

1 INTERACTIONS OF MURINE APOBEC3 AND HUMAN APOBEC3G WITH  
2 MURINE LEUKEMIA VIRUSES

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9 Running Title: Inactivation of MLV by APOBEC3 Proteins

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24 ABSTRACT

25 APOBEC3 proteins are cytidine deaminases which help defend cells against  
26 retroviral infections. One antiviral mechanism involves deaminating dC residues in  
27 minus-strand DNA during reverse transcription, resulting in G:A mutations in the coding  
28 strand. We investigated the effects of mouse APOBEC3 (mA3) and human APOBEC3G  
29 (hA3G) upon Moloney murine leukemia virus (MLV). We find that mA3 inactivates  
30 MLV, but is significantly less effective against MLV than is hA3G. In contrast, mA3 is  
31 as potent against HIV-1 (lacking the protective Vif protein) as hA3G. The two  
32 APOBEC3 proteins are packaged to similar extents in MLV particles. Dose-response  
33 profiles imply that a single APOBEC3 molecule (or oligomer) is sufficient to inactivate  
34 an MLV particle. The inactivation of MLV by mA3 and hA3G is accompanied by  
35 relatively small reductions in the amount of viral DNA in infected cells. Although hA3G  
36 induces significant levels of G:A mutations in both MLV and HIV DNAs, and mA3  
37 induces these mutations in HIV DNA, no such mutations were detected in DNA  
38 synthesized by MLV inactivated by mA3. Thus, MLV has apparently evolved to partially  
39 resist the antiviral effects of mA3, and to totally resist the ability of mA3 to induce G:A  
40 mutation in viral DNA. Unlike the resistance of HIV-1 and human T-cell leukemia virus  
41 type I to hA3G, the resistance of MLV to mA3 is not mediated by exclusion of the  
42 APOBEC from the virus particle. The nature of its resistance, and the mechanism of  
43 inactivation of MLV by mA3, are completely unknown.

44 Mammalian cells contain a number of mechanisms for protection against  
45 retroviral infection and retrotransposition. One of these mechanisms involves members of  
46 the APOBEC3 (APO) family of cytidine deaminases (19, 45). The existence of this  
47 mechanism was first discovered through experiments on the HIV-1 accessory protein Vif;  
48 it has become clear in recent years that the intracellular binding of Vif to human  
49 APOBEC3G (hA3G) results in proteasomal degradation of the APO protein, thus  
50 preventing its incorporation into virions (35, 52). While the antiretroviral activity of  
51 hA3G was initially revealed in studies on HIV-1, it has recently become clear that hA3G  
52 is only one member of a family of cytidine deaminases (26), and that several APOBEC3  
53 family members are able to inhibit infections by a remarkable variety of retroviruses.  
54 Indeed, it appears that endogenous retroviruses and retrotransposons, as well as  
55 exogenous retroviruses, are sensitive to the effects of these cellular proteins (6, 11, 12,  
56 38). Interestingly, another retrovirus, human T-cell leukemia virus type 1 (HTLV-1),  
57 resembles HIV-1 in that it excludes hA3G from virions, but this exclusion involves an  
58 unusual, acidic region in the nucleocapsid domain of its Gag protein (9), rather than a  
59 nonstructural protein like Vif.

60 Although this system has been studied intensively for several years, there are a  
61 number of outstanding questions that remain to be resolved. One of these is how APO  
62 proteins interfere with the viral replication cycle. One obvious factor contributing to the  
63 inhibitory action of hA3G is the hypermutation of G to A residues; these mutations result  
64 from deamination by hA3G of C nucleotides in the single-stranded minus-strand DNA,  
65 the first product of reverse transcription during viral infection (18, 32, 53). However,

66 quantitative studies suggest that this effect is often not sufficient to explain the potent  
67 inhibition of infection by hA3G (22, 40).

68 One viral system for which the effects of APOBEC3 are not yet clear is the  
69 murine leukemia viruses (MLVs). It is known that hA3G is capable of interfering with  
70 MLV infectivity (18, 32). However, as mice and mouse cells are permissive for MLV  
71 replication, one might expect MLV to be resistant to the effect of murine APOBEC3  
72 (mA3), the sole member of the APOBEC3 family in mice. MLV is a “simple” retrovirus;  
73 that is, unlike HIV-1 and HTLV-1, MLV encodes only the three polyproteins that are  
74 assembled to form infectious progeny virions. Thus, if it is indeed resistant to mA3, this  
75 resistance cannot be attributed to an additional protein like Vif. Some reports indicate that  
76 mA3 is not incorporated into MLV particles (10, 29), perhaps because it is degraded by  
77 the viral protease (1), while others present evidence for efficient encapsidation of the  
78 protein without a significant antiviral effect (33).

79 In hopes of clarifying some of these questions, we have performed a quantitative  
80 comparison of the effects of mA3 and hA3G upon MLV and MLV-derived viral vectors.  
81 We now report that the murine protein is encapsidated to virtually the same extent as the  
82 human protein. It possesses significant inhibitory activity against MLV, but this activity  
83 is far lower than that of hA3G. The anti-MLV activity of mA3 does not seem to involve  
84 hypermutation.

85

86 MATERIALS AND METHODS

87

88 Cells and viruses. Unless otherwise noted, all experiments described here were performed  
89 with a full-length, infectious Moloney MLV proviral clone that was identical to the  
90 previously described clone pRR88 (14), except for the removal of 5' cellular flanking  
91 sequences. Virus particles were produced by transient transfection of 293T cells, using  
92 Transit 293 (Mirus) in accord with the manufacturer's instructions. When viruses were to  
93 be analyzed for infectivity, pBABE-Luc, in which the firefly luciferase gene from pGL3  
94 (Promega) has been inserted in the MLV-derived vector pBabe Puro (37, 44), was co-  
95 transfected with the MLV clone and with the appropriate APO plasmid. The total amount  
96 of plasmid DNA transfected into each culture was kept constant by addition of pUC-  
97 CMV plasmid as required; a typical transfection would include, per 10cm culture dish, 5  
98  $\mu\text{g}$  of MLV plasmid and 3  $\mu\text{g}$  of pBABE-Luc DNA in addition to the indicated amounts  
99 of APO expression plasmid.

100 Infectivity was measured by infecting 293T cells expressing the ecotropic MLV  
101 receptor mouse cationic amino acid transporter 1 (mCAT1) (2) (a kind gift of J.  
102 Cunningham [Harvard Medical School]), and assaying cell extracts for luciferase activity  
103 using the Luciferase Assay System (Promega) 48 hours after infection. (Similar results  
104 were also obtained in assays on NIH3T3 cells.) Luciferase assays were always performed  
105 in triplicate. Virions were also assayed for the level of replication-competent MLV using  
106 the S+L- focus assay (3) or for the titer of virus (derived from the pLXSH vector (36))  
107 encoding hygromycin resistance. In the latter assays, NIH3T3 cells were infected with  
108 serial dilutions of the sample. On the day after infection, the medium was replaced with  
109 fresh medium containing 200  $\mu\text{g}/\text{ml}$  hygromycin B (Invitrogen). The plates were fluid-  
110 changed periodically with hygromycin-containing medium, and finally fixed and stained

111 10 days after hygromycin treatment, when colonies of hygromycin-resistant cells were  
112 clearly visible. Except where specified otherwise, all techniques were as described (42,  
113 44).

114 Infectivity of HIV-1-derived viruses was assayed using a luciferase vector exactly  
115 as described (8). The plasmid backbone in the packaging plasmid was pCMVpA (i.e.,  
116 pUC19 containing the cytomegalovirus immediate early promoter and the SV40  
117 polyadenylation signal), and the *vif* gene was destroyed by site-directed mutagenesis,  
118 replacing the methionine codon at position 8 with a termination codon.

