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MOUSE APOBEC3 EXPRESSION IN NIH 3T3 CELLS MEDIATES HYPERMUTATION OF AKV MURINE LEUKEMIA VIRUS

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Abstract

Mouse APOBEC3 (mA3) is a cytidine deaminase that can act on the single-stranded DNA reverse transcripts of retroviruses resulting in G→A hypermutation of proviral DNA. Many mA3 studies have used NIH 3T3 cells assuming that endogenous mA3 production was negligible. We developed a monoclonal antibody specific for mA3 that reveals detectable mA3 in NIH 3T3 cells and we demonstrate that AKV released from the cells undergoes G→A hypermutation. Inactivation of the mA3 gene abolished the deamination confirming that AKV hypermutation was mediated by mA3. The G→A mutations in AKV viral transcripts deviated from a normal distribution with all the mutations contained within only 20% of the transcripts. Single cell analyses revealed that the expression of mA3 in NIH 3T3 cells was limited to 20% of the cells, which likely accounted for the abnormal distribution of mutations. Endogenous NIH 3T3 mA3 was found to restrict AKV replication.

Keywords

Retroviruses; MuLVs; Restriction Factors; APOBEC3; Mutation Rate; Hypermutation

INTRODUCTION

Human APOBEC3G and the mouse counterpart, APOBEC3 (mA3), are innate host factors that restrict the replication of retroviruses. Both exhibit cytidine deaminase activity that acts on single-stranded DNA produced during reverse transcription resulting in G → A hypermutation of retroviral transcripts. (Chiu and Greene, 2008; Langlois et al., 2009). Restriction of endogenous retroviruses by mA3 in mice is thought to involve cytidine deaminase activity (Esnault et al., 2005; Esnault et al., 2006; Jern et al., 2007) but mA3 restriction of many exogenous MuLVs has also been reported to occur in a cytidine

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deaminase-independent fashion (Abudu et al., 2006; Bishop et al., 2004; Boi et al., 2014; Browne and Littman, 2008; Doehle et al., 2005; Langlois et al., 2009).

Ecotropic MuLVs, which infect mice or rat cells but not cells of other species, are found as exogenous viruses as well as endogenous viruses produced in certain mouse strains. AKV is an endogenous ecotropic MuLV isolated from thymoma-prone AKR mice and can be propagated as an exogenous ecotropic MuLV (Rowe et al., 1971; Rowe and Pincus, 1972). In contrast to other MuLVs, which appear to be restricted by mA3 via cytidine deaminase-independent mechanisms, AKV has been reported to undergo G→A hypermutation upon virion capture of mA3 and subsequent infection (Langlois et al., 2009; Rosales Gerpe et al., 2015). In vitro cell lines such as NIH 3T3 cells transfected with mA3 have been used to assess the levels of mutations induced by APOBEC3 proteins (Browne and Littman, 2008; Langlois et al., 2009; Petit et al., 2009) with the assumption that the cells exhibited negligible levels of mA3 (Rosales Gerpe et al., 2015). In this study, we examined the mutations incurred by AKV in NIH 3T3 cells and found that the virus underwent G→A hypermutation without the addition of external mA3 by transfection or expression of an mA3 transgene. We developed a monoclonal antibody reactive with mA3 and assessed the level of expression of mA3 in NIH 3T3 cells by single cell immunoblotting procedures. Our results indicate that hypermutation of AKV in NIH 3T3 cells is the result of mA3 expression in a moderate proportion of the cells.

MATERIALS AND METHODS

Plasmids, cells and viruses

NIH 3T3 cells maintained in our laboratory and new NIH 3T3 cells from the American Type Culture Collection (ATCC CRL-1658) were maintained in Dulbecco's modified Eagle medium with 10% bovine serum and penicillin/streptomycin. CasFr^{KP} is a MuLV derived from the wild mouse ecotropic virus CasBrE and contains a short sequence of the F-MuLV, FB29 (Portis et al., 1994). Infectious AKV was obtained by transfection of NIH 3T3 cells with the AKR MuLV molecular clone 623 (Lowy et al., 1980).

