Mouse APOBEC3 Restricts Friend Leukemia Virus Infection and Pathogenesis In Vivo

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Several members of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like complex 3 (APOBEC3) family in primates act as potent inhibitors of retroviral replication. However, lentiviruses have evolved mechanisms to specifically evade host APOBEC3. Likewise, murine leukemia viruses (MuLV) exclude mouse APOBEC3 from the virions and cleave virion-incorporated APOBEC3. Although the betaretrovirus mouse mammary tumor virus has been shown to be susceptible to mouse APOBEC3, it is not known if APOBEC3 has a physiological role in restricting more widely distributed and long-coevolved mouse gammaretroviruses. The pathogenicity of Friend MuLV (F-MuLV) is influenced by several host genes: some directly restrict the cell entry or integration of the virus, while others influence the host immune responses. Among the latter, the Rf63 gene has been mapped to chromosome 15 in the vicinity of the APOBEC3 locus. Here we have shown that polymorphisms at the mouse APOBEC3 locus indeed influence F-MuLV replication and pathogenesis: the APOBEC3 alleles of F-MuLV-resistant C57BL/6 and -susceptible BALB/c mice differ in their sequences and expression levels in the hematopoietic tissues and in their abilities to restrict F-MuLV replication both in vitro and in vivo. Furthermore, upon infection with the pathogenic Friend virus complex, (BALB/c × C57BL/6)F1 mice displayed an exacerbated erythroid cell proliferation when the mice carried a targeted disruption of the C57BL/6-derived APOBEC3 allele. These results indicate, for the first time, that mouse APOBEC3 is a physiologically functioning restriction factor to mouse gammaretroviruses.

The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3 (APOBEC3) proteins are cellular cytidine deaminases with potent antiretroviral activities (reviewed in reference 8). Thus, after the penetration of retroviral nucleocapsids into target cells of infection and the initiation of reverse transcription, APOBEC3 enzymes can induce the conversion of cytosine to uracil in the minus-sense single-strand viral DNA, leading to G-to-A hypermutations in the subsequent plus-strand viral DNA. The resultant detrimental levels of mutations in the proviral genome, along with a deamination-independent mechanism that works prior to the proviral integration (9), together exert efficient antiretroviral effects in infected target cells. However, retroviruses have evolved to evade their natural hosts’ APOBEC3. Thus, human immunodeficiency virus (HIV) counteracts the action of human APOBEC3G (hAPOBEC3G) through its viral infectivity factor (Vif). The Vif protein expressed in virus-producing cells interacts with hAPOBEC3G to recruit ubiquitin ligase complex and thus mediates polyubiquitination of hAPOBEC3G and Vif, resulting in rapid degradation of hAPOBEC3G (11, 29, 49). Vif is also known to partially impair de novo synthesis of hAPOBEC3G (45). Therefore, hAPOBEC3G is neutralized by HIV Vif, as well as simian immunodeficiency virus (SIV) Vif from the chimpanzee, rhesus macaque, and sooty mangabey (28). However, the antiretroviral effects of human APOBEC3 (hAPOBEC3) proteins are not limited to Vif-deficient lentiviruses of the above-mentioned primate species but are readily exerted with other lentiviruses including SIV from the African green monkey, equine infectious anemia virus, and more distantly related retroviruses such as murine leukemia virus (MuLV), porcine endogenous retrovirus, and foamy viruses (12, 16, 21, 27). The hAPOBEC3G has also been shown to restrict retrotransposition of Alu elements (10), indicating a possible physiological role for APOBEC3 proteins in protecting cells from endogenous retroelements.

Similarly, mouse APOBEC3 (mAPOBEC3) restricts the replication of HIV type 1 (HIV-1) without being countered by Vif (28, 42), whereas mouse gammaretroviruses are relatively resistant to mAPOBEC3 (1, 4, 13). This resistance of mouse gammaretroviruses to the APOBEC3 protein of their natural host seems to be mediated through the exclusion of mAPOBEC3 from MuLV particles and cleavage of virion-incorporated mAPOBEC3 by the viral protease (1, 4, 13). Interestingly, however, mouse mammary tumor virus (MMTV), a betaretrovirus, is susceptible to mAPOBEC3 (39), and evidence has been shown that endogenous polytropic and modified polytropic retroviruses have been genetically modified through the action of mAPOBEC3 (19). Thus, increasing evidence indicates a possible physiological role for mAPOBEC3 in restricting the replication of gammaretroviruses, not just betaretroviruses, of cognate origin; however, direct demonstration of the protective effects exerted by mAPOBEC3 on pathogenic MuLV infection has been lacking (4).

Friend virus (FV) is the pathogenic retrovirus complex composed of replication-competent Friend MuLV (F-MuLV), a
Mice and virus. C57BL/6, BALB/c, B10.A(2R)Sn, and (BALB/c x C57BL/6)F1 (C58F1) mice were purchased from Japan SLC, Inc., Hamamatsu, Japan. A/WSn mice were purchased from the Jackson Laboratory, Bar Harbor, ME. The mAPOBEC3-deficient mice have been described previously (31). They were backcrossed to C57BL/6 mice at least seven times and mated with BALB/c mice. Both male and female mice, 6 to 10 weeks old, were used for virus inoculation. All animals were housed and bred in the experimental animal facilities at Kinki University School of Medicine under a specific-pathogen-free condition, and the experiments described here have been approved by Kinki University. Replication-competent helper virus of the FV complex, F-MuLV, was purified from the culture supernatant of CB6F1 mice that mimics physiological MuLV replication, we established BALB/3T3 cell lines that stably expressed different FV-tagged versions of mAPOBEC3, the short isoform of mAPOBEC3 derived from C57BL/6 mice (mA3b) and the full-length (mA3d) and the short (mA3d) isoforms derived from BALB/c mice (Fig. 1). DNA transfection into BALB/3T3 cells was performed by using Lipofectamine 2000 reagent (Invitrogen). For the establishment of stable transfectants, cells expressing FV flag peptide (FLAG) and FLAG-fusion proteins (FLAG-proteins) were selected in the presence of 6 μg/ml puromycin (Sigma-Aldrich). Colony-forming cells were picked into a well of 96-well culture plates. The expression of FLAG and FLAG-proteins was confirmed by immunoblotting analyses of the cell lysates and with immunofluorescence staining of the cells.