119 Plasmids expressing either hA3G or mA3 were a kind gift from Nathaniel Landau  
120 (New York University School of Medicine). The proteins were both expressed in the  
121 plasmid pcDNA3.1 and were tagged at their C-termini with the hemagglutinin (HA)  
122 epitope (33). The mA3 encoded by the plasmid used here is the isoform lacking exon 5.  
123 For studies involving mutant APO proteins, the coding regions were transferred into  
124 pCMVpA and mutations were created using the QuikChange site-directed mutagenesis  
125 kit (Stratagene) according to the manufacturer's instructions. Wild-type HA-tagged  
126 hA3G in pCMVpA was mutated to produce E67Q, E259Q, and the double mutant  
127 E67Q/E259Q; HA-tagged mA3 was mutated to E73Q, E253Q, and E73Q/E253Q. To  
128 express the mA3 isoform containing exon 5, we purchased a plasmid encoding mA3  
129 cDNA from Open Biosystems Inc. The mA3 coding sequence in the plasmid (clone ID  
130 3155422; accession BC003314) was amplified and subcloned into a CMV-driven  
131 mammalian expression vector with a C-terminal HA epitope tag.  
132 Immunoblotting. Immunoblotting against MLV p30<sup>CA</sup> was performed using rabbit  
133 polyclonal anti-p30<sup>CA</sup> antiserum as described (39). Some immunoblotting experiments

134 also used rabbit polyclonal anti-p15<sup>MA</sup> and rabbit anti-gp70<sup>SU</sup> antisera. Amounts of HA-  
135 tagged APO protein were similarly compared using the mouse anti-HA monoclonal  
136 antibody 16B12 (Covance) as primary antibody and horseradish peroxidase-conjugated  
137 horse anti-mouse IgG (Cell Signaling) as secondary antibody. For comparison of the  
138 amounts of HA-tagged APO protein in transfected cells, the cells were lysed in the  
139 culture dishes with loading buffer and the resulting extracts were sonicated. Total protein  
140 concentration in the lysates was determined using the bicinchoninic acid protein assay  
141 (Pierce). Aliquots of the lysates containing equal amounts of protein were then assayed  
142 by immunoblotting with anti-HA monoclonal antibody as described above. The lysates  
143 were also analyzed by immunoblotting with a monoclonal antibody against  $\beta$ -actin  
144 (Sigma) as primary antibody and the peroxidase-conjugated horse anti-mouse IgG as  
145 secondary antibody.

146 Subtilisin treatment. Virus particles were prepared by centrifugation through 20%  
147 sucrose. They were then treated with subtilisin as described (43).

148 Virus fractionation. Virus particles were first concentrated by centrifugation through 20%  
149 glycerol. Pellets were resuspended in 4 ml phosphate-buffered saline (PBS) and each  
150 sample was divided into two aliquots of 2 ml each. Each aliquot was layered on top of a  
151 step gradient consisting of 1ml 10% sucrose/PBS over 1 ml 30% sucrose/PBS; in one of  
152 each pair of tubes, the 10% sucrose/PBS layer contained 0.1% Igepal CA-630 (Sigma).  
153 Samples were centrifuged in an SW50 rotor at 30,000 rpm for 90 min at 4 °C. Pellets  
154 were then dissolved in loading buffer and analyzed by immunoblotting.

155 Reverse transcriptase activity. In some experiments, amounts of virus were compared  
156 using reverse transcriptase (RT) activity as a quantitative measure of virus particle levels.

157 Two methods were used for RT assays. In one, viruses were pelleted by  
158 ultracentrifugation and resuspended in an assay buffer containing 65 mM Tris (pH 8.0),  
159 65 mM KCl, 0.65 mM MnCl<sub>2</sub>, 10 mM DTT, and 0.65% NP-40 together with 0.5 µCi <sup>3</sup>H-  
160 TTP and primer/template-conjugated scintillation proximity assay beads from the Quan-  
161 T-RT [<sup>3</sup>H]-Reverse Transcriptase Assay Kit (GE Healthcare). After 3 hr at 37 °C, beads  
162 were processed according to the manufacturer's instructions. The signal was linearly  
163 proportional to the amount of sample assayed over at least a 7-fold range, and values  
164 were taken from the middle of this range. Alternatively, samples were analyzed for RT  
165 activity as described (15) following concentration with polyethylene glycol (16). This  
166 assay is linear over a wide range (13).

167 Analysis of reverse transcription complexes: real-time PCR and large-scale sequencing.

168 The ability of MLV-derived virus particles to perform DNA synthesis upon infecting new  
169 host cells was assayed as follows. Viruses were produced by transient transfection of  
170 293T cells that had previously been stably transfected with pLXSH (36) and selected for  
171 hygromycin resistance. The virus particles obtained following the transient transfection  
172 were then used to infect 293T cells expressing mCAT1. 24 hr later, the cells were lysed  
173 by the QIAamp DNA Mini Kit (Qiagen) and the cell extracts were assayed for  
174 hygromycin phosphotransferase (hph) DNA and 2-LTR circular DNA by real-time PCR  
175 as described (21, 44). The results were normalized for minor differences in recovery of  
176 cellular DNA by comparing the copy numbers of the cellular gene CCR5 (44, 48).

177 Real-time PCR enumeration of luciferase DNA copies following infection with  
178 the HIV-1-derived luciferase vector was performed using Luc1006F (5'  
179 ATCAGGCAAGGATATGGGCTCACT 3') and Luc1130R (5'

180 TCCAGATCCACAACCTTCGCTTCA 3') for primers and Luc1079P (5' FAM-  
181 GCGCGGTCCGGTAAAGTTGTTCCATTT-TAMRA 3') for probe. pBabe-Luc plasmid  
182 DNA (44) was used as a standard in these assays. Since these viruses were collected ~ 48  
183 hr after transient transfection with plasmids including the luciferase vector, it was crucial  
184 to eliminate contaminating plasmid DNA from the virus before infecting the target cells  
185 and analyzing the reverse transcription products. Therefore, the virus-containing culture  
186 fluid was pre-incubated with 10 U/ml bovine pancreatic DNase I (Sigma) and 4 mM  
187 MgCl<sub>2</sub> for 1 hr at 37 °C. An aliquot of the DNase-treated virus was inactivated by  
188 incubation at 68 °C for 20 min. Cells were then infected with these virus preparations.  
189 The effectiveness of the DNase treatment was determined by measuring luciferase DNA  
190 sequences in the cells "infected" with the heat-inactivated virus; in all cases, the number  
191 of luciferase copies in these cells was ≤ 2% of that in the cells infected with active virus.  
192 Bulk sequencing of reverse-transcription products. Hph DNA synthesized upon infection  
193 with MLV stocks that included the pLXSH vector was sequenced as follows. Cells were  
194 lysed as described above. Hph sequences from pLXSH were amplified from 0.5 μL to 2  
195 μL of the lysate using primers HPH 2050F (5' AAAGCCTGAACTCACCGCGACGTC  
196 3') and HPH 3030R (5' CACGAGTGCTGGGGCGTCGGTTTC 3') with Pfu Turbo  
197 polymerase (Stratagene) for 20-30 cycles using the following PCR conditions: 95 °C, 30  
198 seconds; 67.5 °C, 1 minute; and 72 °C, 1 minute. Typically the PCR reactions were  
199 fractionated by agarose gel electrophoresis in a ~1.5% agarose gel. The ~1 kb PCR  
200 product was then eluted and purified using a Qiaquick gel extraction kit (Qiagen).  
201 Alternatively, it was purified away from small nonspecific PCR products using a mini  
202 Quick Spin column (Roche) and then ethanol-precipitated. In either case, the purified

203 DNA was then ligated into PCR Blunt II-TOPO (Invitrogen) and transformed into Top 10  
204 cells (Invitrogen), and colonies were selected by growth on kanamycin-containing  
205 medium. In order to minimize repeated amplification and cloning of the same DNA,  
206 transformed bacteria were only grown 20-35 minutes before plating. Selected colonies  
207 were grown in 1 mL Terrific Broth in 96 well plates and DNA was purified using the  
208 Qiagen Biorobot 3000. Fifteen  $\mu$ L of the purified DNAs (representing ~ 15% of the  
209 DNA) were sequenced using the SP6 primer.