Immunoblotting

Cellular mA3 levels were determined in lysates prepared using the CellLytic M Cell Lysis Reagent (Sigma). Cell lysates were centrifuged at $20,000 \times g$ for 10 minutes to separate the soluble and insoluble fractions. The protein concentration of the soluble fraction was determined using a Bradford assay (Pierce) according to the manufacturer's protocol. Samples were normalized according to their protein concentrations and were adjusted to $1 \times$ Laemmli sample buffer containing 5% β -mercaptoethanol and boiled for 10 minutes. The samples were then subjected to SDS-PAGE (10% acrylamide, Life Technologies), followed by transfer onto PVDF membranes. Immunoblots to detect native mA3 were performed on cell extracts utilizing an HRP-conjugated mA3-reactive monoclonal antibody. The antibody was generated in Wistar rats with an mA3 protein encoded by an Ultimate ORF clone (IOM18678) that was inserted into the pDEST-17 vector and expressed in the BL21AI *E. coli* strain according to the manufacturer's instructions (ThermoFisher Scientific/Invitrogen, Cat. # 11803012). Immunization of rats and fusion of rat splenocytes with a murine

myeloma cell line were performed by Green Mountain Antibodies, (Burlington VT). The resulting hybridomas were screened for their suitability for immunoblotting procedures using the *E. coli*-expressed mA3. An antibody released from a hybridoma designated 11B11B4C4 was utilized in these studies.

Immunoblots to detect virion p30 and mA3 were performed on virions sedimented from 4ml of the virus stocks in a Beckman SW 50.1 rotor for 1 hr at 50,000 rpm through 1ml of 20% sucrose cushion. The pelleted virions were resuspended in TSE (100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 7.5), adjusted to 1× Laemmli sample buffer containing 5% β-mercaptoethanol, boiled for 10 minutes, electrophoresed and transferred to PVDF membranes as described above. Immunoblots were performed utilizing a p30-reactive monoclonal antibody, R187, (Chesebro et al., 1983) or the mA3-reactive monoclonal antibody, 11B11B4C4, both of which were directly conjugated to horseradish peroxidase with the Lightning-Link HRP Antibody Labeling Kit (Novus Biologicals). Virion p30 on immunoblots was quantified using the ImageJ software (Schneider et al., 2012).

Mutational Analyses

The effect of virion-associated mA3 on the mutation of viral transcripts was assessed in NIH 3T3 cells infected with viruses harvested from clonal NIH 3T3 cell lines. We derived clonal cell lines by plating approximately 20 cells on a 100mm tissue culture dish after infection of NIH 3T3 cells with the exogenous ecotropic MuLV CasFr^{KP} or AKV at a low multiplicity of infection. Infected colonies were identified by cell-surface expression of their Env protein using an immunofluorescence assay with the monoclonal antibody 573 (Evans et al., 2014). This served to minimize the initial genetic heterogeneity of the viruses. Mutation rates were assessed after infection of NIH 3T3 cells with virus harvested from the infected NIH 3T3 clonal cell lines. Eight hours after infection cellular DNA was isolated using the AquaGenomic DNA isolation kit according to the manufacturer's instructions. Viral DNAs synthesized during the first eight hours of infection were amplified by PCR using Pfx50 polymerase (Life Technologies). Primers within the *env* gene of CasFr^{KP} were CasFrKP6805, TTGAGAGAGTACACTAGTC as the forward primer and CasFrKP7886RC, TCTGTTCCCTGACCTTGATC as the reverse primer. Viral DNAs from AKV-infected cells were amplified using AKV *env*-specific primers, AKV 6704 CCAGCGGGAGTCGAAAACC as the forward primer and AKV 7964RC CCCGGTACTTTCCAGCC as the reverse primer. PCR products were resolved on a 3% 3:1 NuSieve agarose gel and were purified using a Zymoclean Gel DNA Recovery kit (Zymo research) according to the manufacturer's instructions. Purified PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Transformed colonies were isolated from selective LB agar plates containing 50 µg/ml Kanamycin and the selected colonies were grown in 1 ml Kanamycin-containing medium. DNA from the colonies was purified with an Extract-N-AmpTM Tissue PCR Kit (Sigma) according to the manufacturer's instructions and screened by PCR to confirm the presence of the appropriately sized insert. PCR products from 94 confirmed clones were purified with a QIAquick 96 PCR Purification Kit (Qiagen) and sequenced using the primers used to generate the amplicons.