PCR analysis of mAPOBEC3 mRNA and genomic DNA. Endogenous mAPOBEC3, BEC3 and GAPDH mRNA from tissues and cells were detected by reverse transcription-PCR (RT-PCR) using primers 5'-GGGTTACCGGCCAACCCTCAATATCCAGTGACACCATCGC-3' and 5'-GTCGCTAGACATCCGCGTCTCATGGACACCGCT-3', with primary cDNA samples prepared from the spleens of C57BL/6 and BALB/c mice. The hAPOBEC3G and hAPOBEC3F mRNA were amplified by PCR using the primers 5'-GGGTTACCGGCCAACCCTCAATATCCAGTGACACCATCGC-3' and 5'-GTCGCTAGACATCCGCGTCTCATGGACACCGCT-3', with templates prepared from peripheral blood mononuclear cells of a healthy individual. The above-described APOBEC3 cDNA were cloned into the SalI/EcoRI digest (for mAPOBEC3) or HindIII/KpnI digest (for hAPOBEC3) of pFLAG-CMV2 vector.

Endogenous APOBEC3 mRNA was detected by PCR using primers 5'-ATGGGACCATTCTGTCTGGGATGCAGCCATCGC-3' and 5'-GGGGTACCGCCGCCACGG-3' for the mAPOBEC3 exon 5 region; and primers 5'-GCCAAGGCTATCCATGACCGCC-3' and 5'-GTGGGTGTGAACTCTGGGATGAAAT-3' for the mAPOBEC3 exon 5 region; and primers 5'-GCCAAGGCTATCCATGACCGCC-3' and 5'-GTGGGTGTGAACTCTGGGATGAAAT-3'. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out for 15 s at 95°C, followed by 1 min at 60°C. TaqMan rodent GAPDH control reagent (Applied Biosystems) was used as an internal control.

Northern and Western blot analyses. Total RNA was prepared from tissues using TRIzol reagent. Two micrograms of the RNA was denatured.
with RNA loading mixture (GenHunter Corporation, Nashville, TN), separated in a 1% formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with 32P-labeled probes that were prepared by random priming with the templates generated by RT-PCR (mAPOBEC3, 5′/H11032-CCCGTCTCCCTTCAC CATGGGG-3′/H11032 and 5′/H11032-GGGAGACCTTTTGTTAGACAGATATTTGACAGAGTGG-3′/H11032; and mouse /H9252-actin, 5′/H11032-ATGGATGACGATATCGCTGCGCTGGTCGTCGACAACGGCTCCGGC-3′/H11032 and 5′/H11032-GGTCATCTTTTCACGGTTGGCGCTTAGGGTTCAGGGGGGCC-3′/H11032). Specific hybridization was visualized using a BAS-MS imaging plate (Fujifilm Corp., Tokyo, Japan). Densitometric analyses of the detected bands were done by using Image Gauge software (Fujifilm Corp.), and the results were normalized with GAPDH for each sample. Anti-FLAG M2 (Sigma-Aldrich) and anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) Ab were purchased from the above-mentioned suppliers. Horseradish peroxidase-conjugated secondary Ab was purchased from Zymed Laboratories (San Francisco, CA). Detection by immunoblotting of F-MuLV gp70 and p30 gag with monoclonal antibodies (MAb) 720 and R18-7 has been described previously (40).

**FIG. 1. Alleles and isoforms of mAPOBEC3 in FV-resistant C57BL/6 and -susceptible BALB/c mice.** (A) The known genomic organization and splicing pattern of the mouse APOBEC3 gene is shown with short arrows indicating the positions of the PCR primers used. (B) Alignment of the amino acid sequences of mAPOBEC3 for the C57BL/6-derived exon 5-lacking isoform (mA3Δ5) (GenBank accession no. NM_030255), and BALB/c-derived full-length (mA3β) [BC003314] and exon 5-lacking (mA3Δ5) isoforms (GenBank accession no. EDL04624). Open boxes show different amino acid residues, shaded boxes show the regions necessary for CDD activities, and the long horizontal lines indicate two CDDs as described in previous reports (15, 37). B10.A/Snpn mice showed amAPOBEC3 sequence that was completely identical to that of C57BL/6 mice, while A/Wysn mice shared the mAPOBEC3 sequence with BALB/c mice.

with RNA loading mixture (GenHunter Corporation, Nashville, TN), separated in a 1% formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with 32P-labeled probes that were prepared by random priming with the templates generated by RT-PCR (mAPOBEC3, 5′-CCGGTCTCCCTTCAC CATGGGG-3′ and 5′-GGGAGACCTTTTGTTAGACAGATATTTGACAGAGTGG-3′; and mouse /H9252-actin, 5′-ATGGATGACGATATCGCTGCGCTGGTCGTCGACAACGGCTCCGGC-3′ and 5′-GGTCATCTTTTCACGGTTGGCGCTTAGGGTTCAGGGGGGCC-3′). Specific hybridization was visualized using a BAS-MS imaging plate (Fujifilm Corp., Tokyo, Japan). Densitometric analyses of the detected bands were done by using Image Gauge software (Fujifilm Corp.), and the results were normalized with GAPDH for each sample. Anti-FLAG M2 (Sigma-Aldrich) and anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) Ab were purchased from the above-mentioned suppliers. Horseradish peroxidase-conjugated secondary Ab was purchased from Zymed Laboratories (San Francisco, CA). Detection by immunoblotting of F-MuLV gp70 and p30 gag with monoclonal antibodies (MAb) 720 and R18-7 has been described previously (40).