210 DNA synthesized by viruses carrying the HIV-1-derived luciferase reporter vector  
211 was analyzed as follows. DNA was isolated from the infected cells as above, and  
212 luciferase sequences were amplified using the primers Luc 34F (5'  
213 GCGCCATTCTATCCGCTGGAAGAT 3') and Luc 1525R (5'  
214 CGGTTGTTACTTGACTGGCGACGT 3'). The amplification included an initial  
215 treatment at 95 °C for 1 min, followed by 30 cycles of 95 °C, 30 sec; 55 °C, 30 sec; and  
216 72 °C, 2 min; and finally 5 min at 72 °C. The products were then cloned, sequenced, and  
217 analyzed as for the hph DNA.

218 Sequence data were analyzed for mutations by trimming all sequences to the same  
219 length and aligning them using Clustal W (EBI-<http://www.ebi.ac.uk/clustalw/#>) (7) and  
220 Jalview. Typically, each clone yielded at least 800 nucleotides of sequence information,  
221 but only 750 nucleotides were used for analysis.

222

## 223 RESULTS

### 224 Effect of mA3 on MLV infectivity

225 To assess the effect of mA3 and hA3G on MLV infectivity, we transiently  
226 transfected 293T cells (which do not express detectable APOBEC3G) with a mixture of  
227 an infectious, wild-type clone of Moloney MLV; the reporter plasmid pBABE-Luc; and  
228 varying amounts of the APO-expressing plasmids. We harvested the supernatants from  
229 the transfected cultures ~ 48 hr after transfection and measured the levels of infectious  
230 virus capable of infecting 293-mCAT1 cells and expressing the luciferase reporter. Thus,  
231 the particles analyzed here were composed of viral proteins encoded by the MLV helper  
232 plasmid, but some of the particles contained the pBABE-Luc vector genome. Typical  
233 results are shown in Fig. 1. It can be seen that increasing amounts of the mA3 plasmid in  
234 the transfection mixture caused a progressive decrease in the level of luciferase  
235 production in the infected cells. The Figure also shows that hA3G produced a  
236 significantly larger reduction in luciferase expression than mA3: for example, 10 µg of  
237 the hA3G plasmid reduced the luciferase level ~300-fold, while the same level of the  
238 mA3 plasmid only resulted in a ~15-fold decrease.

239 It was conceivable that this effect of mA3 and hA3G on virus infectivity was  
240 specific to the pBABE-Luc vector. To test the generality of the phenomenon, we co-  
241 transfected the MLV plasmid together with pBABE-Luc and the APO expression  
242 plasmids into 293T cells which had previously been stably transfected with another  
243 MLV-derived vector, i.e., pLXSH. Culture fluids harvested from the transfected cells  
244 were then assayed for luciferase-inducing activity as in Fig. 1. In addition, they were  
245 assayed on NIH3T3 cells for their ability to induce hygromycin resistance (a  
246 measurement of pLXSH infectivity) and on S+L- cells (3) for infectious, replication-  
247 competent MLV produced from the wild-type MLV plasmid that rescued pBABE-Luc

248 and pLXSH. The results of these assays are shown in Fig. 2. It is evident that the effects  
249 of hA3G (panel A) and mA3 (panel B) on pLXSH and MLV are quantitatively similar to  
250 their respective effects on pBABE-Luc. These results suggest that mA3 is capable of  
251 inactivating any MLV-derived virus, although its effects are considerably less severe than  
252 those of hA3G.

253 The stronger effect of hA3G than mA3 on the MLV-derived viruses (Fig. 1 and 2)  
254 might simply indicate that hA3G is a more potent general inhibitor of retroviral infections  
255 than mA3. Alternatively, MLV may exhibit specific partial resistance to mA3. To  
256 distinguish between these possibilities, we compared the two APOs with respect to their  
257 effects on an HIV-1 vector encoding luciferase. The HIV-1 packaging plasmid used here  
258 did not encode Vif, which normally protects wild-type HIV-1 against hA3G. As shown in  
259 panel A of Fig. 3, the two APOs were roughly equivalent in their ability to inactivate an  
260 HIV-1-derived vector; in fact, the extent of inactivation by mA3 was consistently slightly  
261 greater than that by hA3G. Thus, the mA3 encoded by the expression plasmid used here  
262 is not deficient with respect to overall antiretroviral activity.

263 It is important to note that the inactivation of MLV and MLV-derived vectors by  
264 both mA3 and hA3G showed a linear dose-response on the semilogarithmic plots in  
265 Figures 1, 2, and 3B, indicating an exponential inactivation pattern. In other words, all of  
266 the data could be fit to the expression  $e^{-kx}$ , where  $x = \mu\text{g}$  of APO plasmid transfected into  
267 the culture. In Fig. 1,  $k = 0.622$  for hA3G and  $0.287$  for mA3. This feature of these  
268 experiments was extremely reproducible: over nine titrations comparing the effects of  
269 hA3G and mA3 upon luciferase activity, the means of the  $R^2$  values for the fits of the  
270 hA3G and mA3 curves to the  $e^{-kx}$  expression were  $0.9878$  and  $0.9825$ , respectively. The

271 average ratio of the hA3G exponent to the mA3 exponent was  $2.41 \pm 0.176$  (mean  $\pm$   
272 standard deviation). The implications of the single-hit exponential character of these  
273 dose-response curves are considered in the Discussion.

274 mA3 exists in two isoforms, differing by the presence or absence of exon 5 (29,  
275 33). We compared the anti-MLV efficacy of these isoforms. As shown in Fig. 4, their  
276 effects on the infectivity of MLV appeared to be very similar.

### 277 Packaging of APO Proteins in MLV Particles

278 One possible explanation for the stronger anti-MLV effects of hA3G relative to  
279 mA3 is that the former protein is more efficiently incorporated into MLV particles during  
280 virus assembly. As both proteins were tagged with the HA epitope, we were able to  
281 compare their levels in MLV virions by immunoblotting with an anti-HA monoclonal  
282 antibody. We compared the packaging of hA3G and mA3 into MLV particles as follows.  
283 Cultures of 293T cells were transfected with 3  $\mu$ g or 10  $\mu$ g of either the hA3G or mA3  
284 expression plasmid (as well as the MLV plasmid). Following harvest of the culture fluid,  
285 the cells were lysed for comparison of their APO levels. As can be seen in Fig. 5A  
286 (middle panel), the steady-state levels of hA3G and mA3 were similar in the cultures  
287 transfected with equivalent amounts of plasmid, and were considerably higher in the  
288 cultures transfected with 10  $\mu$ g than in those receiving only 3  $\mu$ g of plasmid. As shown  
289 in the top panel, similar relationships were observed in the viral samples as well. These  
290 results show that the level of APO encapsidation is, under our experimental conditions,  
291 roughly proportional to the level of APO in the virus-producing cells, and that the  
292 efficiencies of encapsidation of hA3G and mA3 are very similar to each other. In  
293 contrast, Fig. 5B confirms that the encapsidated hA3G and mA3 are drastically different

294 in their abilities to interfere with MLV infectivity, in agreement with the other results in  
295 this report.

