Università di Cagliari

Facoltà di Biologia e Farmacia

Dipartimento di Scienze Biomediche

Sezione di Microbiologia e Virologia

Dottorato di ricerca in

"Sviluppo e Sperimentazione dei Farmaci Antivirali"

XXVI Ciclo

Titolo Tesi

DIFFERENTIAL EFFECTS OF MOUSE APOBEC3 AFTER INCORPORATION INTO MURINE LEUKEMIA VIRUSES

Settore scientifico-disciplinare
BIO/19 – Microbiologia

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Anno Accademico
2012-2013

FOREWORD

During these three years of my PhD course, I had the possibility to work on different projects that I have carried out at the University of Cagliari, Department of Biomedical Sciences, under the supervision of Prof. P. La Colla, and at the Rocky Mountain Laboratories (National Institute of Allergy and Infectious Diseases, Laboratory of Persistent Viral Diseases, Retrovirology Molecular Biology Section) under the supervision of Dr. L.H. Evans. The results obtained during these researches led to the submission of the following manuscripts:

Stefanska, J., D. Szulczyk, A. E. Koziol, E. Augustynowicz-Kopec, A. Napiorkowska, A. Bielenica, M. Struga, G. Giliberti, S. Madeddu, S. Boi, P. La Colla, G. Sanna. 2014. Synthesis and antimicrobial activity of thiourea derivatives of 2-amino-1,3-thiazole. Submitted to Antimicrobial Agents and Chemotherapy.

Szulczyk, D., A. Bielenica, A. E. Koziol, S. Fidecka, E. Kędzierska, J. Stefańska, M. Struga, G. Sanna, S. Madeddu, S. Boi, P. La Colla, G. Giliberti. 2014. Biological evaluation of novel indole-derived thioureas. Submitted to Antimicrobial Agents and Chemotherapy.

Evans, L. H., S. Boi, F. Malik, K. Wehrly, K. E. Peterson, B. Chesebro. 2014. Analysis of two monoclonal antibodies reactive with envelope proteins of murine retroviruses: One pan specific antibody and one specific for Moloney leukemia virus. J Virol Methods. Vol. 200, Pages 47–53. http://dx.doi.org/10.1016/j.jviromet.2014.02.006

Boi, S., A. Kolokithas, J. Shepard, R. Linwood, K. Rosenke, E. Van Dis, F. Malik, and L. Evans. 2014. Incorporation of mouse APOBEC3 into MuLV virions decreases the activity and fidelity of reverse transcriptase. Submitted to Journal of Virology.

ABSTRACT

APOBEC3 proteins are cytidine deaminases that are potent inhibitors of retrovirus replication. These restriction factors induce G→A hypermutation as a result of deamination of cytidine in the single stranded transcripts generated by reverse transcription during replication. Mouse APOBEC3 (mA3) effectively inhibits the replication of endogenous retroelements whereas most exogenous murine leukemia viruses (MuLV) are largely resistant to the action(s) of mA3. However, several studies have reported significant inhibition of infectivity by virion-associated mA3 that did not appear to be the result of G→A hypermutation. In this report the influence of different levels of virion-associated mA3 on the exogenous MuLV, $CasFr^{KP}$ was examined. A correlation was observed between the level of incorporated mA3, the virion specific infectivity, the virion reverse transcriptase (RT) activity and the mutation frequency. Although G→A hypermutation of $CasFr^{KP}$ was not observed, the frequency of other transition mutations was significantly increased. G→A mutations induced by mA3 have been reported to exhibit a strong consensus of two bases flanking the mutation. No strong consensus was observed for mA3-induced mutations of $CasFr^{KP}$ suggesting a general decrease in the fidelity of the RT. The results of this study strongly suggest an interaction between mA3 and the RT of $CasFr^{KP}$ leading to a loss of activity as well as fidelity of the polymerase that ultimately results in a significant loss of infectivity.

In contrast to $CasFr^{KP}$, AKV, an ecotropic MuLV isolated from AKR mice, has been reported to undergo hypermutation mediated by mA3. In this report we also confirm that AKV does undergo G→A hypermutation upon infection of 3T3 cells, however hypermutation is not augmented in viruses that have incorporated HA-tagged mA3. Furthermore, the observed G→A transition mutations are not randomly distributed in that multiple mutations frequently occur in a single

transcript. These observations suggest that the G→A mutations are the result of the incorporation of an unknown restriction factor in only a portion of the released virions.

ACKNOWLEDGEMENTS

I would like to thank Dr. Leonard (Pug) Evans, for allowing me to be part of his laboratory at RML and for helping me during this thesis work.

Thanks to all the members of the lab and all the people and friends who have helped me in Hamilton, they have made the experience that I spent there, unforgettable.

I would also like to thank Prof.ssa Alessandra Pani and Prof. Paolo La Colla for following and encouraged me during these years. Thanks to all the colleagues and friends of our lab here at University of Cagliari for the support, the suggestions, and the good times we spent together.

Finally, I would like to thank Valentina for having been close to me, my Friends and all my wonderful and great family, especially my mother, without whom I would not have been able to reach this goal.

This research was supported by the Intramural Research Program of the NIH, NIAID and the

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Chapter 1: Introduction

1.1. Retroviruses

Retroviruses are enveloped RNA viruses that include different sub-families. They are termed retroviruses because of their means of replication. The viral genome is a linear RNA molecule that, upon infection is transcribed from RNA to linear double-stranded DNA, a process that is termed reverse transcription. The double-stranded DNA is subsequently integrated into the genome of the host cell (72). If this occurs in a germline cell the retrovirus becomes a permanent component of the host genome that is passed down to subsequent generations (129). Over the course of evolution, the human genome has been infected approximately 40,000 times such that about 10% of the genome consists of retroviral sequences (129).

Among the retrovirus family we find important pathogens such as HIV-1, feline leukaemia virus, and several cancer-causing viruses (72). Various retroviruses have been found that infect a large number of organisms, including humans and many other mammals. The earliest retroviruses studied were isolated from mice and birds. Examples of such retroviruses include murine leukemia virus (MuLV), mouse mammary tumor virus (MMTV), and the avian pathogens Rous sarcoma virus (RSV), spleen necrosis virus (SNV), and avian leukosis virus (ALV) (14).

The viral RNA is made up of two identical RNA genomes that are incorporated into the virion and are comparable to a large polycistronic messenger RNA (67). Reverse transcription of the viral RNA results in the generation of DNA transcripts which are ultimately integrated into the target cell genome as a provirus (129). Once integrated, the provirus is able to employ the cell's transcriptional machinery to transcribe its genome into full-length genomic RNA, which also serves as a messenger RNA (129). A portion of the full-length RNA is spliced into a shorter RNA that is translated to yield the Env proteins while the full-length RNA is translated into the

remaining structural proteins of the virus as well as the viral polymerase. The translated proteins and full-length genomic RNA are assembled to create new retroviruses that are subsequently released from the cell through budding. Non-defective viruses are capable of infecting new cells.

1.2. Murine Leukemia viruses

Murine leukemia viruses (MLVs or MuLVs) are retroviruses originally named for the ability of some members of the group to cause tumors in murine hosts. Some MuLVs may infect other vertebrates, including humans, and have been implicated in the induction of leukemias in gene therapy patients (49). MuLVs include both exogenous and endogenous viruses and are classified in the Baltimore classification of viruses as Type VI (+) belonging to the Gammaretroviral genus of the Orthoretroviridae subfamily, Retroviridae family. The viral particles (Figure 1) of replicating MuLVs have what is termed a C-type morphology as determined by electron microscopy (8), with a diameter of ~100–120nm (149) and a spherical inner core composed of the nucleocapsid (NC) and capsid (CA) proteins (8,133). Inside the core there are: RNA genome, reverse transcriptase (RT), integrase (IN), protease (PR), and the $tRNA^{Pro}$ primer (67).