**F-MuLV infection in vitro and APOBEC3 packaging analysis.** BALB/3T3 cells stably expressing FLAG-APOBEC3 were seeded at 3×10⁴ cells/well in 24-well plates and infected with purified F-MuLV at a multiplicity of infection of 2.0 in the presence of 1 μg/ml Polybrene (Sigma-Aldrich). After 2 h of incubation, the cells were washed, fed with fresh medium, and cultured for 2 days. For packaging analyses, the culture supernatants were centrifuged to remove cells, and viral particles were precipitated with polyethylene glycol and step purified into a 15%/85% sucrose interface (33). For flow cytometry analyses of the surface gp70 expression, F-MuLV-infected transfectants were detached from culture wells with a brief trypsin treatment and stained with biotinylated MAb 720 (40), followed by an incubation with allopurinol-conjugated streptavidin (eBio-science Inc., San Diego, CA), and were analyzed with a Becton-Dickinson (Franklin Lakes, NJ) FACScalibur system.

**Sequence analysis of proviral DNA.** F-MuLV infection in vitro and APOBEC3 packaging analysis. BALB/3T3 cells stably expressing FLAG-APOBEC3 were seeded at 3×10⁴ cells/well in 24-well plates and infected with purified F-MuLV at a multiplicity of infection of 2.0 in the presence of 1 μg/ml Polybrene (Sigma-Aldrich). After 2 h of incubation, the cells were washed, fed with fresh medium, and cultured for 2 days. For packaging analyses, the culture supernatants were centrifuged to remove cells, and viral particles were precipitated with polyethylene glycol and step purified into a 15%/85% sucrose interface (33). For flow cytometry analyses of the surface gp70 expression, F-MuLV-infected transfectants were detached from culture wells with a brief trypsin treatment and stained with biotinylated MAb 720 (40), followed by an incubation with allopurinol-conjugated streptavidin (eBio-science Inc., San Diego, CA), and were analyzed with a Becton-Dickinson (Franklin Lakes, NJ) FACScalibur system.

**Sequence analysis of proviral DNA.** F-MuLV in 1 ml of culture medium were inoculated onto a culture of Mus dunni cells and incubated for 18 h. After trypsinization and washing, the cells were treated with RNase I, and their DNA was isolated by using DNeasy (Qiagen, Hilden, Germany). A 1.2-kbp fragment of the F-MuLV proviral genome harboring the U3 and a part of the gag sequence was amplified by PCR using the primers 5′-CGGGATCCAAGGACCTGAACTGACCCTG-3′ and 5′-GAAGAGAGAGGGGAGGTTTAGGG-3′. The amplified fragments were cloned into the pCR-Blunt vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen). Sequencing was performed by using the T7 and T3 primers.

**PCR quantification of F-MuLV genomic RNA and integrated proviral DNA.** F-MuLV viral RNA in culture medium was purified with a QIAamp viral RNA kit (Qiagen) and cDNA generated by RT with SuperScriptIII First-Strand synthesis system (Invitrogen) after a treatment with DNase I (Invitrogen). Genomic DNA was purified from F-MuLV-infected BALB/3T3 cells expressing FLAG or FLAG-protein or Mus dunni cells infected with F-MuLV as described above. Viral DNA was quantified using Platinum Quantitative PCR SuperMix-UDG with ROX and a 7900HT Fast Real-Time PCR system. Primers for the detection of the F-MuLV genome and the FAM-labeled probe were designed for the env region by using the following oligonucleotides. Primers were 5′-AAGTCTCCCG...
with PBS containing 0.05% Tween 20, 2×H9262 F-MuLV particles. The assay signals were measured as optical density at 450 nm, and the gp70 concentration was determined by adjusting it to the standard curve set with purified control reagent was used as an internal control for genomic DNA. Quantification of F-MuLV gp70 in culture supernatant. Ninety-six-well plates were coated with the gp70-specific MAb 48 (7) at 0.5 mg/well in 0.1 M NaHCO3. Wells were blocked with 10% fetal bovine serum and incubated with a culture supernatant containing F-MuLV for 2 h at room temperature. After washing with PBS containing 0.05% Tween 20, 2×g/well biotin-conjugated MAb 720 (40) was added and incubated for 1 h. After washing, the plates were incubated with a 1:30,000 dilution of horseradish peroxidase-conjugated streptavidin (Zymed Laboratories), and the chromogenic reaction was performed with 3,3',5,5'-tetramethylbenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The assay signals were measured as optical density at 450 nm, and the gp70 concentration was determined by adjusting it to the standard curve set with purified F-MuLV particles.

F-MuLV infection in vivo. C57BL/6 mice were inoculated with 1 × 10^6 focus-forming units (FFU) of F-MuLV by injecting 0.5 ml of a dilution via the tail vein. The spleen and bone marrow were removed, and single-cell suspensions were prepared for infectious center assays. CB6F1 mice were inoculated with 1 × 10^6 FFU of F-MuLV.

F-MuLV infectivity and infectious center assays. These assays were performed as described previously (24, 46). In brief, 1 ml of culture supernatant from F-MuLV-infected FLAG- or FLAG-protein-expressing BALB/3T3 cells was diluted serially and plated in duplicate with 1 μg/ml Polybrene on monolayers of Mus dunni cells. For infectious center assays, spleen or bone marrow cell suspensions were serially diluted and plated at concentrations between 1.0 × 10^6 and 1.0 × 10^3 cells/well onto monolayers of Mus dunni cells. After being washed and fixed with methanol on the second day of coculturing, F-MuLV-infected cell foci were visualized with MAb 720 as described previously (40).