296 The viral pellets analyzed in Fig. 5 were purified only by centrifugation through a  
297 layer of 20% sucrose. It seemed possible that the APO proteins detected in these pellets  
298 were not really contained within virions; for example, they could have been released from  
299 the cells in association with large cellular debris. They might also be in the viral pellets  
300 simply because they adhere to virus particles. To critically test these possibilities, we  
301 compared the viral pellets with “mock” viral pellets, which were transfected with the  
302 APO expression plasmids but with an empty plasmid vector rather than MLV; in  
303 addition, we treated the pellets with subtilisin (43) to eliminate proteins that were not  
304 sequestered within particles.

305 The results of this experiment are shown in Fig. 6. There was no APO protein  
306 detectable in the mock viral pellets (lanes 4-5 and 10-11, panel C); thus cells expressing  
307 APO did not release it in pelletable form unless they were also producing virus particles.  
308 Aliquots of the subtilisin-digested and of the mock-digested pellets were tested by  
309 immunoblotting with anti-p30<sup>CA</sup> and anti-gp70<sup>SU</sup> as well as with anti-HA antiserum, in  
310 order to monitor the effects of the subtilisin digestion. Comparison of lanes 7-9 with  
311 lanes 1-3 of panels A and B shows that the subtilisin treatment eliminated the gp70, but  
312 not the p30<sup>CA</sup> from the pellets. This result demonstrates both the structural integrity of  
313 the particles and the efficacy of the digestion, as gp70 is exposed on the surface of intact  
314 virions while p30 is an internal viral protein. Finally, as shown in panel C (lanes 1-2 and  
315 7-8), the digestion also had no effect on the hA3G or mA3 bands. Taken together, these  
316 results confirm that the APO proteins in the viral pellets are contained within virions.

317 It was also possible that mA3 has lower efficacy against MLV than hA3G  
318 because, while it is within the virions (Fig. 6), it is excluded from the nucleoprotein core  
319 of the mature particle. To explore this possibility, we fractionated virions containing mA3  
320 and hA3G by centrifuging them through a solution of 0.1% Igepal. This treatment will  
321 remove the lipid bilayer, matrix protein, and surface glycoprotein from the particles,  
322 while preserving the viral core; the resulting pellet should then contain CA and NC, but  
323 not MA or the Env proteins. As shown in Fig. 7, the pellets in the detergent-treated  
324 samples (lanes 2, 4, and 6) lacked MA and gp70<sup>SU</sup> (second and third panels), but  
325 contained CA (top panel), as expected. Immunoblotting with the anti-HA antiserum (top  
326 panel) showed that, when virions were produced in cells expressing mA3 or hA3G, these  
327 proteins were also present in the pellets. Thus, this experiment did not suggest any  
328 difference in the location of mA3 and hA3G within MLV particles.

#### 329 MLV reverse transcription: Comparison of mA3 and hA3G effects

330 The antiviral effects of hA3G have been characterized in considerable detail.  
331 They include, but may not be limited to, the deamination of cytidine residues in minus-  
332 strand DNA, leading to G:A mutations in the provirus. It was obviously of interest to  
333 determine the mechanism of MLV inactivation by mA3.

334 As a first step in the analysis of the block to MLV infection by the APO proteins,  
335 we measured the level of reverse transcription by the inactivated virions. 293-mCAT1  
336 cells were infected with virus from the experiment described in Figure 2. Twenty-four  
337 hours after infection, the cells were lysed and assayed for the hph DNA that was  
338 synthesized during reverse transcription of the pLXSH particles [as described previously  
339 (44), this experimental design eliminates the possibility that plasmid molecules present in

340 the inoculum will register in the DNA assays]. DNA recovery was monitored by  
341 simultaneously measuring copies of the single-copy cellular gene CCR5. As shown in  
342 Figure 8, synthesis of viral DNA was reduced only about 3-fold in the presence of  
343 maximal levels of mA3 and hA3G, while infectivities were reduced ~ 15-fold by mA3  
344 and ~ 1000-fold by hA3G. Thus, interference with viral DNA synthesis may contribute,  
345 but does not seem to play a major role in MLV inactivation by the APO proteins.

346 We also tested the effects of mA3 and hA3G upon formation of 2-LTR closed  
347 circular viral DNA. These molecules, formed by ligation of the ends of the full-length  
348 viral DNA, are often taken as an indicator of the entry of the viral DNA into the nucleus  
349 of the newly infected cell. Figure 8 shows that the levels of 2-LTR circular DNA were  
350 not dramatically affected by either of the APO proteins tested here (the copy numbers of  
351 2-LTR circle junctions measured here were <1,000; this low level reduces the accuracy of  
352 these measurements and is undoubtedly responsible for the “scatter” in these titrations).

### 353 Sequence Analysis of Reverse Transcription Products

354 APO proteins are cytidine deaminases, and are known to induce G:A  
355 hypermutation by deamination of C residues on the minus strand during reverse  
356 transcription by HIV-1 (18, 32, 53). To determine whether this property was responsible  
357 for inactivation of MLV, we also performed large-scale sequencing on a 750-nt block of  
358 the hph DNA synthesized upon infection with MLVs made as described above. The  
359 results are summarized in Table 1. We found that MLV inactivated by hA3G (rows 4 and  
360 5) had a G:A mutation frequency ~ 50-fold higher than that seen in MLV produced in the  
361 absence of APO (row 2); the latter frequency was indistinguishable from that obtained by  
362 direct amplification and sequencing of pLXSH plasmid DNA (row 1). Interestingly, we

363 observed virtually identical G:A mutation frequencies in the MLVs inactivated by the  
364 intermediate and maximal hA3G doses (rows 4 and 5), despite the fact that the maximal  
365 dose reduced virus-induced luciferase activity nearly 100-fold more than the intermediate  
366 dose. In striking contrast, however, MLV that had been inactivated by mA3 (row 3) did  
367 not show any elevation of G:A mutation frequencies. It is important to note that the  
368 relative infectivity of the mA3-inactivated MLV sample analyzed here (as assessed by its  
369 luciferase titer) was slightly lower than that of the sample inactivated with the  
370 intermediate hA3G dose; thus, if G:A mutation were responsible for MLV inactivation by  
371 mA3, the mutations would certainly have been detectable in the mA3-inactivated viral  
372 DNA. Taken together, the results show that inactivation of MLV by hA3G is  
373 accompanied by G:A mutation, but the mutation frequency is evidently not proportional  
374 to the level of viral inactivation. Inactivation of MLV by mA3 is not associated with  
375 detectable G:A mutations, at least within the “body” of the viral genome.

376 We also tested the ability of mA3 to induce G:A mutation in the  $\Delta$ Vif HIV-1-  
377 derived vector. As shown in Table 1, row 7, DNA synthesized by the HIV-derived  
378 particles carrying mA3 contained a very high level of G:A mutation, in agreement with  
379 previous reports (5, 29, 33, 51). Thus, the inability of mA3 to induce detectable G:A  
380 mutations (Table 1, row 3) appears to be specific to MLV.