Groups of sequences generated in these analyses were compiled and compared in the DNASTar Lasergene program, SeqMan. Mutation rates for each type of point mutation (e.g., G-to-A transitions) were calculated as the frequency per nucleotide of that type of mutation in each sequenced clone. The mutation rates for each type of mutation for each clone were averaged to give the mean mutation rate as well as the standard error for that specific type of mutation. The overall mutation rate per clone was calculated as the frequency per nucleotide of all mutations in each clone. The overall mutation rate for the data set was calculated by averaging the rate for each clone to give the mean overall mutation rate and the standard error.

Derivation of an NIH 3T3mA3 KO cell line

A thymidine at base number 132 was inserted in the APOBEC3 (EU707568) sequences of NIH 3T3 cells (ATCC CRL-1658) using CRISPR/Cas9 by Applied Stem Cell (Milpitas, CA). The insertion resulted in a termination codon and predicted truncated products of 46 amino acids.

Specific infectivity

The infectivity of virions released during an overnight interval from NIH 3T3 cells as well as from NIH 3T3mA3 KO cells was assessed by a focal immunofluorescence assay (FIA) (Sitbon et al., 1985). The specific infectivity's of virions in the samples were calculated by normalizing the infectivity to the levels of the p30 capsid (CA) protein, determined by immunoblotting.

Single cell immunoblots

Single cell (sc) immunoblots were performed using the Milo scBlotting device (ProteinSimple) according to the manufacturer's instructions. Cultured NIH 3T3 cells were trypsinized and then quenched with 10% FBS. The cells were washed with PBS, counted and then diluted to 100,000 cells/ml with 1× suspension buffer. The suspension buffer, scWest chips, antibody dilution buffer, and washing buffer were all obtained from ProteinSimple. The standard medium size scWest chips (ProteinSimple) were rehydrated with the suspension buffer for >10 minutes at room temperature. Approximately 1 ml of the cell suspension was placed on the surface of each rehydrated chip with the polyacrylamide gel side up. The cells were allowed to settle into pre-cut acrylamide microwells for 10 minutes. The unsettled cells were washed off with 1× suspension buffer and each chip was placed into the Milo instrument for lysis, electrophoresis, and fixing. The conditions were set as follows: lysis time 15 seconds, electrophoresis time 45 seconds, and UV exposure 4 minutes. The chip was then incubated with rabbit anti-GAPDH (1:10, Cell Signaling, Cat. 2118S) and anti-mA3 (1:5, mAb 11B11B4C4) diluted in the antibody dilution buffer. It was incubated at room temperature for 2 hours and then washed 3× with the washing buffer. The chip was subsequently incubated with a mixture of fluorescent dye labeled secondary antibodies, AlexaFluor 647 goat anti-rat IgG (1:20, Invitrogen A21247), and AlexaFluor 532 goat anti-rabbit IgG (1:20, Invitrogen A11009), for 1.5 hours protected from light exposure. After 3× washing with the washing buffer, the chip was then briefly rinsed with 1× PBS, and the slide was centrifuged to remove the remaining liquid. The chip was scanned by a Genepix 4000 microarray scanner with the resolution setting at 5 µm.

Real-time PCR analysis of mA3 mRNA

Cells were cultured in 60mm diameter tissue culture dishes and total cellular RNA was isolated using the Quick-RNA™ MiniPrep Plus (Zymo Research) according to the manufacturer's instructions. RNA was treated with DNase I (Ambion) for 30 min at 37°C to remove any genomic DNA contamination and purified using RNA cleanup columns (Zymo Research). RNA concentrations were quantified by measuring optical absorption at 260 nm. Reverse transcription was performed using RT Supermix (Bio-Rad) according to the manufacturer's instructions. The RT reaction mixture was then inactivated at 95°C for 5 min, chilled at 4°C, and the cDNA samples diluted fivefold in RNase-free water. Quantitative real-time PCR reactions of the samples were run in triplicate using a ViiA 7 instrument (Applied Biosystems). SYBR Green dye with ROX (Bio-Rad) was used for measurement of real-time PCR amplification. Each sample was amplified during 40 PCR cycles consisting of denaturation for 15 s at 95°C, annealing and extension for 60 s at 62°C. Data were calculated as the difference in CT value (log2) for β -actin minus the CT value of the mA3 gene for each sample ($CT = CT_{\beta\text{-actin}} - CT_{\text{gene of interest}}$) to control for variations in RNA amounts in each sample. The data are presented as the percentage of expression of the mA3 relative to β -actin. The primers set used to detect β -actin genes were Actb-29f CAGCTTCTTTGCAGCTCCTT and Actb-185r CACGATGGAGGGGAATACAG.