FV complex and assessment of its pathogenicity in vivo. A B-tropic FV complex free of lactate dehydrogenase-elevating virus was kindly provided by K. J. Hasenkrug, Laboratory of Persistent Viral Diseases, NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT. Inoculation of CB6F1 mice with FV complex and assessment of its pathogenicity in vivo was performed as described previously (18, 24).

Statistics. One-way analysis of variance (ANOVA) for the comparison of multiple groups was performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., San Diego, CA), with an indicated posttest. When significant differences were pointed out by the ANOVA analyses, an individual level of significance was calculated for each pair of groups by two-tailed Student’s t test, depending on whether the variances were regarded as equal or not, respectively. Frequencies of mutations were evaluated by two-sided Fisher’s exact test, between selected groups following an extended Fisher’s exact test performed for the entire contingency table.

RESULTS

Allelic differences at the APOBEC3 locus between FV-resistant and -susceptible strains of mice. The Rf/3 gene that influences the duration of viremia after FV infection has been mapped to a segment of mouse chromosome 15 harboring the APOBEC3 gene (17, 23, 36, 47); this led us to explore possible allelic differences in the expression of mAPOBEC3. We found that mAPOBEC3 mRNA expression levels in the hematopoietic tissues from naturally FV-resistant C57BL/6 and B10.A/SgSn mice were higher than those in susceptible BALB/c and A/WySnJ mice (Fig. 2A). Quantitative real-time PCR analyses confirmed that C57BL/6 and B10.A/SgSn mice expressed three- to fourfold higher levels of mAPOBEC3 mRNA than BALB/c and A/WySnJ mice did, both in the spleen and bone marrow (Fig. 2B). In addition, the mAPOBEC3 isoforms derived from C57BL/6 and BALB/c mice differed at several amino acid residues, five of which were located within the CDD1 (Fig. 1). Interestingly, A/WySnJ mice lacking the ability to control viremia (6) and to produce F-MuLV-neutralizing Ab (23) shared the mAPOBEC3 sequence with BALB/c mice.

Additional variability between mouse strains was also observed in part for the relative amounts of the two splice isoforms of mAPOBEC3. The mAPOBEC3 cDNA obtained by RT-PCR amplification from C57BL/6 mice was slightly smaller than that from BALB/c mice (Fig. 2C), and this was due to the lack of a 99-bp stretch corresponding to the exon 5 (Fig. 1). The use of primers placed in exons 4 and 5 showed that the predominant mAPOBEC3 mRNA isoform expressed in C57BL/6 mice lacked exon 5, while BALB/c mice expressed full-length mAPOBEC3 with very low levels of the short isoform (Fig. 2C). CB6F1 mice expressed readily detectable full-length and truncated isoforms, with the latter in excess. Although the observed differences in the intensities of the PCR product bands might reflect different efficiencies in the amplification of the short and long fragments, the observed high intensity of the short-fragment band in C57BL/6 mice and the low intensity of the long-fragment band in BALB/c mice (Fig. 2C) are in agreement with the results of the Northern blotting (Fig. 2A) and real-time PCR (Fig. 2B) analyses. Thus, it is reasonable to conclude that BALB/c mice express a low level of full-length messages and C57BL/6 mice a high level of truncated mAPOBEC3 messages.

In vitro restriction of F-MuLV replication with C57BL/6-derived mAPOBEC3. It has been shown that mAPOBEC3 lacking the exon 5 (mAPOBEC3Δ5) can be packaged into MuLV particles more efficiently than the full-length mAPOBEC3 protein and, thus, can exert partial restriction of MuLV integration (1), although a recent report (4) has indicated similarly efficient incorporation of the full-length and the exon 5-lacking mAPOBEC3 into MuLV virions. However, the previous reports of the possible restricting effects of mAPOBEC3 on MuLV integration employed acute transfection of mAPOBEC3-expressing vector into MuLV packaging cells and examined a single-round integration of the MuLV vector and resultant expression of an inserted indicator gene. We intended to examine the possible restricting effects of mAPOBEC3 isoforms on more physiological replication cycles of infectious MuLV. Thus, we established BALB/3T3 cell lines that stably expressed different FLAG-tagged versions of mAPOBEC3 and measured the infectivity of F-MuLV produced from the transfectants in a focus formation assay that mimics physiological MuLV replication. BALB/3T3 cells stably expressing hAPOBEC3G, hAPOBEC3F, or GFP were also established as controls. These transfectants expressed comparable levels of APOBEC3 mRNA and produced APOBEC3 proteins of the expected sizes (Fig. 2D and F). Acute infection of the BALB/3T3 lines with an infectious molecular clone of F-MuLV resulted in a similar range of the envelope glycoprotein gp70 detected in the lysates, regardless of the APOBEC3 proteins expressed (Fig. 2F). The levels of F-MuLV infectivity and gp70 expression in the transfected lines were also confirmed to be similar by flow cytometric analyses (Fig. 2E). However, when we measured the infectivities of progeny viruses produced from the stable transfectants by focus formation assays on fully permissive Mus dunni cells, we found wide differences depending on the particular APOBEC3 protein expressed (Fig. 2H). Thus, the infectivity of F-MuLV produced from the mA3-Δ5-expressing cells was drastically reduced to a level similar to that obtained with F-MuLV derived from the hAPOBEC3G-expressing cells, whereas some 2.0 × 10^6 FFU/ml of infectious particles were
FIG. 2. Expression of the different alleles and isoforms of mAPOBEC3 in FV-resistant and -susceptible mice and infectivities of F-MuLV virions produced from mAPOBEC3-expressing cells. (A) Comparisons of mAPOBEC3 mRNA expression levels between mouse strains, by Northern blotting. Female mice, 7 to 8 weeks old, were analyzed for endogenous mAPOBEC3 mRNA expression. mAPOBEC3 mRNA was detected in 5 μg total RNA extracted from the spleen and bone marrow of the indicated strains of mice. β-Actin was used as an internal control. The numbers shown below each lane indicate densitometric ratios of expression levels between mAPOBEC3 and β-actin messages, normalized to that in the spleen of C57BL/6 mice. (B) Levels of expression of mAPOBEC3 mRNA relative to GAPDH quantified by real-time PCR are shown. Means of three samples each are shown with bars indicating standard errors of the means. *, statistically significant differences from the expression.
detected when the supernatants from the BALB/3T3 cells expressing FLAG alone or control GFP were tested (Fig. 2H). In contrast, only a marginal reduction in F-MuLV infectivity was observed for the supernatant of mA3dΔ5-expressing cells. Enforcing higher levels of expression of the mA3Δ5 cDNA in the BALB/3T3 cells, compared with a low expression level of the endogenous mA3d allele (Fig. 2D, lanes 1 and 3), did not result in any significant decrease in the infectivity of the virus produced (Fig. 2H), although the detectable amounts of mA3d protein were apparently lower than those of the exon 5-lacking isoforms in several tested clones, which might have caused inefficient incorporation of the full-length protein into the virions (Fig. 2G). Nevertheless, these results indicate strain-dependent differences in F-MuLV-restricting activities of mAPOBEC3, with the C57BL/6-derived short isoform restricting F-MuLV in an efficacy similar to that shown by heterologous hAPOBEC3G.