381 We also determined the immediate sequence context of the G:A mutations  
382 induced in MLV vectors by hA3G. With both hA3G doses, ~ 95% of the mutated  
383 residues were followed by G, while the remainder were followed by A, as expected for  
384 the effects of hA3G, in which the preferred target is CC (data not shown) (20, 51). In  
385 contrast, the great majority of the G:A mutations that appeared in  $\Delta$ Vif HIV-1 DNA

386 synthesized in the presence of mA3 were followed by either G or A; the “hotspots” for  
387 G:A mutation nearly always contained an A residue two bases 3’ to the mutated G (data  
388 not shown), in excellent agreement with previous work on mA3 showing that the  
389 preferred target is T T/C C (5, 28, 51). The simplicity of the target sequences for both  
390 hA3G and mA3 means that the two DNA sequences analyzed here, i.e., hph (in MLV)  
391 and luciferase (in HIV-1), both contain many copies of each target sequence; thus the  
392 lack of mA3-induced mutations in hph is not due to the absence of these targets.

### 393 Anti-MLV activities of mutant mA3 and hA3G molecules

394 APO proteins have two zinc fingers, and in each finger a glutamic acid residue is  
395 the putative active site of the cytidine deaminase enzyme. In order to obtain some insight  
396 into the possible role of deamination in anti-MLV activity, we mutated these residues in  
397 hA3G and mA3 to glutamine and analyzed the effects of these mutant proteins as in Fig.  
398 1. In these experiments, the level of virus in each sample was quantitated by reverse  
399 transcriptase assays, and the luciferase activity was the normalized to the level of virus.  
400 As shown in Fig. 9A, replacing either of the catalytic-site glutamic acids in hA3G with  
401 glutamine greatly reduced the ability of the protein to inhibit MLV infection; we saw no  
402 significant difference in activity between the two single mutants and the double mutant in  
403 which both glutamic acid residues are replaced.

404 The anti-MLV activity of mA3 is, as described above, far lower than that of  
405 hA3G. As shown in Fig. 9B, there was little effect of the mutations upon the anti-MLV  
406 activity of mA3.

407 We also determined the efficiency with which the mutant APO proteins were  
408 encapsidated into MLV virions. Figure 9 shows the results of immunoblotting for the HA

409 tag in the hA3 (panel C) and mA3 (panel D). It can be seen that replacements of glutamic  
410 acid by glutamine had little effect on hA3G packaging (panel C), but encapsidation of  
411 mA3 was significantly reduced by the mutations.

## 412 DISCUSSION

413 Mammalian cells possess a number of defense mechanisms protecting them from  
414 infection by retroviruses. These defenses include the APOBEC3 family of proteins. The  
415 existence of this cellular system was only recently discovered (45), and many  
416 fundamental questions concerning its underlying mechanisms remain unanswered.

417 We have investigated the effects of hA3G and mA3 upon MLV and MLV-derived  
418 vectors. We found that mA3 blocks infection by these viruses, although hA3G induces a  
419 significantly more severe inhibition. In contrast, mA3 was as potent as hA3G as an  
420 inhibitor of infection by a  $\Delta$ Vif HIV-1-derived vector, in agreement with earlier work  
421 (18, 32). Thus, MLV appears to be partially resistant to mA3.

422 In our experiments, inactivation of MLV by either mA3 or hA3G always followed  
423 single-hit kinetics (see Figures 1, 2, 3B, and 5B). This dose-response pattern suggests that  
424 a single APOBEC3 molecule (or oligomer, assuming that intracellular APOBEC3  
425 concentrations are high enough to ensure efficient oligomerization) is sufficient to  
426 inactivate an MLV particle. The ability of a single APOBEC3 molecule or oligomer to  
427 inactivate a virion is quite consistent with the results of Xu et al., who reported that HIV-  
428 1 particles produced in human peripheral blood mononuclear cells contain only ~ 7 hA3G  
429 molecules (50). (While the titration data imply that a single molecule or oligomer can  
430 inactivate a virion, any number of APOBEC3 molecules might be present in each particle  
431 without contributing to the inactivation.) The respective slopes of the mA3 and hA3G

432 titrations imply that per microgram of plasmid transfected, hA3G inactivates MLV ~ 2  
433 1/2 times more efficiently than mA3. As the levels of hA3G and mA3 proteins in cells  
434 and virions are, for a given plasmid dose, similar to each other (Fig. 5), the results  
435 suggest that a molecule of hA3G blocks MLV infection ~2 1/2 times as effectively as a  
436 molecule of mA3.

437         The ability of mA3 to inhibit infection by MLV-related genomes is obviously  
438 significantly lower than that of hA3G, and it might be suggested that this activity is only  
439 an artifact of the high level of expression of mA3 in our transfected cells. However, the  
440 data are not consistent with this suggestion: rather, the steady, progressive decline of  
441 infectivity with increasing dose of mA3 plasmid (Figures 1-5) shows that the inhibition is  
442 not confined to cells with especially high mA3 concentrations, even though it is most  
443 obvious in these cells.

444         It might be imagined that MLV exhibits partial resistance to mA3 by partially  
445 excluding it from assembling virus particles. In fact, both HIV-1 and HTLV-1 have  
446 mechanisms for excluding hA3G from progeny virions, although these mechanisms are  
447 very different from each other: in HIV-1, resistance depends upon the accessory protein  
448 Vif, but in HTLV-1 the exclusion of both hA3G and mA3 involves the nucleocapsid  
449 domain of Gag (9, 35, 52). However, immunoblotting with anti-HA antibody (for  
450 detection of the HA-tagged APO proteins) showed that mA3 was incorporated into MLV  
451 particles as efficiently as hA3G (Figure 5). Further analysis showed that mA3, like  
452 hA3G, is within the detergent-resistant ribonucleoprotein in the interior of MLV particles  
453 (Figure 7).

454 Despite intensive investigations in a number of laboratories, the mechanism by  
455 which APOBEC3 molecules inactivate retroviruses is still not well understood. The  
456 cytidine deaminase activity of hA3G is well documented (17, 25, 47), and it is very clear  
457 that APOBEC3 proteins can induce G:A mutations in retroviral genomes (18, 30, 32, 33,  
458 53). However, several situations have been described in which this activity does not seem  
459 sufficient to explain the inactivation (reviewed in (23)). Evidence has been presented for  
460 additional inactivation mechanisms including interference with reverse transcription or  
461 with the stability of the newly synthesized viral DNA (4, 23, 24, 31-34, 46, 49) and with  
462 integration of the viral DNA into cellular DNA (23, 34), possibly by impairing the  
463 normal removal of primer tRNA after the second strand-transfer step during reverse  
464 transcription (34) or by interfering with the function of a viral component such as IN or  
465 NC in the pre-integration complex (23).

466 In the present work, we also addressed the mechanism by which mA3 and hA3G  
467 interfere with MLV infection. We found a slight reduction in the production of viral  
468 DNA following infection with APOBEC3-inactivated MLV (Figure 8), but the  
469 magnitude of this effect was far too low to account for the loss of infectivity (23, 34).  
470 There was no significant effect on the formation of 2-LTR circular viral DNA (Figure 8),  
471 implying that transport to the nucleus was unimpaired.

472 Most interestingly, we found no G:A mutations in the DNA synthesized upon  
473 infection with MLV inactivated by mA3, while these mutations were readily detected if  
474 the MLV contained hA3G, or in mA3-containing  $\Delta$ Vif HIV-1-derived vector particles  
475 (Table 1). The same frequency of G:A mutations was observed in DNA from MLV  
476 generated with two different doses of hA3G expression plasmid (3  $\mu$ g and 10  $\mu$ g, Table

477 1), although the specific infectivities of these two virus preparations differed by almost  
478 100-fold. As 10  $\mu$ g of mA3 plasmid inactivated the MLV as effectively as 3  $\mu$ g of hA3G  
479 plasmid, G:A mutations should have been detectable in the former sample if virus  
480 inactivation proceeds *via* cytidine deamination. Both the absence of the mutations in  
481 mA3 and the discordance between mutation frequency and infectivity in hA3G strongly  
482 suggest that cytidine deamination cannot fully explain the inactivation of MLV by  
483 APOBEC3 proteins, as previously proposed by others for HIV-1 (see above). In addition,  
484 we observed that each of the APO proteins inactivates pLXSH and replication-competent  
485 MLV with similar efficiencies (Figure 2); this is also difficult to reconcile with a simple  
486 deamination mechanism, since the minus-strand DNA of the latter virus would present a  
487 target for deamination roughly twice the size of that of the former.