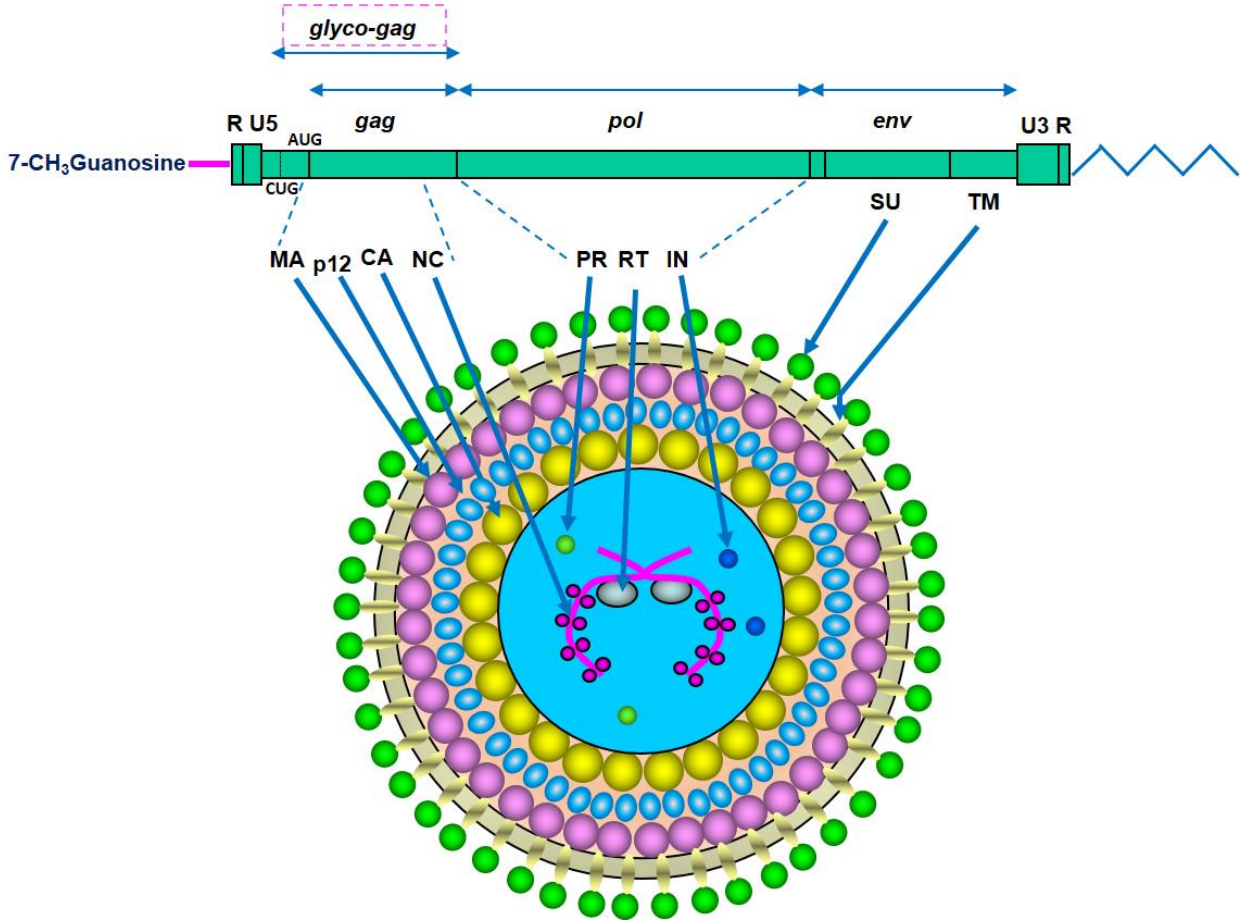


Figure 1. Structure of a MuLV particle. TM and SU subunits are located at the outer surface of the virus, while the inner part of a virion, known as the core, contains the RNA genome, NC, IN and PR surrounded by the CA. The CA is surrounded by the p12 and the MA protein, which helps anchor the lipid membrane. Figure credit: Dr. Leonard Evans.

The virus is encapsulated in a lipid envelope that is coated with glycoprotein spikes similar to that of the human immunodeficiency virus (HIV) (111). The spikes consist of the glycoprotein SU (surface) which includes regions binding the cellular receptor and determines the host range of the virus and the TM (transmembrane) protein, which anchors the SU protein to the virion by disulfide bridges. The TM protein is involved in fusion of the viral membrane to cellular membrane. In contrast to other types of retroviruses, type C particles appear to be assembled largely at the plasma membrane during budding.

MuLVs can be transmitted from one host to another by horizontal transmission of exogenous viruses or transmitted from parent to offspring by vertical transmission of endogenous MuLVs integrated into the host genome (67). The major classification of MuLVs has been based on host specificity or tropism which reflects different cell-suface receptor usage. Focusing on this type of classification we can distinguish between ecotropic (77) MuLVs that infect only mice, and utilize the mCAT-1 receptor (4); amphotropic MuLVs that infect mice and cells of many other species using the Pit-2 receptor (52,144); xenotropic MuLVs, that are endogenous to mice, can infect cells of many species but do not infect mouse cells, and utilize the Xpr-1 receptor (77), and polytropic viruses that can infect many species including mice and humans and also use Xpr-1 as a receptor (139).

MuLVs are comprised of a large number of mouse gammaretroviruses. Some MuLVs that cause proliferative diseases in mice are named after their discoverers: Gross MuLV by Dr. Ludwig Gross (lymphocytic leukemia) (47); Graffi MuLV by Dr. Arnold Graffi (erythroleukemia) (42); Friend MuLV (F-MuLV) by Dr. Charlotte Friend (erythroleukemia) (41); Moloney (M-MuLV) by Dr. John B. Moloney (lymphocytic leukemia) (87); Rauscher MuLV (R-MuLV) by Dr. Frank J. Rauscher (erythroleukemia) (110); Kirsten MuLV (Ki-MuLV) by Dr. Warner H. Kirsten (lymphocytic leukemia) (66); and Abelson MuLV (A-MuLV) by Dr. Herbert T. Abelson (B-cell leukemia) (1).

1.3. MuLV Genome and Proteins

Viral genomic RNAs resemble large mRNAs (~8.3 kb) containing a 5' cap (113) and are polyadenylated at the 3' end (45). The RNA genome begins with a 5’ short terminal repeat, R (53,125), followed by a short region which is termed U5 (61). Near the 3’ terminus of the viral

genome is a region termed U3 region, which contains the transcriptional regulatory elements of the virus (20). The U3 is followed by the 3' short terminal repeat (R) (53). The U3 region and U5 region are so named because they are unique 3' and 5' sequences that are incorporated along with the R region into the long terminal repeat (LTR) found in proviral DNA after reverse transcription (Fig. 2).