When F-MuLV particles were purified from the culture supernatant of acutely infected transfectants, virion-incorporated mAPOBEC3 lacking the exon 5, but not the full-length mAPOBEC3, was readily detectable along with viral gp70 and p30prov, regardless of their strains of origin (Fig. 2G). Interestingly, although mA3Δ5 derived from BALB/c mice was incorporated into F-MuLV as efficiently as C57BL/6-derived mA3Δ5 (Fig. 2G), only mA3Δ5 inhibited F-MuLV replication as strongly as hAPOBEC3G did in vitro (Fig. 2H). To exclude the possibility that the difference observed for the effects of mA3Δ5 and mA3Δ5 in restricting F-MuLV replication in vitro was caused by the slightly smaller amount of mA3Δ5 than mA3Δ5 detected in the transfectants used (Fig. 2F), we examined separate pairs of the stable transfectants. As shown in lanes 8 and 9 in Fig. 2 F and G, a higher level of mA3Δ5 was detected in the separate clone of stable transfectant, and the detected levels of virion-incorporated mAPOBEC3Δ5 proteins were similar. Nevertheless, the progeny virus produced from the mA3Δ5-expressing cells showed only about 60% reduc-

| Table 1. Number of F-MuLV provirus, infectious particles, and provirus in cells infected with progeny virus |
|---|---|---|---|---|
| Stable transfectant | Proviral copy no. (mean ± SEM) in producer cells (10⁵)ᵃ | Concen (μg/ml) of gp70 (mean ± SEM) in culture supernatantᵇ | Viral genomic copy no. (mean ± SEM) in culture supernatant (10²)ᶜ | Proviral copy no. (mean ± SEM) in indicator cells (10⁴)ᵈ |
| FLAG | 99.47 ± 21.23 | 87.61 ± 4.76 | 5.43 ± 0.47 | 14.38 ± 4.79 |
| GFP | 156.80 ± 31.84 | 70.97 ± 11.17 | 4.06 ± 0.29 | 18.89 ± 4.00 |
| mA3Δ5 | 140.19 ± 23.25 | 73.01 ± 7.63 | 3.17 ± 0.40 | 0.57 ± 0.05³ |
| mA3d | 94.57 ± 24.01 | 70.71 ± 15.41 | 5.05 ± 0.71 | 16.45 ± 1.74 |
| mA3Δ5 | 128.18 ± 23.60 | 72.57 ± 13.27 | 4.18 ± 0.31 | 4.59 ± 1.25³ |
| hAPOBEC3G | 53.78 ± 4.43 | 53.35 ± 8.44 | 2.82 ± 0.33⁴ | 0.35 ± 0.05⁴ |
| hAPOBEC3F | 99.80 ± 2.99 | 65.95 ± 0.60 | 3.54 ± 0.78 | 2.55 ± 0.40 |

ᵃ F-MuLV proviral DNA was quantified in the genomic DNA of the infected cells by quantitative real-time PCR using an F-MuLV-specific probe and primers. Data shown are means ± standard errors of the means (SEM) of copies per 1 ml of culture supernatant calculated from three repeated experiments. One-way ANOVA with Tukey's posttest for multiple comparisons indicated a significant difference only between GFP and hAPOBEC3G, but this was not significant (P = 0.08) by individual analysis using Welch's t test.

ᵇ Concentration of F-MuLV structural protein gp70 in culture supernatant from the acutely infected stable transfectants was analyzed by captured enzyme-linked immunosorbent assay using anti-gp70 MAb. Data shown here are means ± SEM calculated from three repeated experiments. No significant differences between the groups were found by one-way ANOVA with Tukey's posttest.

ᶜ Viral genomic RNA in the culture supernatant from the acutely infected stable transfectants was quantified by quantitative RT-PCR. Data shown here are means ± SEM of copies per 1 ml culture supernatant calculated from 3 repeated experiments. One-way ANOVA with Tukey's posttest indicated a significant group-wise difference only between the FLAG and hA3G.

ᵈ Student's t test for individual level of significance gave a P value of 0.011.