488 We also tested the anti-MLV activity of mutant hA3G and mA3 molecules in  
489 which the glutamic acids at the deaminase active sites are replaced by glutamines. In  
490 hA3G, it was clear (Figure 9A) that both glutamic acids are essential for the efficient  
491 inactivation of MLV. In contrast, the relatively inefficient inactivation of MLV induced  
492 by wild-type mA3 was only slightly reduced by elimination of either or both of the  
493 glutamic acid residues (Figure 9B). We also noted that the mutations significantly  
494 reduced the amount of mA3 protein packaged into MLV (Figure 9D). One explanation of  
495 these results might be that under our conditions, most of the wild-type mA3 molecules  
496 packaged in virions are not participating in its antiviral activity. However, it is also  
497 possible that the mutations do significantly affect the antiviral activity of mA3, but that  
498 this effect is difficult to detect because the activity of wild-type mA3 is relatively low. In

499 this case, the antiviral effects seen at the higher doses of the mA3 mutants may represent  
500 artifacts of high expression levels.

501 In summary, our data indicate that MLV is partially resistant to the antiviral  
502 effects of mA3, and completely resistant to the G:A hypermutation seen with many other  
503 retrovirus-APO combinations. Unlike the previously described resistance mechanisms (9,  
504 35, 52), the mechanism by which MLV resists mA3 does not involve exclusion of the  
505 latter from the virion. We have no direct information on the nature of this mechanism; it  
506 seems possible that a constituent of MLV particles specifically inhibits the enzymatic  
507 activity of mA3, or the access of the latter to intracellular structures, such as the  
508 replication complex or the pre-integration complex, that arise from the viral core during  
509 infection. Exploration of these possibilities is under way.

510 Other investigators have previously reported that MLV is unaffected by mA3 (10, 29,  
511 33). Some reports have also indicated that mA3 is excluded from MLV particles (1, 10,  
512 29). We do not know why our results are different from those of these other investigators.  
513 Nearly all of our experiments used the mA3 isoform lacking exon 5, and it has been  
514 reported that MLV is more resistant to mA3 containing this exon than to the more spliced  
515 isoform, evidently because the exon contains a site which is cleaved by MLV protease  
516 (1). However, MLV appeared to exhibit similar resistance to both isoforms in our hands  
517 (Figure 4). Further work will be required to fully resolve the differences between the  
518 various experimental systems used to analyze mA3-MLV interactions.

519 Like MLV, mouse mammary tumor virus (MMTV) has been grown for many years in  
520 mouse cells. It is interesting to note the recent report by Okeoma et al. (41), indicating  
521 that MMTV replication is impaired, although not completely prevented, by mA3, both in

522 cultured cells and in mice. No G:A mutations were detected in the DNA synthesized by  
523 the MMTV inactivated by mA3. The similarity of these results and ours with MLV  
524 presumably reflects the similarity in the selective pressures to which the two viruses have  
525 been subjected. In contrast, G:A mutations are present in the genomes of some  
526 endogenous MLVs, but not in others (27). The mutations bear all the hallmarks of mA3  
527 action. Thus, it appears that some, but not all, of the gammaretroviruses that have  
528 infected the mouse germline were susceptible to deamination by mA3.

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#### 538 NOTE ADDED IN PROOF

540 While this manuscript was in revision, a paper by Browne and Littman appeared,  
541 with results in general agreement with ours (J. Virology 82: 1305-1313, 2008).

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## FIGURE LEGENDS

715

716 Figure 1. Effects of hA3G and mA3 on infectivity of an MLV-derived luciferase vector.

717 293T cells were transiently transfected with an infectious MLV clone, the pBABE-Luc

718 plasmid, and different doses of expression plasmids for hA3G or mA3. 293-mCAT1 cells

719 were then infected with culture fluids from the transfectants, and lysates of these cells

720 were assayed for luciferase activity. The error-bars show the standard deviations on the

721 luciferase assays.

722

723 Figure 2. Comparison of effects of hA3G and mA3 on infectivity of a luciferase vector, a

724 hygromycin phosphotransferase vector, and MLV itself. 293T cells that had been stably

725 transfected with pLXSH were transiently transfected as in Fig. 1. The culture fluids from

726 the transfectants were then assayed for pBABE-Luc infectivity, pLXSH infectivity, and

727 MLV infectivity as described in Materials and Methods. (A), hA3G; (B), mA3.

728

729 Figure 3. Comparison of effects of hA3G and mA3 on infectivity of HIV-1-derived and

730 MLV-derived luciferase vectors. (A) HIV-1 vector; (B) MLV vector.

731

732 Figure 4. Comparison of effects of mA3 isoforms with and without exon 5 on infectivity

733 of an MLV-derived luciferase vector. Virions were produced as in the experiment shown

734 in Fig. 1, using plasmids encoding mA3 lacking exon 5 (as in all other experiments in this

735 report), mA3 containing exon 5, or hA3G. The graph shows the luciferase-inducing

736 activity divided by the virus particle concentration (as determined by RT activity  
737 following polyethylene glycol precipitation), so that the data represents the specific  
738 infectivity of the samples.

739

740 Figure 5. Comparison of encapsidation of mA3 and hA3G in MLV virions. Virus  
741 particles were produced by transient transfection in the presence of either 3  $\mu$ g or 10  $\mu$ g  
742 of either mA3 or hA3G expression plasmids, or in the presence of an empty vector (-).  
743 Virions were assayed for RT activity following precipitation from the culture fluids with  
744 polyethylene glycol. (A) Equal amounts of virus were loaded in the five lanes and  
745 analyzed by immunoblotting with antiserum against the HA epitope tag on the APO  
746 proteins. The cells were also lysed, and equal amounts of lysate (as determined by protein  
747 concentration) were analyzed by immunoblotting with the anti-HA antiserum. As an  
748 additional loading control, equal amounts of the lysates were also analyzed by  
749 immunoblotting with anti- $\beta$ -actin antiserum. The sample in lane 1 was produced without  
750 an APOBEC expression plasmid; lanes 2 and 3 with 3 and 10  $\mu$ g of mA3 plasmid,  
751 respectively; and 4 and 5 with 3 and 10  $\mu$ g of hA3G plasmid. In addition to  
752 autoradiography, the chemiluminescence of the immunoblots of the viral samples was  
753 measured on an Alpha Innotech ChemiImager 5500. Two different exposures were  
754 analyzed in this way, and the % Integrated Density Values of the viral samples (means  $\pm$   
755 standard deviations) were as follows: 3  $\mu$ g mA3,  $13.6 \pm 1.2$ ; 10  $\mu$ g mA3,  $32.1 \pm 1.0$ ; 3  
756  $\mu$ g hA3G,  $11.4 \pm 2.8$ ; 10  $\mu$ g hA3G,  $43.0 \pm 3.0$ . (B) The infectivity of the viruses shown in  
757 (A) was analyzed by luciferase assays. The graph shows luciferase-inducing activity

758 divided by virus particle concentration, and thus the data represent the specific infectivity  
759 of the samples.