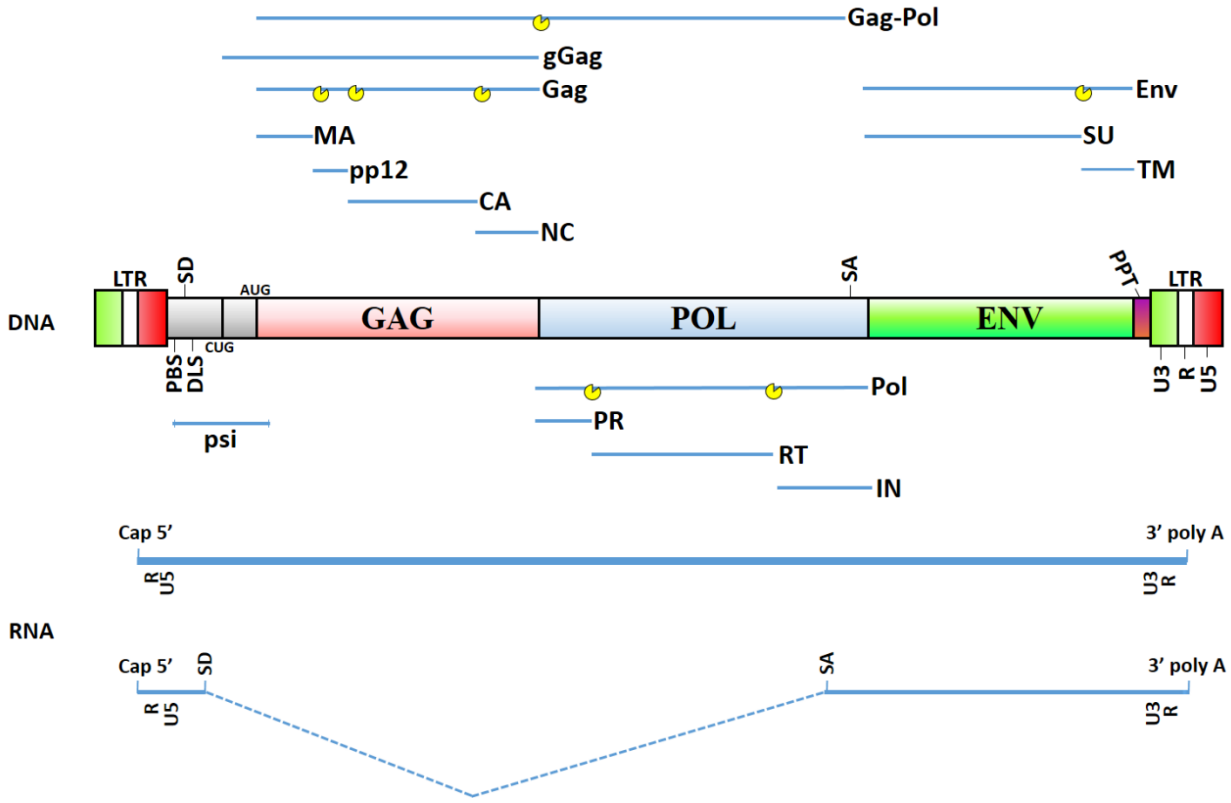


Figure 2. MuLV genomic structure and organization. The proviral DNA is flanked by two LTRs, both of which contain a U3, R and U5 region. Following the 5' LTR are signals that function in primer binding (PBS), splicing (SD), dimerization of the RNA genome (DLS), and packaging (psi). Next are the genes that code for internal structural components (Gag), replication (Pol) and receptor recognition (Env). These are followed by a sequence required for DNA synthesis (PPT) and an LTR which contains transcriptional regulatory sequences. Transcription of proviral DNA into RNA (▬) leads to two major species, the full-length genome, and the spliced mRNA that encodes the envelope polyprotein. Translation of the full-length RNA yields the polyproteins (▬) gGag, Gag and Gag-Pol. Translation of the spliced mRNA yields the Env polyprotein. These polyproteins are further processed by the viral protease (◔) to yield their mature forms.

Three major polyproteins are encoded by the MuLV genome. The Gag polyprotein is comprised of the major structural components of the viral core and includes the hydrophobic matrix protein (MA, $p15^{gag}$) (6), the acidic phosphorylated protein (pp12) (98), the neutral capsid protein (CA, p30) (97), and the basic nucleocapsid protein (NC, p10) (40). The Pol polyprotein is comprised of the protease (PR, p14) (100), the reverse transcriptase (RT, p80) (5), and integrase enzymes (IN, p46) (43). The Env polyprotein encodes the surface glycoprotein (SU, $gp70^{env}$) (59) and transmembrane proteins (TM, $p15^{env}$) (61) that jointly mediate the entry of an infectious virus particle into a new host cell to initiate infection (127). Many gammaretroviruses, including M-MuLV and F-MuLV, as well as feline leukemia virus, encode an additional glycosylated form of the Gag protein termed glyco-Gag (36). "glyco-Gag" or gPr80Gag differs in sequence from "standard" Gag in that it is contains additional N-terminal amino acids. Synthesis of glyco- Gag is initiated at a CUG codon in a favorable context for translation initiation, 264 bases 5' of the normal Gag AUG initiation codon (108,36).

As in all orthoretroviruses, the three coding regions are organized, from 5' to 3', *gag*, *pol* and *env*. The Pol proteins are firstly synthesized together with Gag, in a large Gag-Pol merged polyprotein. Gag and Gag-Pol are both translated from full-length viral RNA, identical in sequence to the genomic RNA present in the virion. It seems likely that the Gag-Pol polyprotein is incorporated into assembling virions due to "coassembly" of its Gag moiety with Gag polyprotein molecules (37). In the gammaretroviruses such as MuLV, *gag* and *pol* are in the same reading frame, divided by a single termination codon. MuLV RNA contains a 57-base cis-acting signal immediately 3' of the termination codon (38) that induces the insertion of glutamine (normally encoded by CAG), rather than UAG codon termination in about 5% of the translation products. The resulting product is extended by translation of the entire Pol coding region (153).

The Env protein of MuLV, like that of other orthoretroviruses, is translated from a singly spliced mRNA. There is an overlap of 58 bases between the end of the Pol coding region and the beginning of the Env coding region (111).

There are noncoding regions in the MuLV RNA that contains a set of cis-acting signals that are essential for its function as a viral genome. These include the "primer binding site" (PBS), the polypurine tract (PPT) which is necessary for second strand synthesis of DNA and participates in the insertion of the DNA form of the viral genome into cellular DNA by the IN protein, the "packaging signal" sequences (Ψ, psi) required for packaging of the viral RNA into virions, and the promoter and enhancer sequences within the LTR. The PBS is an 18-base stretch that is complementary to the last 18 bases of a cellular tRNA molecule. In exogenous MuLVs, this is usually $tRNA^{Pro}$, however endogenous MuLVs frequently utilize $tRNA^{Glu}$. Within the virion, the tRNA is hybridized to the viral RNA and upon infectious entry, the tRNA serves as the primer for reverse transcription. The PBS is located ~145 bases from the 5' end of the RNA and ~460 bases 5' of the start of the Gag coding region (111).

In general, during reverse transcription the RNA is copied by the polymerase activity of RT and is progressively degraded, shortly after being copied, by the RNase H activity of RT. However, an exceptional stretch of ~15 purines near the 3' end of retroviral RNAs (the PPT) is specifically resistant to this degradation. After reverse transcription, this fragment of the viral RNA serves as the primer for synthesis of the second (plus) strand of DNA. The 3' terminal base of the PPT encodes the first base of the plus strand of the DNA copy, that is, the 5' end of the plus strand or "left" end of the double-stranded DNA (111). These sequences at the two ends of the final DNA product are the sequences joined by IN to host-cell chromosomal DNA during the integration reaction (12).

Between the PBS and the packaging signal (psi) there is the splice donor (SD) site (126) that is joined to a splice acceptor (SA) site that precedes the *env* gene. The spliced *env* gene mRNA lacks the packaging signal and thus avoids packaging into the virion. The psi region also contains the dimer linkage site (DLS) that is required for RNA dimerization (89,117). In all orthoretroviruses, the viral RNA is actually packaged in dimeric form, with two molecules of the viral RNA linked by a limited number of intermolecular base pairs. The primary location of these base pairs is in the "leader", between the PBS and the beginning of the Gag coding sequence (69).