ﾟ F-MuLV proviral DNA was quantified in infected Mus dunni cells as described in the text. One-way ANOVA with Tukey's posttest for multiple comparisons indicated significant group-wise differences, which were then individually analyzed by t test.

⁴ P < 0.05, in comparison with that of GFP.

levels in B10.A/SgSn mice, indicated by one-way ANOVA with Dunnett's posttest for multiple comparisons (P < 0.05). (C) Splicing variants of the APOBEC3 gene expressed in C57BL/6 and BALB/c mice. The known genomic organization and splicing pattern of the APOBEC3 gene along with the positions of the primers used are shown in Fig. 1. The primers a and b amplified the entire mAPOBEC3-coding region, while primers c and d encompassed exons 4 and 6. GAPDH was used as an internal control. (D) Expression of APOBEC3 mRNA in the BALB/3T3 cells stably transfectected with each APOBEC3 gene was analyzed by RT-PCR. The same primers for mAPOBEC3 were used for samples in lanes 1 to 4. Samples in lanes 5 to 8 were amplified with each specific primer set. GAPDH was used as an internal control. Note the faint band of endogenous mA3Δ5 cDNA in lane 1. (E) Flow cytometric analyses of the cell surface expression of F-MuLV gp70 on acutely infected stable transfectants are shown. Cells expressing the indicated genes were infected with F-MuLV at a multiplicity of infection of 2.0 and analyzed for surface gp70 expression with MAb 720 2 days later. (F and G) Proteins detected in cell lysate (F) and virus particles in the culture supernatant (G) from the infected BALB/3T3 cells expressing FLAG and FLAG-proteins are shown. Immunoblot detection was performed with the anti-FLAG, anti-gp70, anti-p30 or anti-actin Ab. (H and I) Infectivities of progeny F-MuLV produced from APOBEC3-expressing BALB/3T3 cells. Mus dunni cells were infected with the progeny virus produced from the indicated transfectants, and foci of infected cells were stained with anti-gp70 MAb for enumeration. The vertical axis in panel I shows F-MuLV infectivity as in panel H. The infectivities are shown as an equivalent of infectious virus per 1 ml of culture supernatant (n = 3, mean ± standard deviation; *, P < 0.05; †, P < 0.01; ‡, P < 0.005). The F-MuLV infectivity detected in the supernatant of hAPOBEC3G-expressing cells was drastically reduced, while only a moderate reduction in F-MuLV infectivity was observed when the indicator cells were inoculated with the supernatant from the hAPOBEC3F-expressing cells, consistent with the previous reports (1, 4, 13). All the experiments shown in panels C to I were performed with at least two representative clones of stable transfectants for each gene, and the results obtained with the independent clones were in agreement with the data shown.
tion in F-MuLV infectivity, while the progeny virus produced from the mA3b/H90045-expressing cells showed vastly reduced infectivity, which was significantly lower than that shown by the mA3d/H90045-containing F-MuLV. The effectiveness of virion-incorporated mAPOBEC3 in restricting F-MuLV replication was further confirmed by quantitative analyses of viral copy numbers. Whereas neither the numbers of F-MuLV proviruses within the acutely infected stable transfectants nor the amounts of viral gp70 and genomic RNA in the supernatants were significantly different, regardless of the APOBEC3 or control genes expressed by the transfectants (Table 1), with the exception of a slight reduction in viral RNA in supernatants from the hAPOBEC3G-expressing cells, the number of F-MuLV proviral copies detected in infected Mus dunni indicator cells was reduced to less than 1/20 of the number detected in the cells infected with the control preparations when progeny virions were produced from mA3b/H90045- or hAPOBEC3G-expressing cells. The number of proviral copies in the indicator cells was only moderately reduced when infected with progeny viruses produced from the mA3d/H90045-expressing cells, and no reduction in the proviral copy numbers was observed for the cells infected with the virus produced from cells expressing the full-length mA3d (Table 1). Thus, in agreement with the results of the infectious focus formation assays (Fig. 2H and I), mA3d/H90045 restricts F-MuLV proviral integration more efficiently than does mA3b/H90045.

C57BL/6-derived mAPOBEC3 restricts F-MuLV replication in the absence of the deaminase catalytic site. The above-
clones. F-MuLV proviral DNA was cloned, and its sequence was analyzed for at least 90
comparsion with that of FLAG.

A pairwise difference was then analyzed by Fisher's exact test.

tween mA3b and mA3d 1). To analyze this, we constructed reciprocal chimeras be-

In Fig. 3B. These results clearly localized the
dependent functional difference of mAPOBEC3 to the

It is not clear to what extent APOBEC3 proteins restrict
retroviral integration through their deaminase activity as op-
posed to through a deaminase-independent mechanism (1, 4,
38, 39, 43). Sequencing of the multiple proviral genomes re-
posed to through a deaminase-independent mechanism (1, 4,
retroviral integration through their deaminase activity as op-

TABLE 2. Sequence variations of F-MuLV proviral genome
observed for infected cells

<table>
<thead>
<tr>
<th>Stable transfectant</th>
<th>Total no. of nucleotides analyzed</th>
<th>No. of nucleotide exchanges</th>
<th>G/A</th>
<th>C/T</th>
<th>Other mutations</th>
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<tr>
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<td>0</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

a A 1,061-nucleotide fragment between the U3 and gag sequences from the F-MuLV proviral DNA was cloned, and its sequence was analyzed for at least 90 clones.

b The entire contingency table was analyzed for the possible presence of group-wise difference, which indicated a P value of <0.001. An individual group-wise difference was then analyzed by Fisher's exact test.

c P = 0.026 in comparison with that of GFP but was not significant in comparison with that of FLAG.