760

761 Figure 6. Subtilisin resistance of mA3 in MLV virions. MLV particles produced in the  
762 presence of hA3G or mA3 plasmids were subjected to subtilisin digestion as described in  
763 Materials and Methods, and then analyzed by immunoblotting for intact (A) p30<sup>CA</sup>, (B)  
764 gp70<sup>SU</sup>, or (C) HA-tagged APO protein. Lanes 1 and 7, virions produced by cells  
765 expressing MLV and hA3G; 2 and 8, virions produced by cells expressing MLV and  
766 mA3; 3 and 9, virions produced by cells expressing MLV; 4 and 10, “virions” produced  
767 by cells expressing hA3G; 5 and 11, “virions” produced by cells expressing mA3. The  
768 samples in lanes 7-11 were digested with subtilisin before immunoblotting.

769

770 Figure 7. Localization of APO proteins in the interior of MLV particles. MLV particles  
771 were prepared by transient transfection in the presence of 10 µg of hA3G (lanes 1 and 2),  
772 mA3 (lanes 3 and 4), or empty (lanes 5 and 6) expression plasmid. The virions in the  
773 culture fluids were fractionated as described in Materials and Methods, and the pellets  
774 analyzed by immunoblotting against the HA epitope tag or p30<sup>CA</sup> (top panel), gp70<sup>SU</sup>  
775 (middle panel), or p15<sup>MA</sup> (bottom panel). In lanes 1, 3, and 5, the virions were  
776 sedimented with no detergent, while in lanes 2, 4, and 6, Igepal was present in the 10%  
777 sucrose layer during centrifugation.

778

779 Figure 8. DNA synthesis by APO-inactivated MLV. 293T-mCAT1 cells were infected  
780 with the culture fluids that were assayed for infectivity in Figure 2. They were lysed 24 hr

781 later and assayed by real-time PCR for *hph* DNA and 2-LTR circles as described in  
782 Materials and Methods. Values are corrected for differences in CCR5 DNA copy  
783 numbers. (A), hA3G; (B), mA3.

784

785 Figure 9. Effects of mutants of hA3G and mA3 on infectivity of an MLV-derived  
786 luciferase vector. Mutant and wild-type APOs were assayed as in Figure 1, except that  
787 the luciferase activities are corrected for variations in the amount of virus, so that the  
788 graphs represent the specific infectivity of the virus preparations. Virus preparations were  
789 also analyzed by immunoblotting against the HA tag in the APO proteins. (A) and (C),  
790 hA3G; (B) and (D), mA3. In (C) and (D), lanes 1, 3, 5, and 7 are the samples transfected  
791 with 2.5  $\mu$ g of APO plasmid, lanes 2, 4, 6, and 8 are the samples produced with 7.5  $\mu$ g,  
792 and lane 9 is the sample produced with no APO.

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Table 1

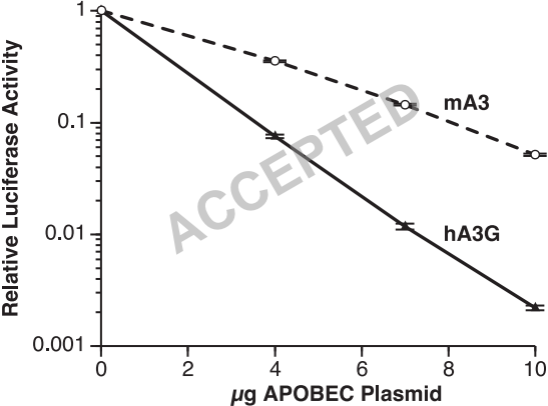
794

Source of DNA	Treatment	Relative Titer	Bases sequenced	G:A mutations	All other mutations	Frequency of G:A mutations
pLXSH plasmid	None	N.A.	5,746	1	0	$1.74 \times 10^{-4}$
MLV-infected cells	No Apobec	1	56,481	8	22	$1.42 \times 10^{-4}$
MLV-infected cells	10 $\mu$ g mA3	~0.08	43,556	6	8	$1.32 \times 10^{-4}$
MLV-infected cells	3 $\mu$ g hA3G	~ 0.17	22,176	165	5	$7.44 \times 10^{-3}$
MLV-infected cells	10 $\mu$ g hA3G	~ 0.0035	31,017	227	32	$7.32 \times 10^{-3}$
HIV-infected cells	No Apobec	1	22,022	8	---	$3.6 \times 10^{-4}$
HIV-infected cells	3 $\mu$ g mA3	0.008	25,304	209	--	$8.3 \times 10^{-3}$

795

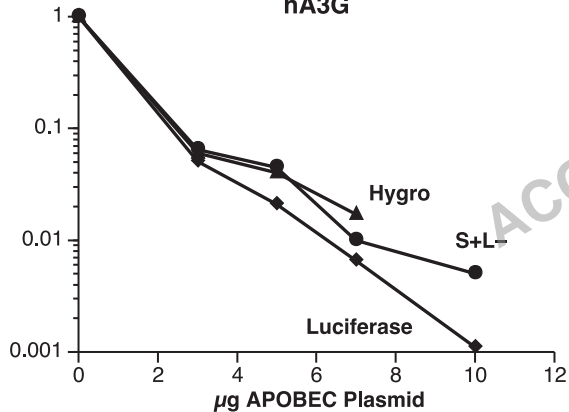
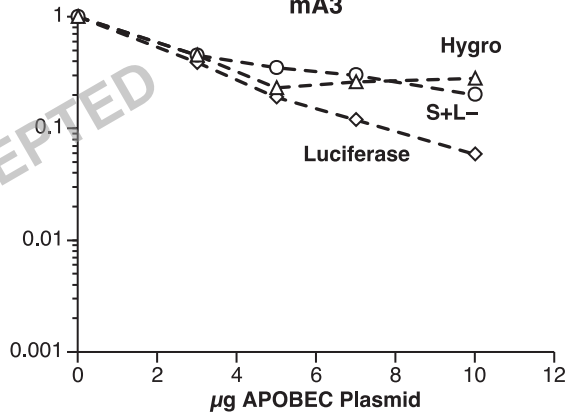
796 Table 1. Induction of mutations by APO proteins. DNA was isolated from cells infected  
797 with MLV that had been produced by cotransfection with APO-expressing plasmids. *Hph*  
798 DNA was amplified and sequenced as described in Materials and Methods. A similar  
799 analysis was also performed directly on pLXSH plasmid DNA. DNA was also isolated  
800 from cells infected with  $\Delta$ Vif HIV, and luciferase DNA was similarly amplified and  
801 sequenced. “Relative titers” are approximate for MLV because samples from two  
802 experiments with very similar results were analyzed and the results are pooled here.  
803 Mutations other than G:A transitions were not tabulated in the HIV samples.