1.4. Replication Cycle

1.4.1. Binding and entry

As with all orthoretroviruses, infection is initiated by the binding of the SU (gp70) glycoprotein on the exterior of the mature, infectious virion to a receptor on the surface of the target cell (56). This binding event triggers dramatic changes in Env, leading to the release of the SU component and conformational rearrangement of TM. The ultimate result is the fusion of the viral membrane with a cellular membrane. In some cases, fusion may occur within endosomes that have captured virions (85). The fusion of the two membranes leads to the deposition of the contents of the virion in the cytoplasm of the cell (67). The replication cycle of MuLV is depicted in Fig. 3.

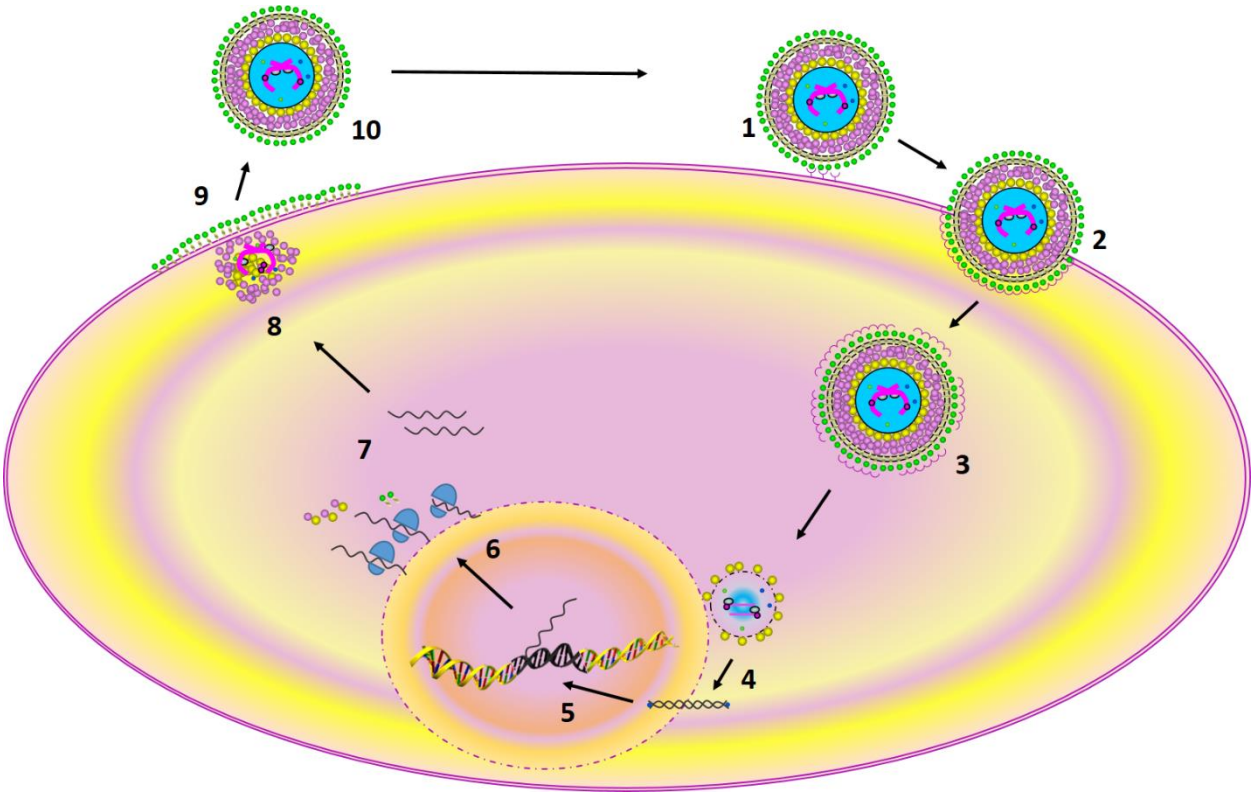


Figure 3. Replication cycle of MuLV. 1) Envelope glycoproteins bind to the specific cell receptors required for viral entry. 2) Fusion and entry into the cell. 3) Uncoating of virion 4) Reverse transcription. 5) Preintegration complex enters nucleus, integrates proviral DNA into host genome. 6) Transcription of provirus. 7) Translation of virion components. 8) Assembly at the cell membrane. 9) Budding of the virion. 10) Maturation and release of the virion.

1.4.2. Reverse transcription and integration

After fusion of the virion with the cellular membrane, the viral capsid uncoats and the RNA genome is reverse transcribed into DNA (Fig. 4). Reverse transcription may proceed as soon as intact capsids have access to nucleotides following fusion (72). The provirus is synthesized from the RNA genome by the virion reverse transcriptase (RT). At the beginning of

the process the $tRNA^{pro}$, which is carried into the virion, binds the primer binding site of the viral RNA (53) and the RT begins to transcribe the cDNA in the 3' to 5' direction ending at the R region (53). At the same time, the RNase H activity of RT degrades the RNA portion of the RNA/DNA Hybrid (44). The complementary DNA just formed, termed strong stop DNA, then binds to the 3' complementary R region at the end of the viral RNA (23,24). The first strand of complementary DNA (cDNA) is extended and the majority of viral RNA is degraded by RNAse H except for the PPT region (32). This region is then used as a primer by RT to synthesize the plus strand DNA in 5' to 3' direction, using as a template the cDNA (131). The RNase H removes the primer tRNA, exposing sequences in +DNA that are complementary to sequences at or near the 3′end of plus-strand DNA. Annealing of the complementary PBS segments in +DNA and minus-strand DNA constitutes the second strand transfer. Plus and minus-strand syntheses are then completed, with the plus and minus strands of DNA each serving as a template for the other strand (71,22).