d P = 5.77 × 10⁻¹⁷ in comparison with the data for FLAG, 4.55 × 10⁻¹⁰, in comparison with that of GFP.

described results indicate that the major difference between the efficiencies of the C57BL/6- and BALB/c-derived mAPOBEC3 proteins in restricting F-MuLV infection in vitro likely stems from the differences in their amino acid sequences (Fig. 1). To analyze this, we constructed reciprocal chimeras between mA3bΔ5 and mA3dΔ5 by mutually exchanging the N-terminal portion at 196Q (Fig. 1) and established BALB/3T3 lines stably expressing the chimeric mAPOBEC3 (Fig. 3A). The provirus virus produced from the cells expressing the chimeric mA3bΔ5/mA3dΔ5 protein, with its N-terminal portion encoded by the BALB/c-derived mA3bΔ5, failed to fully restrict F-MuLV replication, while the reciprocal mA3bΔ5/mA3dΔ5 construct restricted F-MuLV replication as efficiently as mA3dΔ5 did (Fig. 3B). These results clearly localized the strain-dependent functional difference of mAPOBEC3Δ5 to the N-terminal portion harboring the CDD1.

It is not clear to what extent APOBEC3 proteins restrict retroviral integration through their deaminase activity as opposed to through a deaminase-independent mechanism (1, 4, 38, 39, 43). Sequencing of the multiple proviral genomes revealed a significant increase in G-to-A mutations in comparison with that of GFP.

The observed restriction of F-MuLV replication by mA3b
produced from the stably transfected cell lines might have resulted from an excessive amount of mAPOBEC3 protein that was forcibly incorporated into the virion. To examine directly the possible physiological effect of the putative resistant allele, mA3b, on F-MuLV infection in vivo, we introduced the targeted disruption of the APOBEC3 gene (31) into C57BL/6 mice by backcrossing. The progenies of heterozygous breeding pairs were genotyped (Fig. 4A), and the expression or lack of expression of the mA3b allele in the spleen and bone marrow was confirmed by RT-PCR (Fig. 4B). Infectious center assays revealed that C57BL/6 mice deficient in mAPOBEC3 possessed nearly 100-fold higher numbers of F-MuLV-producing cells at postinfection day (PID) 6, both in the spleen and bone marrow, in comparison with that of the wild-type or heterozygous counterparts (Fig. 4C). To further determine if the allelic difference in the APOBEC3 locus influences resistance to F-MuLV infection, heterozygous C57BL/6 mA3b+/− mice were mated with BALB/c mice, and the resultant CB6F1 mice were genotyped and infected with F-MuLV. As expected, CB6F1 mice of the mA3b+/− genotype expressed low levels of the full-length, as well as the short mA3b mRNA, in the bone marrow, while the mA3b/− mice expressed a higher level of the mA3bΔ5 message along with a low level of the full-length mA3b mRNA (Fig. 4D). Importantly, mA3b−/− mice deficient in the C57BL/6-derived APOBEC3 protein harbored more than 100-times-larger numbers of F-MuLV-producing cells in their bone marrow than the wild-type mA3b+/− mice at PID 6, despite the expression of the BALB/c-derived mA3d allele (Fig. 4E).

Thus, the C57BL/6-derived mA3b allele dominantly confers resistance to F-MuLV infection in the presence of the mA3d allele.

C57BL/6-derived mAPOBEC3 restricts erythroid cell prolif-
eration in mice infected with the pathogenic FV complex. To further determine if the mA3b allele physiologically functions in conferring resistance to FV-induced pathogenesis, FV-susceptible CB6F1 mice possessing or lacking the mA3b allele were infected with FV. CB6F1 mA3b/− mice possessed on average 13.1% ± 9.2% gp70-positive (gp70+) cells in their bone marrow at PID 7 (Fig. 4F), a large majority of which belonged to the TER119+ erythroblast population (25). On the other hand, when CB6F1 mice lacking the mA3b allele were infected with FV, significantly increased numbers (35.6% ± 11.9%, P = 0.00015) of bone marrow cells became positive for gp70, and these included more immature TER-119 cells. Reflecting the above differences, CB6F1 mA3b/− mice uniformly possessed extremely high hematocrit values at PID 21, while hematocrit
FIG. 4. Replication of F-MuLV in mAPOBEC3-deficient mice and the development of FV-induced disease in vivo. (A) Representative results for genotyping of the APOBEC3 alleles by PCR. (B) Expression of mAPOBEC3 mRNA in the bone marrow and spleen of the APOBEC3-deficient C57BL/6 mice analyzed by RT-PCR is shown. (C) C57BL/6 mice possessing either the APOBEC3<sup>+/+</sup> <sup>-/-</sup> (<i>n</i> = 8), <sup>+/-</sup> <sup>-/-</sup> (n = 10), or <sup>+/-</sup> <sup>-/-</sup> (n = 9) alleles were inoculated intravenously with 10<sup>5</sup> FFU of purified F-MuLV. F-MuLV infectious centers were enumerated on <i>Mus dunni</i> cells with anti-gp70 MAb on PID 6. (D) Expression of APOBEC3 mRNA in mAPOBEC3-deficient CB6F<sub>1</sub> mice analyzed by RT-PCR. (E) CB6F<sub>1</sub> mice possessing either the APOBEC3<sup>d/b</sup> (<i>d/-</i>) (n = 10) or d/b (n = 10) alleles were inoculated intravenously with 10<sup>4</sup> FFU of purified F-MuLV. F-MuLV infectious centers in the bone marrow were enumerated by coculturing <i>Mus dunni</i> cells and staining with anti-gp70 MAb on PID 6. (F and G) CB6F<sub>1</sub> mice possessing either the APOBEC3<sup>d/b</sup> (<i>d/-</i>) or d/b alleles were inoculated intravenously with 150 spleen FFU of FV complex. On PID 7, the cells prepared from the bone marrow were stained for TER119 and gp70 and were analyzed by flow cytometry (F). Five mice of each genotype were examined to calculate the means described in the text, and the dot graphs shown are of representative animals. On PID 21, hematocrit values were determined in the peripheral blood (G).
values for the mA3b allele were significantly lower (Fig. 4G). Thus, the mA3b allele does confer resistance to FV-induced erythroid pathology.