804



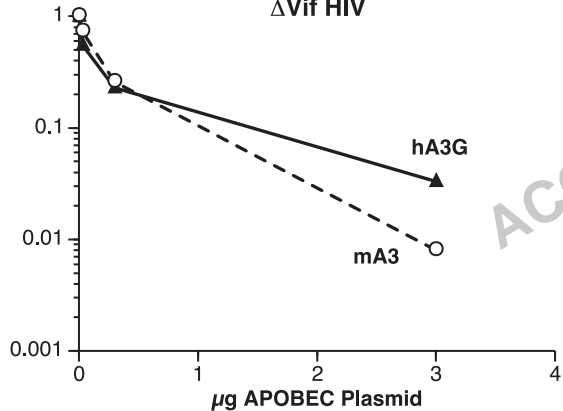
**A****hA3G**

Relative Infectivity

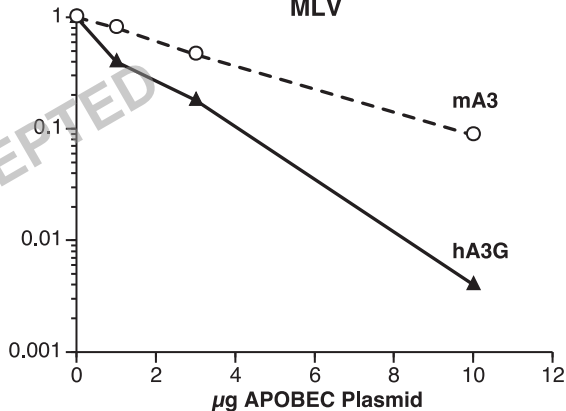
**B****mA3**

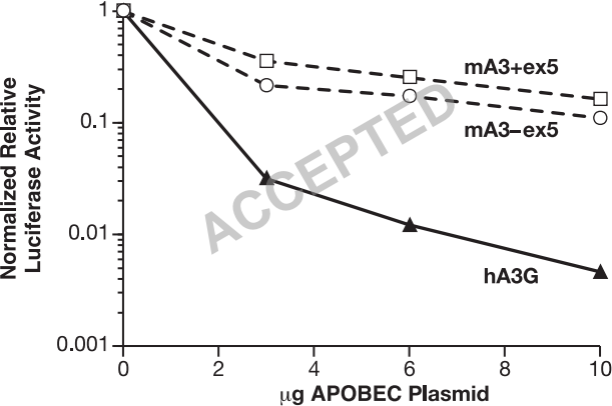
**A** $\Delta$ Vif HIV

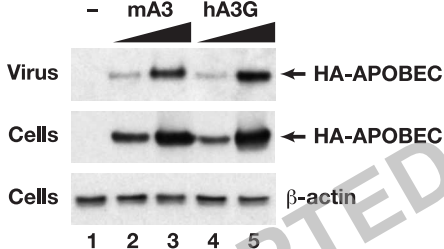
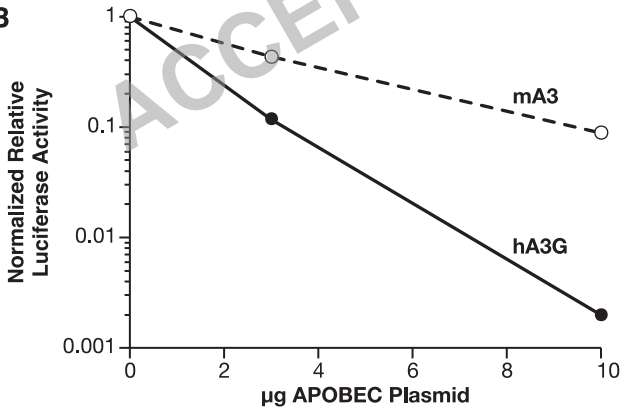
Relative Luciferase Activity

**B**

MLV





**A****B**

MLV:	+	+	+	-	-	-	+	+	+	-	-
hA3G:	+	-	-	+	-	-	+	-	-	+	-
mA3:	-	+	-	-	+	-	-	+	-	-	+
SUB:	-	-	-	-	-	-	+	+	+	+	+

**A**



← p30<sup>CA</sup>

**B**



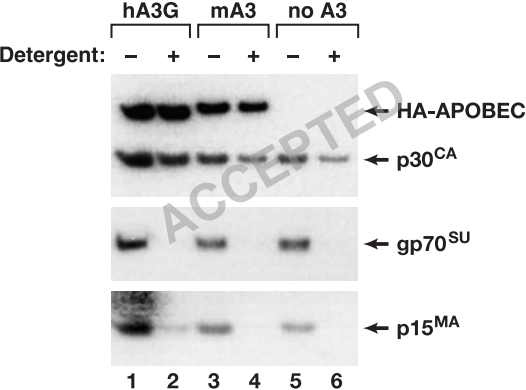
← gp70<sup>SU</sup>

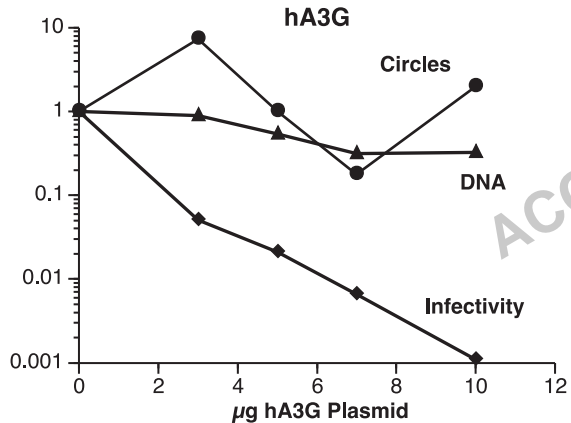
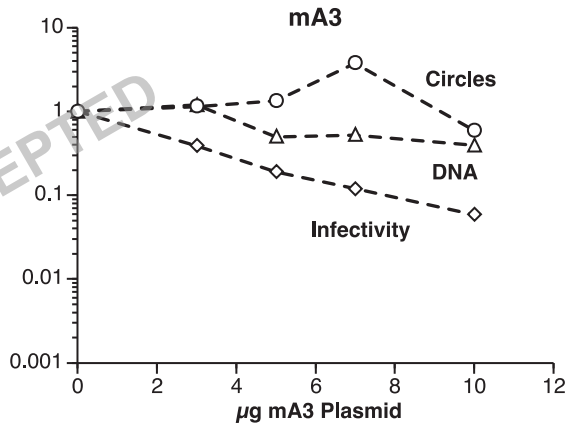
**C**

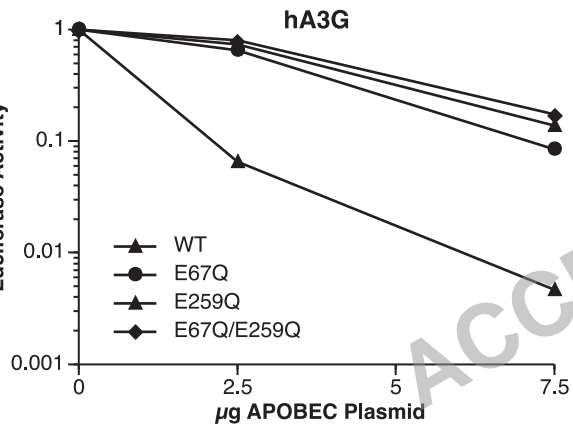
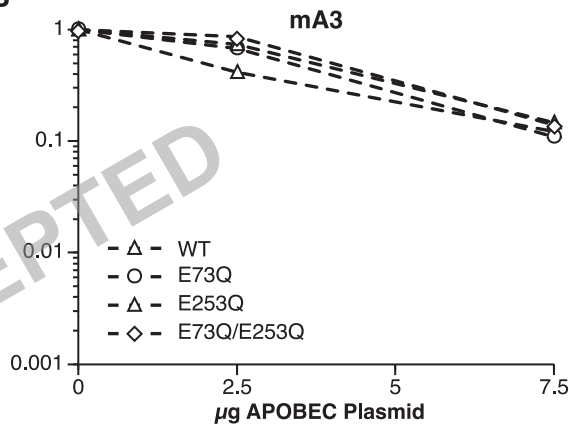
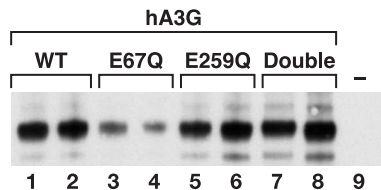


← HA-APO

1 2 3 4 5 6 7 8 9 10 11



**A****B**

**A****B****C****D**