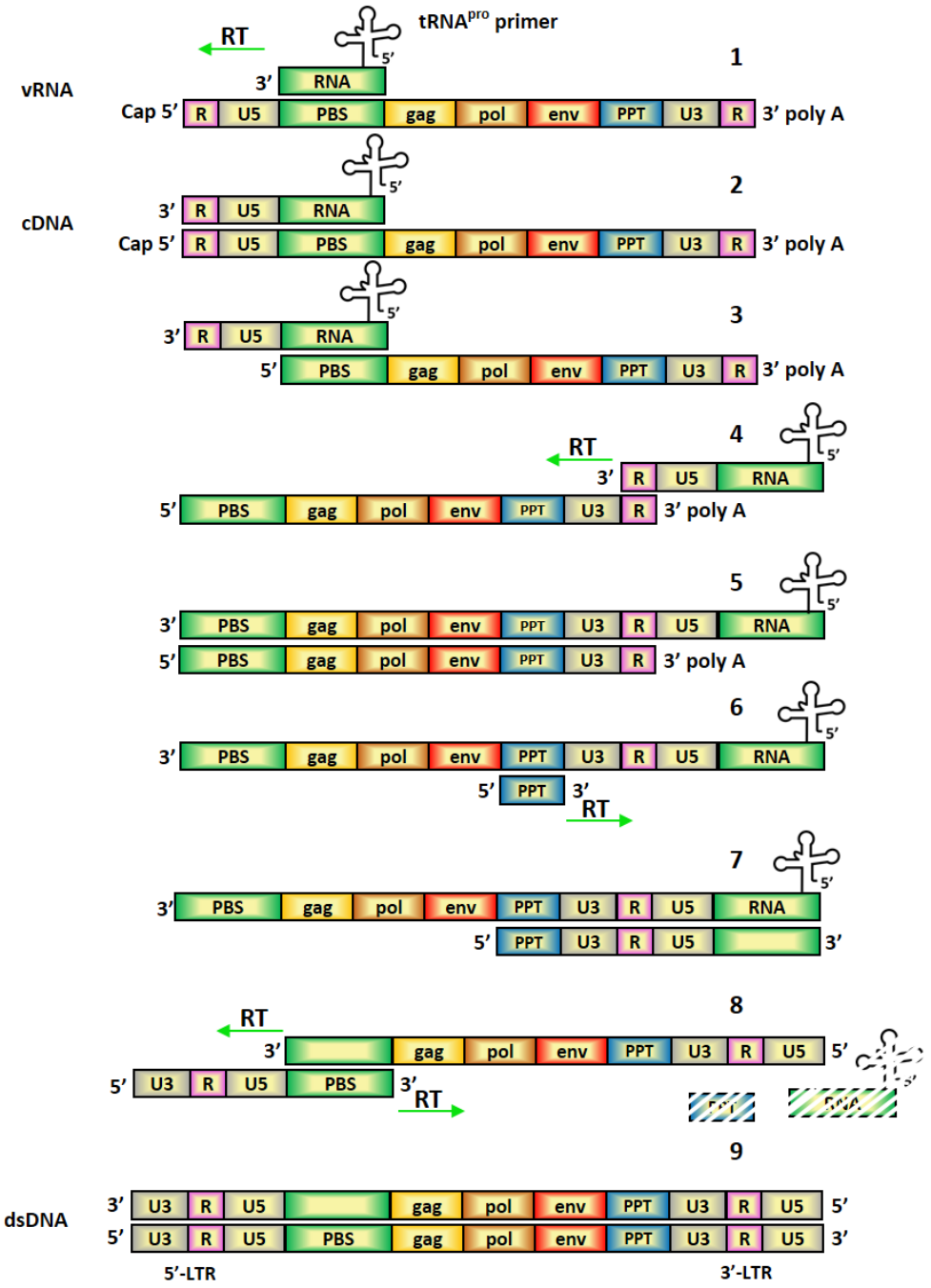
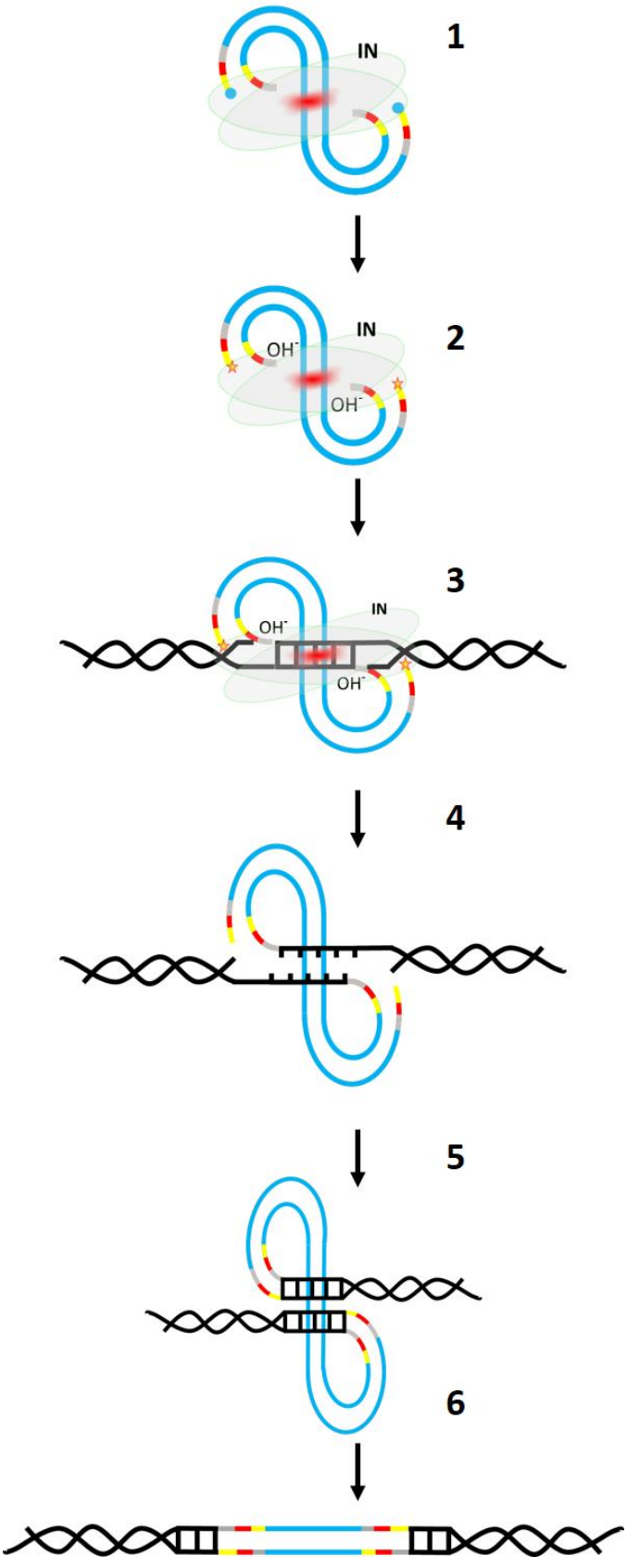


Figure 4. Schematic description of Reverse transcription 1). The $tRNA^{Pro}$ binds to the PBS of the RNA genome **2).** RT transcribes minus (-) strand DNA in the 3' to 5' direction ending at the R region. **3).** RT's RNase H activity degrades the RNA portion of the RNA/DNA hybrid. **4).** The newly synthesized DNA and tRNA primer dissociates from the 5' end of the RNA genome and hybridizes to the 3' R region of the genome. **5).**Minus strand DNA synthesis continues to the PBS at the 5' end of the molecule. **6).** RNase H activity of RT degrades the RNA genome, except for the PPT region. **7).** RT uses the PPT RNA as a primer to synthesize (in the 5' to 3' direction) the plus (+) strand of DNA to the PBS contained within the strong stop (-) DNA, using the minus strand as a template. The resultant strand is called strong stop plus (+) strand DNA. **8).** The $tRNA^{Pro}$ primer, PPT RNA and all remaining RNA are removed. The PBS of the (+) strand can anneal to the PBS of the minus strand and RT can further synthesize DNA **9).** Resulting in a double stranded proviral DNA genome.

After reverse transcription is complete, the resulting double stranded DNA transcript is associated with cellular and viral proteins into a preintegration complex (PIC) that is subsequently translocated to the nucleus (Fig. 5). MuLV PICs are translocated upon disintegration of the nuclear membrane during mitosis while PICs of HIV and other lentiviruses are actively translocated through pores in the nuclear membrane (30). Insertion of the viral DNA into the host cell DNA is catalyzed by a viral enzyme called integrase (IN). This enzyme recognizes and nicks the two ends of viral DNA, and the new 3′-ends are then joined covalently to the host DNA at staggered nicks made by integrase. Although retroviruses may integrate at many different sites in the genome, the process is not entirely random. MuLVs exhibit preferential integration sites near transcriptional start sites and are influenced by MuLV Gag and IN proteins, while lentiviruses exhibit preferential integration within actively transcribed gene sequences (30). Once the preintegration complex associates with the host chromosome, viral IN catalyzes the insertion of the viral sequences into the host DNA. The two LTR ends of the linear viral DNA are brought together into a ternary complex with IN and host DNA, where the insertion occurs in a coordinated or concerted reaction (58). A 2-bp sequence is lost from each end of the viral DNA, and a short duplication of 4 to 7 bp from the host, depending upon the viral IN, is introduced into the host sequence flanking the viral DNA. Integrated viral DNA is termed the provirus (58). Integration occurs in two well-characterized catalytic steps, referred to as end processing and joining, respectively. End processing involves removal of a dinucleotide, adjacent to a highly conserved CA dinucleotide, from the 39 bp strand of the U3 and U5 viral DNA LTRs in a reaction involving a water molecule or other nucleophile. This exposes a hydroxyl group, whose oxygen is used as an attacking nucleophile on the target DNA during the joining reaction, in which the viral DNA is inserted into the cellular DNA (58).

