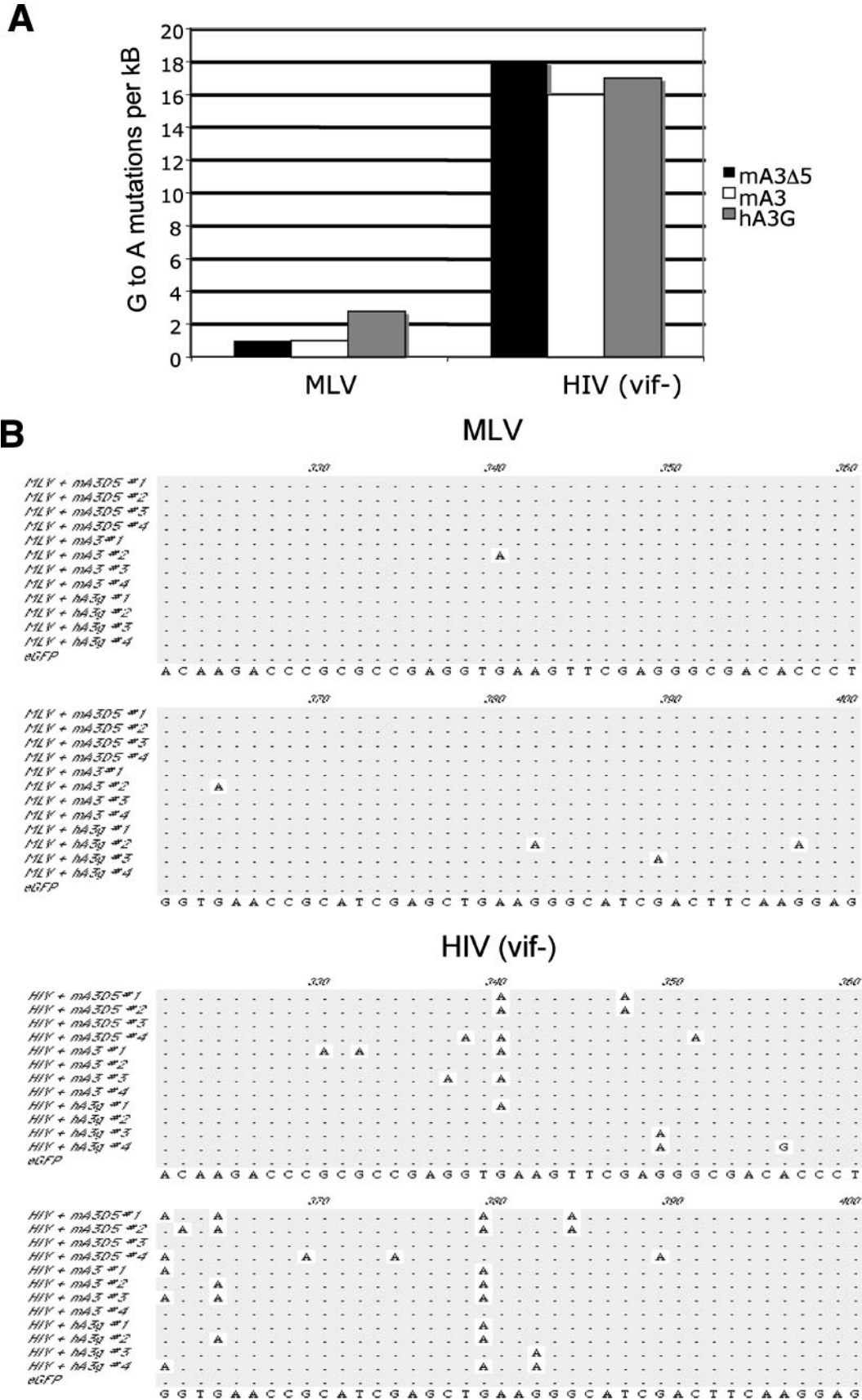
MLV blocks Apobec mediated hypermutation. Since Apobec family members are able to cause extensive hypermutation to cDNA derived from numerous retroviruses, we sought to determine whether differences in deamination could underlie the resistance of MLV to m3 and m3Δ5. We generated stocks of HIV (*vif*⁻) or MLV in the presence of m3Δ5, m3, or hA3G and then used these to infect cells. Cellular DNA was isolated from infected cells, and viral sequences were amplified by PCR, cloned, sequenced, and examined for mutations. Clones were considered hypermutated if they contained more than two G-to-A mutations in the amplified region (566 nucleotides). We confirmed by PCR for plasmid sequences that the cellular DNA sample was not contaminated with plasmid DNA carried over from the transfection. Sequencing of viral DNA from virus-infected cells revealed that m3Δ5, m3, and hA3G caused extensive G-to-A hypermutation of HIV (*vif*⁻) (Fig. 4). The level of mutation we observed in *vif*⁻ HIV (17 per kb or 1.7%) in the presence of hA3G was consistent with previous reports (16). Surprisingly, clones of MLV DNA made in the presence of m3Δ5 or m3 showed few or no G-to-A mutations. Furthermore, although hA3G was able to cause some G-to-A hypermutation of MLV DNA, the proportion of MLV viral DNA clones containing hypermutation (3 of 20 clones) was significantly lower than for HIV (*vif*⁻), in which virtually all clones exhibited hypermutation. This suggests that m3 or m3Δ5 that is incorporated into MLV is in a complex or compartment in which its ability to cause deamination is prevented and that this feature of MLV may have evolved as a defensive strategy to avoid the antiviral activity of Apobec