Two different sets of primers were used for the detection of mA3, Apobec3.1-804f ACCAGCTGGAGCAGTTCAAT associated with Apobec3.1-914r GCTCCATGGACCGAATCTTA and Apobec3.2-452f TTCCTGGCTACACACCACAA associated with Apobec3.2-551r GAACCAGCCTGCAAAGATTC.

Statistical analysis

The significance of differences between mutation rates was calculated by the Student's unpaired *t* test. The significance of the sequence preference of bases near mutation sites was calculated by the two-tailed Binomial Test. The significance of the distribution of mutations on retroviral transcripts was calculated using the Chi-square test comparing observed and expected frequencies. Probability values <0.05 were considered significant for all tests. All statistical tests were computed using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

RESULTS

AKV undergoes G→A hypermutation in NIH 3T3 cells

AKV has been reported to undergo G→A hypermutation upon incorporation of mA3 into virions while Mo-MuLV did not undergo hypermutation in parallel experiments (Langlois et al., 2009). We have reported that another MuLV, CasFr^{KP}, does not undergo G→A hypermutation upon incorporation of mA3 (Boi et al., 2014) but does exhibit a loss of RT specific activity and fidelity upon incorporation of high levels of mA3. To study the differences between the two MuLVs regarding their interactions with mA3, we wished to obtain baseline mutation rates of both viruses in the absence of mA3. Clonal NIH 3T3 cell lines infected at a low multiplicity of infection (MOI) with either AKV or CasFr^{KP} were established to minimize genetic heterogeneity of the viruses (Fig. 1). Viruses released from

the clonal cell lines were used to infect target NIH 3T3 cells to assess mutations. Mutations occurring after infection of new NIH 3T3 target cells are the result of mA3 acquired upon release of progeny virions from the clonal line. Cellular DNA from the target NIH 3T3 cells was isolated at 8 hours after infection and a region of the *env* gene of the proviral DNAs transcribed during this interval was amplified by PCR. An 8-hour interval was chosen to preclude a second round of infection and to minimize the loss of deaminated transcripts through cellular degradative pathways (Chiu and Greene, 2008; Sova and Volsky, 1993). The amplicons were subsequently cloned and sequenced to detect and quantify mutations. Quite surprisingly, these analyses revealed a marked difference in the patterns of mutations observed between the two MuLVs (Fig. 2A). AKV, in the apparent absence of mA3, exhibited hypermutation with a G→A mutation rate much higher than the G→A mutation rate of CasFr^{KP}. This resulted in a significantly higher overall mutation rate for AKV (Fig. 2B). Similar mutation rates were observed using a newly obtained NIH 3T3 cell line (ATTC CRL-1658) indicating that the hypermutation activity was not unique to the NIH 3T3 cells maintained in our laboratory (data not shown).

Hypermutation of AKV from NIH 3T3 cells is the result of virion incorporation of an endogenous restriction factor

M. dunni cells are devoid of some major classes of endogenous MuLVs and are highly permissive to infection by many exogenous retroviruses (Lander and Chattopadhyay, 1984). This could be the result of differences in the levels of MuLV restriction factors such as mA3 in M. dunni cells compared to NIH 3T3 cells. If this were the case, AKV released from M. dunni cells might not undergo hypermutation. To investigate this possibility, M. dunni cells were infected with AKV at a low MOI and an infected clonal cell line was established. AKV released from this line was used to infect NIH 3T3 cells to determine rates of mutation (Fig. 3A). We found that AKV released from M. dunni cells did not undergo G→A hypermutation upon primary infection of the NIH 3T3 cells (Fig. 3B). However, G→A hypermutation activity was restored to progeny virions released from the infected NIH 3T3 cells upon secondary infection of new NIH 3T3 cells (Fig. 3B) and exhibited an overall mutation rate not significantly different than originally observed with AKV from NIH 3T3 cells (Figs. 2B and 3C). These results confirmed that AKV incorporated a restriction factor expressed in NIH 3T3 cells, but not M. dunni cells, that induced G→A mutations.