**DISCUSSION**

It has been generally accepted that retroviruses have evolved to evade the antiviral activities of their natural host's APOBEC proteins. In fact, primate lentiviruses counter the action of primate A3G with their Vif protein, while Vif is unable to counteract mAPOBEC3 and HIV-1 is highly susceptible to restriction by mAPOBEC3 (28, 42). The primate APOBEC3G gene predates HIV-related lentiviruses and has been under strong selective pressure for its conserved functionality (41). Thus, physiological targets of primate APOBEC3G might be endogenous retroviruses and nonautonomous retroelements (10, 14). Similarly, MuLV has also evolved mechanisms to block mAPOBEC3. These include the competitive exclusion of mAPOBEC3 from MuLV particles (1, 13), cleavage of virion-incorporated mAPOBEC3 by viral protease (1), and inhibition of the deaminase activity (4). However, possible inhibitory roles of APOBEC3 proteins on exogenous retroviral infection in vivo have become evident through the study of human lentiviruses. Thus, the patterns of hypermutation of HIV from infected individuals have indicated that the hAPOBEC3 proteins fulfill an inhibitory role (2, 26), and the levels of hAPOBEC3G expression have been associated with suppression of HIV-1 viremia and HIV-1-exposed but -uninfected status (3, 20). Regarding the mouse, however, the only previously demonstrated target for mAPOBEC3 in vivo was the betaretrovirus MMTV, with mAPOBEC3-deficient mice giving increased viral replication (39). Although putatively residual activities exerted by mAPOBEC3Δ5 in inhibiting MuLV integration have been reported (1, 4), these might have been caused by an excessive amount of mAPOBEC3 protein forcibly incorp- rated into the virion, especially when the experiments were performed by transfecting MuLV packaging cells with an mAPOBEC3-expressing plasmid vector.

Based on our previous demonstration that the FV resistance gene Rfv3 colocalized with the APOBEC3 locus (23, 36), we have shown here that the mouse gammaretrovirus F-MuLV is a target for mAPOBEC3 and, further, that mAPOBEC3 acts to restrict viral pathogenesis in vivo (Fig. 4). Gammaretroviruses have coevolved with their natural hosts (48), with MuLV and related endogenous retroviruses distributed more widely than MMTV among murine strains and species. Nevertheless, C57BL/6 and closely related C57BL/10 (B10) mice possess multiple host factors that make these strains resistant to FV-induced disease development (5, 22, 36). We have shown in the present paper that differences in the sequence of mAPOBEC3 (Fig. 1), along with different expression levels in the hematopoietic tissues (Fig. 2A to C) account for part of this polymorphism. Further, we have also localized the functional difference between F-MuLV-restricting mA3bΔ5 and less-restricting mA3Δ5 to the N-terminal portion other than the deaminase catalytic site (Fig. 3). Thus, F-MuLV infection in mice may not only provide a tractable model for the study of the in vivo mechanism of APOBEC3-mediated retroviral restriction, it may also provide insight into mechanisms of virus-host coevolution.

Finally, whether or not the Rfv3 locus is identical to the APOBEC3 locus must be discussed. The Rfv3 gene was first described by comparing the persistence of viremia after FV infection between the prototypic FV-resistant B10.A/SgSn and the susceptible A/WySn mice that share the same H-2d haplotype (6). A/WySn mice remained viremic at more than 30 days after FV infection, while B10.A/SgSn mice had cleared viremia by PID 30. Since Fv crosses between these two strains were not viremic and about half of the (B10.A X /A/WySn) X /A/WySn backcross mice showed viremia at PID 30, the presence of a recessive host gene in A/WySn mice was postulated in association with the persistence of viremia and was designated the Rfv3 allele. Thus, B10.A/SgSn mice possess a dominant allele, the Rfv3, conferring the early clearance of viremia. The Rfv3 locus was later mapped to within chromosome 15 (17, 47). As we have shown here (Fig. 1), B10.A/SgSn mice share the APOBEC3 sequence with B10.A/FvJ and A/WySn with BALB/c. Therefore, it is conceivable that the FV-restricting mA3b allele in B10.A/SgSn mice functioned to limit the replication of FV and thus contributed to the observed earlier clearance of viremia. However, for the clearance of viremia in FV-infected mice, the host immune responses are also required. In fact, B-cell-deficient C57BL/6 mice possessed higher levels of viremia than their wild-type counterparts at PID 7 (30), and FV-producing cells in the bone marrow and spleen could not be eliminated, even after effective priming of T cells with the viral antigens, in the absence of Ab-producing cells (24, 30). Thus, although the mA3b allele does contribute to the reduction in the number of virus-producing cells in the early stage of FV infection (Fig. 4), it must influence the immune responses, either directly or indirectly, to explain the phenotypes influ- enced by the Rfv3 gene. In this regard, (B10.A X /A/WySn)FvJ mice do produce F-MuLV-neutralizing Ab earlier than A/WySn mice do (23). The less massive expansion of FV-infected erythroid cells in the mA3b possessing mice than in those lacking this resistant genotype (Fig. 4) might result in the possible preservation of the stromal architecture that is re- quired for cell-to-cell interactions involved in lymphocyte priming and B-cell activation. Further studies are required to clarify the presumable identity of the mouse APOBEC3 gene as the Rfv3 gene.

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