proteins. Furthermore, these results indicate that the ability of MLV to restrict Apobec mediated hypermutation also extends, somewhat, to hA3G.

Accumulation of MLV DNA is reduced by m3. Although m3 and m3Δ5 inhibit MLV less potently than HIV, they nevertheless possess some residual anti-MLV activity that could play a significant role in inhibiting MLV replication in vivo (Fig. 1). Since inhibition of MLV by m3Δ5 and m3 does not involve extensive hypermutation of viral cDNA, we examined whether other early steps in infection of target cells are affected. We measured the accumulation of viral DNA in target cells by real-time PCR of infected cells at 48 h postinfection. The data were normalized to a cellular DNA control. We found that for MLV made in the presence of m3Δ5, m3 or hA3G, accumulation of viral DNA in target cells was significantly reduced at 48 h postinfection (Fig. 5A). We also examined the effect of m3Δ5 on accumulation of MLV reverse transcripts earlier in infection (12 h postinfection). Both "early" and "late" MLV reverse transcription products were reduced in the presence of m3Δ5 (Fig. 5B). We conclude that m3Δ5 affects MLV either very early in reverse transcription or prior to reverse transcription. This reduction in viral DNA accumulation for MLV was quantitatively equivalent to the reduction in viral infectivity caused by m3Δ5, suggesting that it is sufficient to explain the antiviral properties.

DISCUSSION

Several Apobec proteins exhibit the ability to cause extensive hypermutation of retroviral cDNA, and this activity very likely has a key role in inhibition of viral infectivity. However, it has recently become increasingly clear that some Apobec proteins are also capable of deaminase-independent modes of viral inhibition, although the details of the molecular mechanism(s) by which this occurs remain unclear. Several groups have shown that reverse transcripts fail to accumulate in the absence of deaminase activity (2, 9, 12, 14, 18), suggesting that this antiviral activity of Apobec occurs early in the retroviral replication cycle.