The sequence context of G→A mutations in AKV is similar to the sequence context of mA3-induced G→A mutations

G→A mutations in AKV as well as other retroviruses susceptible to mA3, e. g., HIV, exhibit a preferred target sequence corresponding to TTC in the negative sense transcript with C the base that becomes deaminated (Bishop et al., 2004; Jern et al., 2007; Langlois et al., 2009; Petit et al., 2009). To determine if the sequence context of G→A mutations in AKV transcripts revealed a strong consensus target sequence consistent with the cytidine deaminase target of mA3, the flanking sequences of the AKV G→A mutations were analyzed (Fig. 4). Indeed, a very conserved consensus sequence flanking the 5' and 3' ends of the cytidine target was observed corresponding to TTCAA in the negative sense transcript. A strong consensus flanking both the 5' and 3' sides of the cytidine deaminase target has not previously been reported.

NIH 3T3 cells express a low level of mA3 that is responsible for G→A hypermutation of AKV

The findings that G→A hypermutation of AKV was observed in cells not known to express mA3 could have reflected the presence of another protein with cytidine deaminase activity similar to mA3 or, alternatively, have been due to expression of endogenous mA3 in the 3T3 cells sufficient to cause hypermutation. To analyze endogenous mA3 expression we developed a monoclonal antibody reactive to native mA3 (mAb 11B11B4C4) effective in immunoblotting procedures. In addition, we produced an NIH 3T3 cell line in which the genes encoding mA3 were knocked out by CRISPR/Cas 9 techniques (NIH 3T3mA3KO). The mA3 antibody was used to examine the levels of mA3 expression in wild-type (wt) NIH 3T3 cells, NIH 3T3mA3KO cells and *M. dunni* cells. As expected, mA3 was not detected in the NIH 3T3mA3KO cell line, or in *M. dunni* cells. However, a low but detectable level of mA3 was found in NIH 3T3 cells (Fig. 5A). Analysis of mRNA in the cell lines revealed detectable levels of mRNA in both the NIH 3T3 cells as well as the NIH 3T3 mA3KO cells but negligible levels in *M. dunni* cells (Fig. 5B). The finding that mA3 mRNA was not statistically different between NIH 3T3 and NIH 3T3 mA3KO cells was expected in that the KO cell line was generated by introducing a termination codon that resulted in a truncated mA3 protein translated from a full-length mRNA. We found that AKV released from the mA3-KO cell line did not exhibit G→A hypermutation (Fig. 5C) and exhibited a low overall mutation rate (Fig. 5D). These results indicated that the expression level of mA3 in the NIH 3T3 cells was sufficient to induce hypermutation of AKV.

G→A mutations are disproportionate among AKV transcripts and correlate with heterogeneity in the expression of mA3 in NIH 3T3 cells

When compiling the results of the AKV mutations it was noted that most of the transcripts were devoid of mutations while some of the transcripts exhibited multiple mutations (Table 1). We found that all the mutations occurred in approximately 20% of the transcripts. This observation is similar to previously reported observations (Langlois et al., 2009; Paprotka et al., 2010). The results of our analyses were tabulated and subjected to a chi-square test for compliance to a random distribution. It was found that the data were inconsistent with a random distribution of mutations with a P value of <0.0001.

The non-random distribution of G→A mutations among the AKV transcripts could have been the result of limiting levels of mA3 in NIH 3T3 cells such that only a portion of the progeny virions incorporated mA3, or alternatively, that only a fraction of the NIH 3T3 cells expressed the restriction factor. To investigate this issue, we examined the expression of mA3 in NIH 3T3 cells using a single cell Western blotting procedure (Sinkala and Herr, 2015). Cells were distributed at less than one cell per well in microwells of scWest chips (ProteinSimple) and processed in the Milo scBlotting device (ProteinSimple). The chips were immunoblotted with antibodies to GAPDH to identify the locations of microwells containing a cell (Fig. 6A) and with the mA3 monoclonal antibody to determine which of the cells expressed mA3 (Fig. 6B). Analysis of over 1000 single cells revealed that only 20% of the cells expressed detectable levels of mA3 in close agreement with the percentage of virions that generated G→A mutations.

