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Incorporation of Mouse APOBEC3 into Murine Leukemia Virus Virions Decreases the Activity and Fidelity of Reverse Transcriptase

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APOBEC3 proteins are restriction factors that induce G→A hypermutation in retroviruses during replication as a result of cytidine deamination of minus-strand DNA transcripts. However, the mechanism of APOBEC inhibition of murine leukemia viruses (MuLVs) does not appear to be G→A hypermutation and is unclear. In this report, the incorporation of mA3 in virions resulted in a loss in virion reverse transcriptase (RT) activity and RT fidelity that correlated with the loss of virion-specific infectivity.

APOBEC3G (hA3G) in humans and APOBEC3 (mA3) in mice are cytidine deaminases that act on single-stranded DNA during reverse transcription, resulting in G→A hypermutation of newly synthesized proviral DNA (1, 2). Although exogenous murine leukemia viruses (MuLVs) are relatively insensitive to the actions of mA3 (2–7), several studies have reported partial inhibition of exogenous MuLVs after incorporation of mA3 (2, 3, 6, 8–11). Furthermore, the finding that *Rfv3*, a resistance gene for Friend erythroleukemia, encodes mA3 and is responsible for a decreased infectious titer of the Friend MuLV (Fr-MuLV) (11–13) strongly suggests that mA3 inhibits the replication of exogenous MuLVs *in vivo*. Exogenous ecotropic MuLVs, such as the Fr-MuLV and Moloney MuLV (Mo-MuLV), are inhibited through mechanisms that do not appear to involve cytidine deamination (2, 9, 10). In this study, the effects of mA3 on the infectivity, reverse transcriptase (RT) activity, and frequency of mutations of the ecotropic MuLV CasFr^{KP} were examined.

Virion-associated mA3 suppresses CasFr^{KP} MuLV infectivity. In order to examine the effects of mA3 incorporated into MuLV virions, we derived clonal cell lines infected with CasFr^{KP} (14). The 3T3mA3 cells were derived by transfection of a plasmid encoding the full-length mA3 derived from the BALB/c mouse strain and was tagged at the C termini with hemagglutinin (HA) (5). Infected clonal cell lines were obtained from 3T3 cells as well as from 3T3 cells expressing mA3 (3T3mA3) and were designated 3T3/CasFr^{KP} and 3T3mA3/CasFr^{KP}, respectively. In agreement with earlier reports (2, 7, 9, 13, 15), the clonal cell line expressing mA3 (3T3mA3/CasFr^{KP}) released virions that had incorporated an easily detectable level of mA3 (Fig. 1).

The infectivity of CasFr^{KP} containing mA3 was compared to that of CasFr^{KP} devoid of mA3 by a focal immunofluorescence assay (FIA) (16) and normalized for virion number using the level of p30 CA protein (Fig. 2A). The specific infectivity of virions released from cells expressing mA3 exhibited over a 90% reduction in infectivity (Fig. 2B), corroborating earlier studies of inhibition by mA3 (2, 3, 6, 8–11).

Decrease in RT activity in virions containing mA3. A recent study examined the efficiency of virion reverse transcription by monitoring the appearance of strong-stop DNA during the course of the RT reaction using virions isolated from C57BL/6 and BALB/c mice as well as those from mA3 knockout (KO) mice (17). Both mouse strains exhibited a similar decrease in RT activity compared to KO mice, indicating an effect of endogenous mA3. C57BL/6 and BALB/c mice express different allelic forms of mA3, suggesting that both forms inhibit RT to a similar extent. These results may reflect a direct effect of mA3 on the enzymatic activity of RT or an indirect effect, such as interference of primer binding