Figure 5. Integration of proviral DNA. 1). The proviral cDNA (in blue) is bound to the integrase portion of the pre-integration complex (PIC). 2). The 3' ends of the DNA are nicked by IN to produce 3' hydroxyl ends. 3). The PIC is then shuttled into the nucleus and the IN produces a 4-6 bp staggered cut in the host genome (in black) which acts as a target for the proviral genomes free hydroxyl groups to integrate into. 4). The pairing of host DNA is dissolved. 5). The gaps left behind and the non-complementary ends of the proviral genome are 6). repaired and removed, respectively.



1.4.3. Transcription of the provirus

Once the viral DNA is integrated into host DNA, it is transcribed and translated by normal host-cell machinery (111). Specifically, the provirus is transcribed as a cellular gene by RNA polymerase II and mediated by transcriptional control elements contained within the proviral LTR (48). Expression directed by the viral LTR signals is carried out entirely by host cell enzymes (RNA pol II, poly A synthetase, guanyl transferase). The enhancer and other transcription regulatory signals are contained in the U3 region of the 5' LTR, and the TATA box is located roughly 25 bp from the beginning of the R sequence. The integrated provirus has two LTRs, and the 5' LTR normally acts as an RNA pol II promoter (48,27). The transcript begins, at the beginning of R, and proceeds through U5 and the rest of the provirus, usually terminating by the addition of a poly A tract just after the R sequence in the 3' LTR (48). The mRNA generated during the transcription can be spliced or unspliced and then translated to produce the viral proteins.

1.4.4. Translation and maturation

Cells infected by a retrovirus synthesize virus-specific RNAs by transcription of the integrated proviral DNA. The primary transcript is 5’ capped and 3’ polyadenylated and can be either spliced to give the subgenomic RNAs or transported to the cytoplasm where it serves as an mRNA for the synthesis of gag and gag-pol polyproteins and/or as the pregenomic RNA. The gag and gag-pol polyproteins are the precursors to the major proteins and enzymes, respectively, of the virion core (21). The genetic organization of the 5’ sequences of the genomic RNA is complex and contains a long multifunctional 5’ untranslated domain, the leader, that precedes the gag gene. The 5’ leader is formed of independent well structured domains involved in key steps of the viral life cycle such as the initiation of proviral DNA synthesis (the PBS and R sequences),

genomic RNA dimerization and encapsidation (the dimerization-encapsidation signal E/DLS), and the initiation of gag precursor translation (AUG-619 gag initiation codon). The genomic RNA of MuLVs directs the synthesis of two gag-related proteins: $Pr65^{gag}$, the precursor to the virion core structural proteins, and $Pr75^{glyco\text{-}gag}$, the precursor to the glycosylated gag protein found on the surface of MuLV-infected cells (36,121). Translation of Pr65gag starts at an AUG while $Pr75^{glyco\text{-}gag}$ is initiated at a CUG located within the 5' leader and in frame with AUG^{gag}. (108). During virus maturation $Pr65^{gag}$ is proteolytically cleaved into the final products designated p15, pp12, p30, and p10 (176). The processing is accomplished by a virion-associated protease (150), which first cleaves $Pr65^{gag}$ into $Pr27^{gag}$ (p15 + pp12) and $Pr40^{gag}$ (p30 + p10), the two major intermediate cleavage products (145,151). The MA protein (p15) is the most hydrophobic protein of the virus (6). The CA protein (p30) is neutral and is the major structural protein of the virus (96). Bound to the genomic RNA is the basic nucleocapsid protein (NC or p10) which is a major component of the ribonuclear protein complex (40).

The Gag-Pol polyprotein, in addition to Gag includes PR, RT, and IN. The PR protein (p14) catalyzes the cleavages leading to virus maturation. Like all retroviral PRs, it is an aspartic protease, which is only active as a dimer (84,138). RT (p80) is packaged into the core of the virion and synthesizes the DNA copy of the viral genome during infection. MuLV RT is active as a monomeric protein (28,86). MuLV IN (p46) is also incorporated into the core of the virion and has not been characterized in detail but is presumed to function as a tetramer (142,51).

The MuLV Env gene product is synthesized in the rough endoplasmic reticulum and glycosylated in the Golgi apparatus. It is also cleaved in the Golgi by a cellular furin-like protease into two fragments, the large, N-terminal surface glycoprotein (SU or gp70) and the C-terminal transmembrane protein (TM or p15E). A trimer of these heterodimeric SU-TM

complexes is then trafficked to the cell surface. Once the envelope protein has reached the cell surface, it is incorporated into a budding C-type retroviral particle (57, 124). SU and TM proteins are linked through a labile disulfide bond (103,105) with the SU on the outside of the particle and the TM exhibiting an extraparticle domain, a transmembrane domain, and a 35-amino acid domain that resides within the particle (104). During a late stage of viral particle maturation the carboxyl-terminal 16 amino acid residues of the TM protein are removed by the retroviral PR resulting in a 12-kDa envelope transmembrane protein (135,127,57,26,64,124). The Env protein present on the membrane of an infected cell is therefore structurally different from that found in a mature particle. Removal of the carboxyl-terminal region during maturation activates the Env protein so that it is capable of fusing membranes in a receptor-dependent manner (109,112,141). The immature virion particles are released from the host cell through a budding mechanism and subsequently undergo maturation as the PR in the virus cleaves the viral polyproteins.

1.5. Restriction Factors

Over the course of the retroviral replication cycle, viral cDNA is introduced into host chromosomal DNA to establish the provirus. If this occurs in a germline cell the process results in a permanent insertional mutation in the host cell genome. In this regard approximately 10% of the human and mouse genomes are derived from retroviruses that have been inserted over the course of evolution (129). Host cells have evolved intracellular factors that block the spread of retroviral infection. Some of these antiviral factors act prior to integration and therefore also block the mutagenic potential of infection. Several such factors have been identified, including Fv1, the APOBEC3 complex, and TRIM5. These host factors potently block HIV-1 and other retroviruses from establishment of the provirus (72).

Fv1 restriction was the first system for resistance to MuLV to be described in mice. Fv1 confers

resistance to MuLVs, blocking progression of the viral life cycle after reverse transcription, but before integration into the host chromosome. It is known that the specificity of restriction is determined by both the restriction factor and the viral capsid CA (78,63,137). Identification of the Fv1 gene as an endogenous virus-derived *gag* gene has led to speculation that its gene product may interact with the Gag proteins of an infecting MuLV preventing a crucial interaction necessary for efficient infection. However a direct interaction between Fv1 and MuLV CA has not yet been demonstrated (134).

The human gene encoding an activity similar to Fv1 was originally called Ref1 and is now known as TRIM5α (55,65,101,148). TRIM5α is a member of the tripartite motif (TRIM) protein family, TRIM5α binds to the retroviral capsid lattice in the cytoplasm of an infected cell and accelerates the uncoating process of retroviral capsid, thus providing a potent restriction to HIV-1 and other retrovirus infections (29). Another restriction factor called tetherin (CD317/BST-2) blocks the release of viral budding particles from the cell membrane by tethering newly forming viruses to host cells (54,76).