AKV replication is restricted by endogenous mA3 in NIH 3T3 cells

Previous reports of mA3 restriction of AKV have been based on studies in which virions containing mA3 were obtained after co-transfection of 293T cells with a plasmid encoding an AKV provirus and a plasmid encoding mA3. Subsequently, the infectivity of the released virions was evaluated on NIH 3T3 cells and found to decrease with increasing levels of mA3. To determine if endogenous mA3 was restricting AKV replication in NIH 3T3 cells we evaluated the specific infectivity's of virions released from NIH 3T3 and from NIH 3T3mA3KO cells. We found that the specific infectivity of AKV virions released from NIH 3T3mA3KO cells was significantly higher than those released from the wild-type NIH 3T3 cells indicating that the endogenous mA3 expressed in NIH 3T3 cells was exerting a restrictive effect on AKV (Fig. 7A). The specific infectivity's of CasFr^{KP} virions released from NIH 3T3 and NIH 3T3mA3KO cells were not significantly different (Fig. 7B).

DISCUSSION

NIH 3T3 cells have been employed for many investigations of mA3 with the implicit assumption that the expression of mA3 in this cell line was negligible. The finding that mA3 activity is evident, even when expressed at very low levels in these cells, is an important consideration when utilizing the cells for mutation studies. This observation is reminiscent of a study utilizing 293T cells in which low levels of endogenous human APOBEC3G obscured the low intrinsic mutation rate of foamy virus (Gartner et al., 2009). It has been reported that incorporation of mA3 into AKV virions harvested from 293T cells resulted in G→A hypermutation upon subsequent infection of NIH 3T3 cells (Langlois et al., 2009). The extent to which endogenous NIH 3T3 mA3 contributed to this result is unclear, considering that the longer time interval between infection and analysis of DNA transcripts (48 hrs compared to 8 hrs in our analyses) allowed the spread of virus through secondary infection of the NIH 3T3 cells (Rosales Gerpe et al., 2015). Our data suggest that many of the observed mutations were the result of endogenous mA3 in the NIH 3T3 cells. Although this discrepancy could be attributed to differences in the respective NIH 3T3 cell lines utilized in the laboratories, our results were confirmed using newly acquired NIH 3T3 cells from the ATCC.

It was possible that the RT of AKV exhibited an elevated spontaneous G→A mutation rate compared to other MuLVs. In this regard, G→A hypermutation observed in retroviral infections has been suggested to be the result of error-prone polymerases (Pathak and Temin, 1990). This possibility was excluded in the present study by the finding that AKV harvested from a *M. dunnii* cell line did not exhibit hypermutation upon initial infection of NIH 3T3 cells, but recovered this activity upon subsequent passage on the cells (Fig. 3). It was also possible that NIH 3T3 cells expressed a restriction factor distinct from mA3. This seemed unlikely considering the similarity of the sequence context for deamination by mA3 (Fig. 4) and the finding that hypermutation was observed in AKV but not CasFr^{KP}, (Fig. 2), in agreement with earlier reports of mA3 restriction. The involvement of mA3 in hypermutation of AKV in NIH 3T3 cells was confirmed by the demonstration that NIH 3T3mA3KO cells, in which mA3 had been genetically inactivated, no longer supported hypermutation (Fig. 5B). This conclusion was further supported by the detection of low

levels of mA3 in NIH 3T3 cells using our newly developed mA3-specific antibody (Fig. 5A).

The finding that the distribution of G→A mutations was not random among the sequenced transcripts could have been due to heterogeneity in the virions or in the cells. It was possible that the low level of mA3 expressed in the NIH 3T3 cells was insufficient to populate all released viruses leaving most virions devoid of the restriction factor. However, we found that endogenous expression of mA3 by the NIH 3T3 cells was limited to only about 20% of the cells, correlating very closely to the proportion of transcripts containing G→A mutations. It is unclear whether the expression of mA3 changes with different cellular states such as progression through the cell replication cycle or if the expression is a static property of individual cells. The latter seems unlikely considering that distinct clonal cell lines exhibit nearly identical patterns of heterogeneity of mA3 expression (data not shown). The availability of a monoclonal antibody to mA3 will facilitate further studies on mA3 expression and function.

Our findings support the conclusion that low levels of mA3 are sufficient to drive hypermutation of AKV. In this regard, it has been estimated that very low levels (4 to 7 molecules) of hA3G are sufficient to inactivate an HIV-1 virion (Xu et al., 2007) and that as few as one mA3 molecule may be sufficient to inactivate a MuLV virion (Rulli et al., 2008). The extent of hypermutation reported earlier (Langlois et al., 2009) was approximately 4–5-fold higher than that reported here. However, our results indicating that only 20% of the NIH 3T3 cells express mA3 suggest that only 20% of the virions in our system contain the restriction factor. Thus, the incidence of G→A mutations per mA3-containing virion would be about 5-fold higher than we observed when considering 100% of the virions and would be consistent with the earlier report (Langlois et al., 2009).