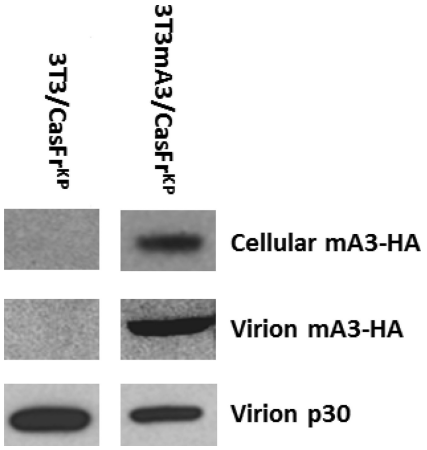

FIG 1 Incorporation of mA3 into virions released from clonal cell lines. The expression of mA3-HA in the clonal cell line 3T3mA3/CasFr^{KP} was determined from an immunoblot of a cellular extract containing 15 μg protein and developed using an anti-HA monoclonal antibody. Virion mA3-HA released from 3T3mA3/CasFr^{KP} cells was determined from an immunoblot of a gel of virion proteins contained in 1.5 ml of culture supernatant using the anti-HA antibody, while virion p30 was determined from immunoblots of gels of virion proteins contained in 0.075 ml of culture supernatant developed with a monoclonal antibody to p30. Exposure times for the immunoblots of virion p30 were 1 h, while immunoblots of virion mA3-HA were exposed for 5 min. Parallel analyses of 3T3/CasFr^{KP} cells and virions devoid of mA3-HA were included as controls.

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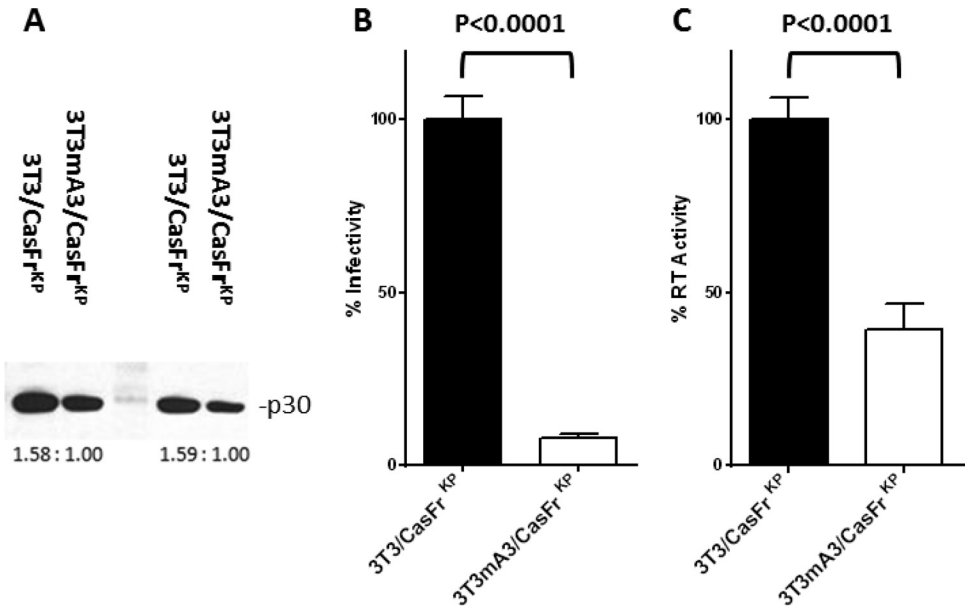

FIG 2 Influence of virion-incorporated mA3 on specific infectivity and virion RT activity. (A) Virion p30 was determined from immunoblots of gels of virion proteins contained culture supernatant from 3T3/CasFr^{KP} and 3T3mA3/CasFr^{KP} cells. Ratios of the intensities of the bands determined by ImageJ densitometry are shown below the sets of bands. Two different levels of each sample were loaded, resulting in sets of bands with nearly identical ratios of intensity. The average of the ratios was used to normalize the infectivity and RT activity determinations. (B) The infectivity of virions released from cells during an 8-h interval was determined by the FIA and normalized to the levels of p30 present in the samples. The determinations are expressed as a percentage of the mean specific infectivity exhibited by virions released from cells not expressing mA3 (3T3/CasFr^{KP}). Error bars represent the standard errors of 3 to 6 determinations. The *P* value determined by Student's unpaired *t* test is indicated. *P* values of <0.05 were considered significant. (C). The RT activities of virions released from cells during an 8-h interval were determined and normalized to the levels of p30 present in the samples. RT activity was determined for virions contained in 2.5 ml of supernatant media of the samples assayed for infectivity and p30. The determinations are expressed as a percentage of the mean specific infectivity exhibited by virions released from cells not expressing mA3 (3T3/CasFr^{KP}). Error bars represent the standard errors of 3 to 6 determinations. The *P* value determined by Student's unpaired *t* test is indicated. *P* values of <0.05 were considered significant.