1.5.1. APOBEC3

APOBEC3 (Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) proteins are a sub-family of intrinsic restriction factors of the innate immune system (25). They are able to block retroviruses from different species after the entry into the host cells (75). APOBEC3 proteins were originally discovered by Sheehy et al. in 2002 during their investigations of the HIV-1 (18). In this study they observed that when the Vif gene is removed from HIV-1, the resulting HIVΔvif viruses do not produce infectious particles in some cells (Fig. 6) (18). Their studies concluded that there were cellular factors acting against HIV-1 that could

inhibit the infectious cycle of the virus in the absence of the vif protein (18). It was subsequent discovered that the human APOBEC3G (hA3G) was a target of the Vif protein.

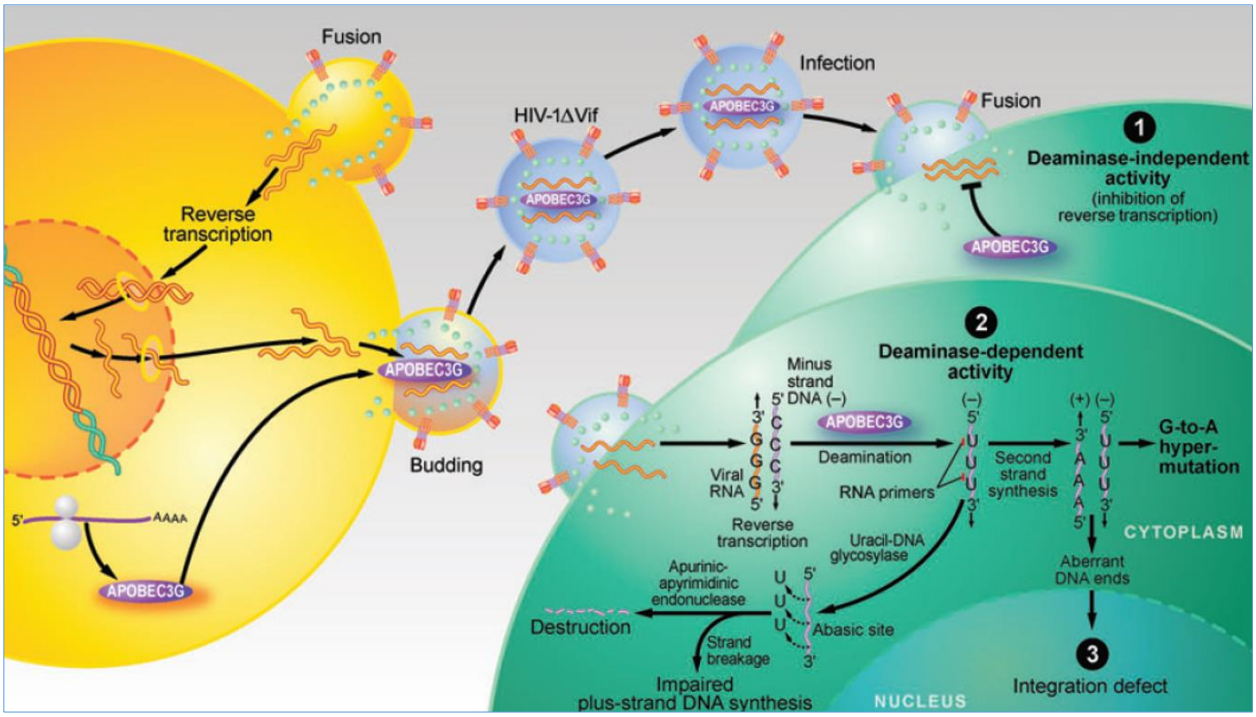


Figure 6. APOBEC3 activity. hA3G-mediated restriction of HIV-1Δvif. hA3G proteins are incorporated into the budding particles during assembly at the cellular inner membrane, after the first round of infection. hA3G is able to inhibit the replication cycle of the retroviral pathogens during the second round of infection. Restriction of retroviral pathogens is mediated by deamination-dependent and –independent restriction pathways of the protein. Figure credit: Chiu and Greene. 2008

APOBEC3 proteins possess enzymatic activity that targets single-stranded DNA. They deaminate cytidines to uridines, disrupting the single-stranded DNA sequences and ultimately induce guanosine to adenosine hypermutation of the proviral genome (18,90). Since the reverse transcription of the viral genomic RNA occurs in the host cell cytoplasm, retroviral DNA intermediates become a major target for APOBEC3 proteins (3,18,90).

Different studies revealed that humans have seven APOBEC3 genes (A3A, A3B, A3C, A3DE, A3F, A3G and A3H) all of which are located within a 150kb region on chromosome 22 (318,90,93). The feline genome has four A3 genes, the equine genome has six, and the mouse

genome has only a single copy of the A3 gene (75,114). Human A3A, A3C, and A3H proteins contain one catalytic domain (CD). In contrast A3B, A3DE, A3G, and A3F and the mouse A3 protein have two catalytic domains (3,18,60,74,90). The most extensively studied member of the APOBEC3 family is the hA3G protein because of its potency as an antiretroviral restriction factor and its importance for HIV-1 infection. hA3G is incorporated into virions and acts through cytidine deamination of single stranded DNA during reverse transcription. The inhibitory activity of hA3G is dependent on two catalytic domains (CDs). CD1 mediates RNA binding and incorporation into the virion (15) and the C-terminal (CD2) domain catalyzes deamination (91). Encapsidation of hA3G requires the nucleocapsid (NC) region of HIV-1 Gag (136), and as few as 7 APOBEC molecules incorporated into a HIV-1 ΔVif virion are sufficient to inhibit the virus in the next round of infection (146).

After the entry in uninfected cells, the viral particles containing hA3G are uncoated and the the viral capsid is liberated into the cytoplasm of the cell. The reverse transcription process starts with the elongation of tRNA attached to the viral genomic RNA, allowing for the production of the minus strand strong-stop DNA. The strong-stop DNA is translocated to the 3' end to generate the whole minus strand (153) where hA3G acts to deaminate cytidines to uridines. The mutation rates of these sequences increase with longer exposure time to A3 proteins. Therefore, the number of mutations are directly related with the exposure time prior to plus strand DNA synthesis and the distribution of mutations is skewed (153). DNA strands containing uridine may be degraded by uracil DNA glycosylase and apurinic-apyrimidinic endonuclease (147). Strands that are not degraded and serve as a template for plus-strand synthesis incorporate A residues rather than G residues at the deaminated positions. Extensive G-to-A mutations render the provirus inactive. Unfortunately, retroviruses such as HIV-1 have evolved mechanisms to escape

the restriction activity of APOBEC3 proteins. HIV-1 encodes a protein called Vif (virion infectivity factor) that can bind to APOBEC3 proteins, and determine their degradation through the 26S proteasome (18,83). Vif binds the N-terminal region of APOBEC3 and links it to the ubiquitin ligase (E3) complex. This complex will consequently promote the polyubiquitylation of APOBEC3 and its subsequent degraded by the proteasome (3,18). In the absence of Vif, hA3G acts to inhibit retroviral replication by mechanisms other than deamination. It is has been proposed that the RNA binding domain of CD1 physically interacts with the RT and inhibits the infectivity of the virus without editing any residues on the single stranded DNA intermediate (3, 18,19,25,60). Furthermore, as discussed below, MuLVs have evolved mechanisms to evade the actions of mA3 not involving cytosine deamination.