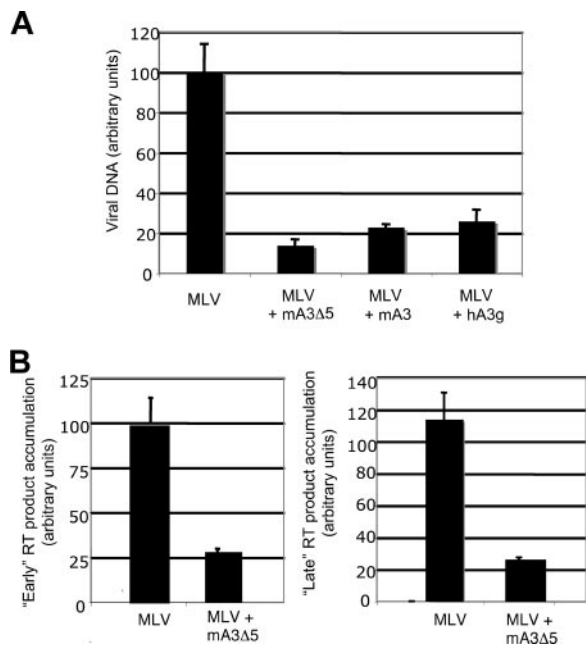


FIG. 5. Accumulation of MLV DNA is reduced by mA3. (A) MLV particles were produced by transfection of 293T cells with 10 μg of MLV DNA alone or in the presence of mA3Δ5, mA3 or hA3G. Viral supernatants were then used to infect NIH 3T3 cells. At 48 h postinfection, cellular DNA was isolated, and the level of viral DNA was measured by real-time PCR. This measurement was normalized to a cellular genomic DNA control. (B) Accumulation of MLV "early" and "late" reverse transcripts was analyzed identically to the procedure describe for panel A except that cellular DNA was isolated at 12 h postinfection.

Both HIV and MLV have evolved strategies that facilitate species-specific resistance to their corresponding Apobec3 proteins. HIV is able to protect itself from hA3G by targeting it for proteasomal degradation through the actions of the virally encoded Vif protein. It has been previously reported that MLV resists the antiviral mA3 protein by two mechanisms (1, 11). First, the viral RNA prevents incorporation of mA3 into virus particles. Second, the viral protease causes cleavage of mA3 within the sequence encoded by exon 5. The mA3Δ5 splice variant was found to be resistant to this proteolysis and was consequently more active against MLV (1). We found that the mA3Δ5 variant is slightly more potent than mA3 in its antiviral activity against MLV, although we did not observe significant levels of proteolysis of either mA3 or mA3Δ5.

We found that, in contrast to these studies, the mouse Apobec3 protein can be packaged efficiently into MLV particles but is restricted from carrying out hypermutation of retroviral cDNA, and we propose that this accounts for the relative resistance of MLV to mA3. It is possible that technical details

in how these experiments were performed could explain the difference in the results.

The lack of hypermutation we observe with MLV and mA3 is consistent with a previous report (3), although that study did not examine the level of mA3 incorporation into virus particles. mA3 and mA3Δ5 are fully capable of deaminase activity and cause extensive hypermutation when they are incorporated into HIV. It will be important to determine the reasons for why mA3 and mA3Δ5 behave differently in different viruses. We cannot exclude, however, the possibility that hypermutated MLV cDNA is degraded more rapidly or efficiently than hypermutated HIV cDNA, thereby preventing the integration of mutated MLV proviruses and thus eluding experimental detection.

This finding is also reminiscent of the failure of an A3F-A3G chimera to cause lethal hypermutation to HIV, despite being encapsidated efficiently and being fully capable of deamination in *Escherichia coli* (10). These results suggests that Apobec protein activity is regulated by a step that occurs between encapsidation and hypermutation and that MLV may defend itself from mA3 by blocking this step.

It is possible that, inside MLV particles, mA3 is in a complex or compartment that inhibits its deaminase activity or limits its access to the viral nucleic acid substrate. We found that the majority of mA3 inside MLV particles was not removed from virus particles by Triton X-100, indicating its presence inside the viral core. It is possible that it fails to interact with the viral RNA or interacts with the RNA in a way that permits inhibition of reverse transcription but does not permit deamination-dependent hypermutation.