(18). To assess the effect of virion-incorporated mA3 on the enzymatic activity of RT, we compared the RT activity using a colorimetric assay (Roche Applied Science; no. 11468120910) with exogenous poly(A)/oligo(dT) as the substrate/primer. As was the case with infectivity, the RT activity was significantly lower in virions containing mA3 released from 3T3mA3/CasFr^{KP} cells than that in virions released from 3T3/CasFr^{KP} cells (Fig. 2C). These results suggest an inhibition of the specific activity of the enzyme, although a decrease in the level of fully active RT in the virion cannot be excluded.

Incorporation of mA3 into virions results in a decrease of RT fidelity. The clonal cell lines utilized were obtained at a low multiplicity of infection, such that only a small percentage of the clonal lines were infected. This procedure served to minimize virion genomic heterogeneity as well as yield infected clones with and without mA3 expression. Viruses released from the clonal cell lines (3T3/CasFr^{KP} and 3T3mA3/CasFr^{KP}) were used to infect new 3T3 cells, and 8 h after infection, cellular DNA was isolated and amplified by PCR using Pfx50 polymerase, which exhibits a very low error rate ($\sim 2 \times 10^{-6}$) (Invitrogen). Amplicons from the *env* gene region were cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and subsequently sequenced using the amplification primers CasFrKP6805FOR (TTGAGAGAGTACAC TAGTC) and CasFrKP7886RC (TCTGTTCCTGACCTTGATC).

In agreement with other reports (2, 3, 6, 8–11), there was no significant increase in G→A mutations attributed to mA3 (Fig. 3A). Transversion mutations were infrequent; however, increases in transition mutations other than G→A mutations were observed. Notably A→G mutations were significantly higher in infections by the mA3-containing virus. C→T and T→C transition mutations also appeared elevated but did not reach significance ($P < 0.05$). The inclusion of all of the mutations to calculate overall mutation rates yielded a very significant difference attributable to mA3 (Fig. 3B).

A bias in the mutation rate in retroviruses has been reported in which the frequency of incurred G→A mutations differs in different regions of the viral genome (19–21). We compared the overall mutation rates in transcripts of regions of the *gag*, *pol*, and *env* genes generated after infection of 3T3 cells with mA3-containing viruses. No significant differential effect of mA3 was observed, suggesting the absence of G→A hypermutation in other regions.

Sequence context of mutations effected by mA3 in CasFr^{KP}. Flanking sequences of mutations effected by mA3 were examined to determine if any sequence consensus was evident. The analyses revealed only four instances of flanking sequences that were significantly different from the expected frequency (Fig. 4). It is unclear if these deviations reflect preferred targets of mA3. The context of the G→A mutations of CasFr^{KP} (GGN) was distinct from that observed with mA3-induced G→A hypermutation (GAA) (2, 4, 22, 23) and is unlikely to involve cytidine deaminase activity.

The incidence of G→A transition mutations was unchanged, while other transition mutations were elevated in the presence of mA3. A paucity of target sequences in CasFr^{KP} could reflect the low G→A mutation rate. In contrast to other MuLVs, AKV undergoes mA3-mediated G→A hypermutation (2). A comparison