Our results indicate that the low level of endogenous mA3 expression is sufficient to restrict the replication of AKV (Fig. 7A). This observation is in agreement with previous studies of the action of mA3 on AKV indicating that restriction of AKV infectivity correlated with increasing levels of the mA3. However, in contrast to AKV, CasFr^{KP} was not mutated or restricted in response to endogenous NIH 3T3 mA3 (Figs. 2 and 7).

In an earlier study with the CasFr^{KP} virus, we found that augmented expression of mA3 by stable transfection of NIH 3T3 cells was accompanied by a marked decrease in virion specific infectivity, a decrease in reverse transcriptase (RT) specific activity and an increase in the overall incidence of mutations. However, the increased mutations were not the result of mA3 signature G→A hypermutation (Boi et al., 2014). The observation that endogenous levels of mA3 in NIH 3T3 cells were not sufficient to increase the overall incidence of mutations or to restrict infectivity of CasFr^{KP}, while it was sufficient to hypermutate and restrict AKV, suggests the existence of two distinct mechanisms of action by mA3 that exhibit different stoichiometry's.

The MuLV glycosylated Gag protein (glyco-Gag) has been implicated in the resistance of MuLVs to the deaminase-independent action of mA3 (Kolokithas et al., 2010; Stavrou et al., 2013), and it has been recently reported that glyco-Gag also confers resistance to deaminase-

dependent G→A hypermutation of AKV (Rosales Gerpe et al., 2015). This could reflect a mechanism of inhibition by glyco-Gag that acts in an indirect manner, upstream of two distinct mA3 activities. In this regard, it has been suggested that glyco-Gag acts by blocking access of mA3 to the RT complex (Stavrou et al., 2013). The basis for the differences in susceptibility of AKV and CasFr^{KP} to hypermutation remains unclear. It is possible that the glyco-Gag plays a more direct role in blocking hypermutation than simply blocking access to the RT complex, perhaps by direct interaction with mA3 and/or RT. If this were the case, the AKV glyco-Gag may be less efficient in this function than the glyco-Gag proteins of exogenous MuLVs. In support of this idea, alterations in the glycosylation of Moloney MuLV have been reported to facilitate G→A hypermutation (Rosales Gerpe et al., 2015). Studies of possible interactions in this process should be facilitated by the development of antibodies to the components.

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Highlights

- NIH 3T3 cells express very low levels of mA3 that cause hypermutation of AKV
- Expression of mA3 is heterogeneous leading to an abnormal distribution of mutations
- Development of an mA3-specific and sensitive monoclonal antibody
- Development of an NIH 3T3 line with a genetically inactivated mA3 gene

Figure 1. Analysis of proviral mutations

Cell lines were infected at a low MOI with either AKV or CasFr^{KP}. To minimize genetic heterogeneity in the viruses, infected colonies were identified by cell-surface expression of the Env protein using an envelope-specific monoclonal antibody (red) and expanded to establish clonal cell lines. Viruses released from the lines were used to infect NIH 3T3 cells. DNA isolated 8 hours post infection (p.i) was amplified by PCR to obtain amplicons of the env region of the early proviral transcripts. Individual amplicons were cloned and sequenced for mutational analyses.

AKV and CasFr^{KP} released from NIH 3T3 cells

Figure 2. Mutation rates of AKV and CasFr^{KP} released from NIH 3T3 cells

NIH 3T3 cells were infected at a low MOI with either AKV or CasFr^{KP} and subcloned to establish infected clonal cell lines. Viruses released from the lines were used to infect new NIH 3T3 cells for analyses of mutation rates as illustrated in Fig. 1. **A).** Individual mutation rates of transcripts 8 hours after infection of NIH 3T3 cells with AKV (black bars) and CasFr^{KP} (white bars) released from infected NIH 3T3 clonal cell lines. **B).** The overall mutation rates calculated by averaging the rate of all mutations for each transcript generated by viruses from the clonal lines. Error bars represent standard errors of the mean (SEM) and significant differences were evaluated by the Student's unpaired *t* test.