Surprisingly, we also observed that hA3G exhibited a significantly lower ability to cause hypermutation in MLV relative to HIV. Although virtually all HIV DNA sequences generated in the presence of hA3G exhibited the G-to-A hypermutation, only a minority of MLV sequences were mutated. This indicates that the ability of MLV to inhibit the deaminase activity of mA3 applies to hA3G as well, although this block is not strong enough to eliminate all hypermutation. In light of this observation, it is interesting that hA3G strongly restricts MLV replication. It is possible that the level of mutations caused by hA3G in MLV is sufficient to potently interfere with viral replication, while the level of mutations seen in MLV is not. hA3G inhibited MLV DNA accumulation at a similar level to mA3 and mA3Δ5 (Fig. 5), and perhaps its antiviral activity is due to the combination of hypermutation with the inhibition of viral DNA synthesis. It is also possible that hA3G possesses additional hypermutation-independent antiviral activities, such as inhibiting integration (19).

Despite their failure to cause deamination, mA3 and mA3Δ5 do retain a modest ability to inhibit MLV replication, albeit less potently than their ability to inhibit HIV replication, and

FIG. 4. MLV blocks Apobec-mediated hypermutation. (A) MLV or HIV (*vif*⁻) particles were generated by transfecting 293 cells with 10 μg of viral DNA and 3 μg of mA3, mA3Δ5, or hA3g and used to infect 3T3 cells. At 48 h postinfection, cellular DNA was isolated from infected cells, and the viral GFP cassette (566 nucleotides) was amplified by PCR and TA cloned. A pool of 15 to 20 clones (8 to 12 kb) were sequenced for each virus-Apobec combination, and the number of G-to-A mutations was counted. (B) A representative panel of mutations for four clones from each virus-Apobec combination are shown.

we observed that MLV particles made in the presence of mA3Δ5 and mA3 exhibited a reduced ability to generate reverse transcripts in infected cells. We also found that, in 293 cells, mA3Δ5 exhibits a distinct deaminase-independent activity that involves the inhibition of the processing reaction of the viral Gag protein. Although such an activity may contribute to the antiviral properties of mA3Δ5 with respect to MLV, it remains unclear whether the activity is explicitly required. Indeed, this activity was not observed in 3T3 cells, suggesting that it may be restricted to 293 cells. The relative abundance of mA3Δ5 and mA3 *in vivo* is unclear, and it is unknown whether mA3 splicing is regulated in any way.

Mice lacking mA3 expression were recently shown to exhibit a higher susceptibility to MMTV infection and more rapid spread of virus to the spleen and lymph nodes that did not drain the site of inoculation (23). That study also showed that MMTV made in the presence of mA3 did not exhibit hypermutation, indicating that this virus may also have devised a strategy to evade deamination. It is unknown whether mA3-deficient mice exhibit enhanced susceptibility to MLV infection, but it is noteworthy that a previously described Friend MLV susceptibility locus, *Rfv-3*, was previously mapped to a 0.83-centimorgan region of chromosome 15 that contains the mouse *ApoBec3* gene (29).

It has been shown that hA3G causes a reduction in HIV reverse transcripts in infected cells (2), and a mutant hA3G that lacks the ability to cause deamination and hypermutation was still able to inhibit HIV replication (22). It seems likely, however, that both deamination-dependent and -independent activities contribute to the antiviral properties of ApoBec proteins. We also observed reduced MLV viral DNA accumulation in the presence of mA3Δ5, mA3, and hA3G, although it is unclear whether mA3Δ5 and mA3 are directly inhibiting the reverse transcription process or whether this is simply a consequence of a block at an earlier step in infection, such as maturation, entry, or uncoating. We also cannot exclude the possibility that mA3Δ5 and mA3 accelerate the degradation or decay of viral cDNA before integration. Furthermore, it is possible that they possess other deaminase-independent activities.

Together, these results suggest that existing models for the species-specific relative resistance of MLV to mA3 and mA3Δ5 may be incomplete and indicate that MLV is able to block or restrict the ability of these proteins to mediate deamination of the viral cDNA. Furthermore, we find that these proteins do exhibit some deamination-independent antiviral activity against MLV.

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