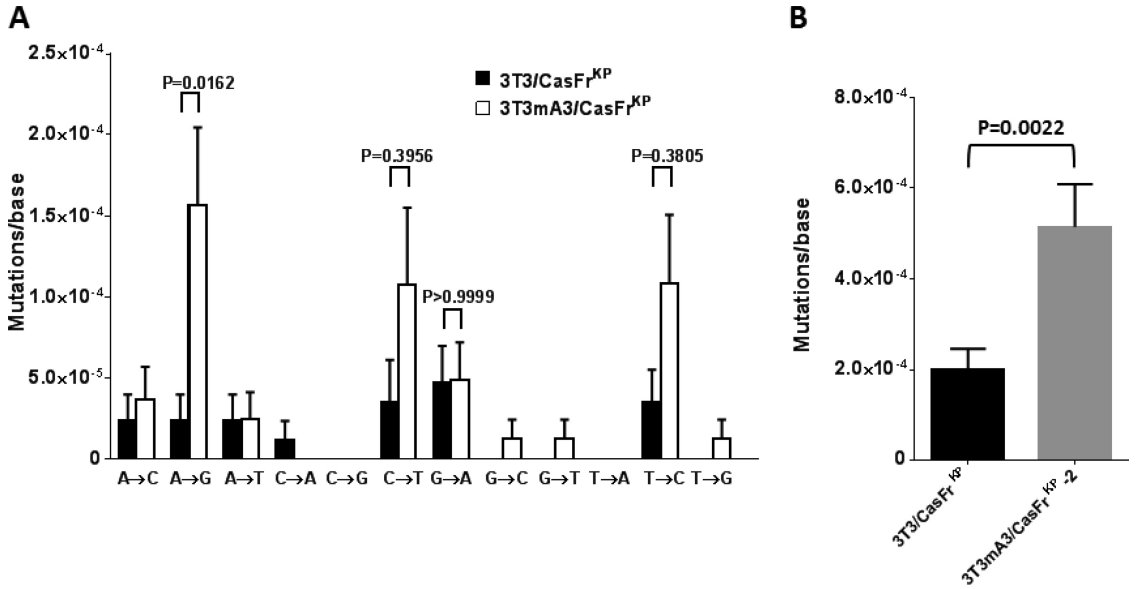

FIG 3 Influence of mA3 on RT fidelity. 3T3 cells were infected with viruses released from each of the clonal cell lines, and the nucleotide sequences of *env* gene transcripts synthesized during the first 8 h were determined. (A) The rates of all transition and transversion mutations are shown. Error bars represent the standard errors of detected mutations on approximately 90 transcripts from each of the infections, totaling about 85,000 bases analyzed for each virus. *P* values determined by one-way ANOVA using Sidak's multiple comparisons test are indicated for the transition mutations. *P* values of <0.05 were considered significant. (B) The rates of all transition and transversion mutations shown in panel A were combined to calculate the overall mutation rates. Error bars represent the standard errors of detected mutations. The *P* value determined by Student's unpaired *t* test is indicated. *P* values of <0.05 were considered significant.

of putative mA3 target sequences from the AKV genome to those of the CasFr^{KP} sequence did not reveal substantial differences in the number of preferred sites. It remains unclear why the incidence of G→A mutations was unchanged, while the incidence of other transition mutations was increased in transcripts from the mA3-containing virions.

The results of this study suggest an effect of virion-incorporated mA3 on the RT of the virus that affects the activity of the transcription process as well as the fidelity of the enzyme. A loss of fidelity of RT as a result of mA3 incorporation has not been previously reported and may represent another cytidine deaminase-independent mechanism by which APOBEC proteins act to inhibit retroviral replication. CasFr^{KP}, like other exogenous MuLVs, encodes a glycosylated Gag protein (gGag) that partially counteracts the action(s) of mA3. Stavrou et al. (17) have recently observed an inhibition of RT activity by mA3 that is counteracted by gGag. It would be of interest to determine if the gGag protein influences RT fidelity.

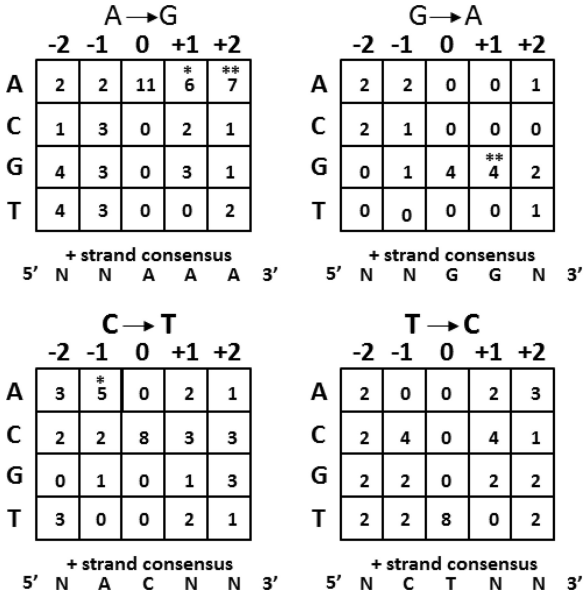

FIG 4 Consensus 3′ and 5′ sequences of mA3-induced transition mutations. The incidence of flanking bases for each of the transition mutations observed in transcripts obtained 8 h after infection by mA3-containing viruses is shown. Analyses include mutations detected in approximately 90 transcripts totaling about 85,000 bases. Significant elevation of the frequency of bases at each position compared to the expected frequency was calculated by the two-tailed binomial test with probability values of <0.05% considered significant (*, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01).

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