Figure 3. Loss of hypermutation of AKV released from *M. dunni* cells and reacquisition of hypermutation activity upon passage in NIH 3T3 cells

A.) A schematic depicting the infections and mutational analyses. **B.)** Individual mutation rates of viruses released from a clonal AKV-infected *M. dunni* cell line were analyzed for mutations after primary infection of NIH 3T3 cells (white bars). Following a single subsequent passage in NIH 3T3 cells, the released viruses were reanalyzed after secondary infection of new NIH 3T3 cells (black bars). **C.)** Overall mutation rates of AKV released from *M. dunni* cells or after passage on NIH 3T3 cells. The error bars represent SEM values and significant differences were evaluated by the Student's unpaired *t* test.

Figure 4. Sequence context of induced G→A mutations in AKV

The sequence context of G→A mutations in transcripts generated after an 8 hr infection were determined for the two bases flanking the 3' and 5' side of the mutation site. The consensus sequences of the observed G→A mutations as well as the consensus of the negative strand DNA flanking the cytidine deaminase target C residue are shown. A total of 64 mutations were detected in 168 transcripts totaling approximately 1.5×10^5 bases that were sequenced. The numbers in the grid represent the number of instances when the indicated base occupied that position with respect to a G→A mutation. The frequency of the flanking bases at a particular position compared to the expected frequency was calculated by

the two-tailed binomial test with probability values of <0.05 considered significant. The significance of frequencies in the positive strand consensus strand are indicated (****, P 0.0001). The consensus strands include both positive and negative sense to indicate the context of the cytidine targeted for deamination.

Figure 5. Detection of mA3 in NIH 3T3 and mutation rates of AKV from NIH 3T3/mA3KO cells
A). An immunoblot of NIH 3T3 cells, NIH 3T3mA3KO cells and Mus. Dunni cells using a rat monoclonal antibody reactive with mA3 directly conjugated with horseradish peroxidase.
B). Levels of mA3 mRNA determined by RT PCR and expressed as the percentage of β-Actin mRNA detected in the samples. The results are the average values obtained from two different primer sets, each performed in triplicate. Error bars represent the SEM for each determination.
C). Individual mutation rates of 8 hour transcripts after infection of NIH 3T3 cells with AKV released from NIH 3T3mA3KO cells. A total of 90 transcripts totaling approximately 8.2×10^4 bases were sequenced.
D). The overall mutation rate was calculated by averaging the rate of all mutations for each transcript. Error bars represent the SEM for each determination.

Figure 6. Representative single cell blotting to detect heterogeneity of mA3 expression in NIH 3T3 cells

A). An scWest Chip (ProteinSimple) was immunoblotted with an antibody to GAPDH to identify the locations of microwells containing individual cells. **B).** The same chip immunoblotted with the anti-mA3 monoclonal antibody to determine which of the cells expressed mA3. White arrows indicate cells expressing mA3. In this field, 6 of 24 NIH 3T3 cells expressed mA3. Analyses of additional fields revealed that 201 of 1002 NIH 3T3 cells expressed detectable mA3.

Figure 7. Suppression of AKV infectivity by endogenous mA3 in NIH 3T3 cells

The infectivity's of AKV and CasFr^{KP} virions released from NIH 3T3 cells as well as from NIH 3T3mA3KO cells during an overnight interval were determined by the FIA and normalized to the levels of p30 present in the samples. **A).** Specific infectivity of AKV on NIH 3T3 and NIH 3T3mA3KO cells. **B).** Specific infectivity of CasFr^{KP} on NIH 3T3 and NIH 3T3mA3KO cells. The error bars represent SEM values and significant differences were evaluated by the Student's unpaired *t* test. Immunoblots of p30 of the virions used for infections are shown below each graph.

Table 1

Distribution of G→A mutations in AKV transcripts *

Number of transcripts	G→A Mutations per Transcript	Total Number of G→A Mutations	% G→A Mutations	% Transcripts
133	0	0	0.0	79.2
19	1	19	29.7	11.3
8	2	16	25.0	4.8
4	3	12	18.8	2.4
3	4	12	18.8	1.8
1	5	5	7.8	0.6

* Viral transcripts in NIH 3T3 cells infected with viruses released from infected clonal cell lines of NIH 3T3 cells were analyzed for G→A mutations. The number of transcripts containing the indicated number of mutations were compared to the expected frequencies by the Chi-square test. The discrepancy between the observed frequencies compared to the expected frequencies were highly significant ($P < 0.0001$).