A3 enzymatic activity is closely regulated to protect the cell's genomic DNA from APOBEC3 catalytic activity during cell division (18). A3 proteins are generally localized in the cytoplasm; however, some family members like hA3B reside in both the cytoplasm and nucleus (18). Depending on the current activation status of the cell, A3 proteins either form 5-15 MDa high molecular mass (HMM) ribonucleoprotein complexes or 150 kDa low molecular mass (LMM) ribonucleoprotein complexes (18). It is possible that some retroviruses may exploit these mechanisms to circumvent the actions of A3 proteins.

1.5.2. Murine APOBEC3 (mA3)

There is only one A3 gene in the mouse genome and is situated on mouse chromosome 15. This is in contrast to the seven A3 genes in humans. (18). mA3, like hA3G, has two catalytic domains however the structural organization and functional roles of the catalytic domains are dissimilar than its human counterpart (13,18,50,140). mA3 was reported to have a reversed

structural organization compared to the human A3G protein (50). The deaminase activity of mA3 resides in the CD1 while the RNA binding and encapsulation into the virion resides in CD2 (50). Sequence analysis of a variety of mouse strains and species show that mA3 is expressed from two major mouse alleles. Different inbred mouse strains express either a C57BL/6 or BALB/c mA3 allele (95) that differ by their splicing patterns as well as some polymorphic differences. The full-length mRNA transcript of mA3 contains 9 exons and both major mA3 alleles have related mRNA splicing isoforms (95). However, two splice variants have been identified for mA3 (delta-exon 2 and delta-exon 5) (114,120), and it has been shown that almost all of the mRNA transcripts from the C57BL/6 allele exclude exon 5 (over 95%) (13,73,95). In recent studies by Santiago et al., it has been show that mA3 is synonymous with Rfv3 (Recovery from Friend virus 3) (120); a gene implicated in the resistance of certain mouse strains to the induction of acute erythroleukemia in adult mice by the Friend virus complex of the F-MuLV and a replication-defective pathogenic virus termed the spleen focus-forming virus (SFFV) (120).

1.5.3. mA3 as retroviral restriction factor

Many retroviruses and endogenous retroelements are restricted by the cytidine-deaminase activity of mA3 (73,79,140). Several studies have also reported that mA3 can partially inhibit the replication of exogenous MuLVs such as F-MuLV or M-MuLV although none have been shown to exhibit G to A hypermutation with the exception of AKV (119,2,31,46,120,73). AKV MuLV was identified as an endogenous ecotropic virus in the thymoma-prone mouse strain AKR (115). Although it is endogenous to this mouse strain, it is capable of infecting and replicating as an exogenous virus and is susceptible to the cytidine-deaminase activity of mA3 (73).

A number of studies have reported that mA3, like hA3G can cause high levels of G→A mutations in the HIVΔvif proviral genome. Furthermore, mA3 can also mutate- wild-type HIV-1 because Vif is not able to interact with murine A3 protein (39,95,153). Conversely, hA3g induces high levels of G→A mutations in murine retroviruses however, as noted above, mA3 does not.

MuLVs do not encode a Vif like accessory protein, however they have evolved a mechanism(s) to evade the action of mA3 (11,13,31,73). Very recent studies have reported that MuLV glyco-Gag is involved it the resistance to mA3 during the retroviral replication (68,92,132). It is not completely clear how mA3 is able to restrict MuLVs, and why in some cases, the inhibition proceeds by a deaminase-independent mechanism and in others with a deaminase-dependent mechanism (18,19,79,94,140).

One of major aims of the current studies is to elucidate mechanisms by which mA3 is able to inhibit the replication of MuLVs. Studies have focused on the inhibition of MuLVs by deaminase-independent mechanisms of mA3 as well as the inhibition of AKV by a deaminase-dependent mechanism.

1.6. Importance of the work

These studies focus on understanding how mA3 influences MuLV replication. The APOBEC proteins are found throughout the mammalian system and are important in many physiological processes such as the maturation of immunoglobulins, as well as the control of retroviral endogenous elements. In addition they serve as part of the innate immune system to protect hosts against exogenous retrovirus infection. The mechanisms employed by the APOBEC proteins to protect against retrovirus infection are varied as are the mechanisms evolved by retroviruses to circumvent these defenses. A thorough understanding of the interplay between

these factions in different systems may facilitate the development of intervening pharmaceuticals against retroviral infection such as HIV or HTLV in humans. Further, some MuLVs are capable of infecting the human population. In this regard, proliferative diseases in humans have been attributed to MuLV infection acquired during gene therapy trials (49). A thorough knowledge of factors that may influence this interspecies infection are critical for anticipating possible consequences.

Chapter 2: Incorporation of mouse APOBEC3 into MuLV virions decreases the activity and fidelity of reverse transcriptase

Part of the data reported in this chapter have been submitted to Journal of Virology:

Stefano Boi, Angelo Kolokithas, Joyce Shepard, Rebbeca Linwood, Kyle Rosenke, Erik VanDis, Frank Malik and Leonard H. Evans. Incorporation Of Mouse APOBEC3 Into MuLV Virions Decreases The Activity And Fidelity Of Reverse Transcriptase. J Virol. Sub. Feb 10 2014

2.1. Introduction

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 (18,73). Although APOBEC3 family members from both humans and mice inhibit endogenous LTR retrotransposons and retroviruses (11,34,33), exogenous murine leukemia viruses (MuLVs) are relatively insensitive to the actions of murine mA3 (2,11, 13,31,68,73). Several studies, however, have reported partial inhibition of exogenous MuLVs after incorporation of mA3 ranging from 10 to ~90% when assayed in vitro (2,31,46,73,118,119,120). Furthermore, the finding that Rfv3, a resistance gene for Friend erythroleukemia, encodes mA3 and is responsible for a decreased infectious titer of the Friend (Fr-) MuLV (120,130,140) strongly suggests that mA3 inhibits the replication of exogenous MuLVs in vivo.

Exogenous ecotropic MuLVs such as the Fr-MuLV and Moloney (Mo-) MuLV are inhibited through mechanisms that do not appear to involve cytidine deamination (46,73,102). In contrast, the ecotropic MuLV, AKV, isolated from AKR mice has been reported to undergo G→A hypermutation after virion incorporation of mA3 (73). In an earlier report (68) we found that the infectivity of CasFr^{KP}, an ecotropic MuLV which induces a rapid neurological disease in susceptible mice, was not significantly inhibited by mA3 incorporated into virions harvested from an mA3-expressing cell line. However it was not known how the levels of mA3 incorporation in our experimental protocol compared to the levels achieved by co-transfection of cells with plasmids encoding mA3 and plasmids encoding MuLVs; a protocol commonly employed in in vitro studies of mA3 and MuLVs (11,13,18,46,68,118). In the present study we have examined the infectivity of CasFr^{KP} released from cells expressing varying levels of mA3. We have observed that a

higher level of mA3 incorporation does indeed result in a loss of infectivity which is accompanied by a significant loss of virion reverse transcriptase (RT) activity. Moreover, although G→A hypermutation was not observed, we found a highly significant increase in the overall mutation rate of $CasFr^{KP}$ in the presence of mA3, reflecting a decrease in the fidelity of the enzyme. It seems probable that the loss of activity and fidelity of RT facilitates the observed loss of infectivity. It is likely that the inhibitory action of mA3 is a result of interaction of the restriction factor with the viral polymerase.

2.2. Materials and Methods

2.2.1. Plasmids, cells and viruses.

The plasmid encoding the full length mA3 derived from the BALB/c mouse strain was a kind gift from Dan Littman (13). It was provided in the pcDNA3 vector and was tagged at the C-terminus with hemagglutinin (HA) (83). NIH 3T3 (3T3) were maintained in Dulbecco's modified Eagle medium with 10% bovine serum and penicillin/streptomycin. Quantitative RT PCR assays using the forward primer (ACCTGAGCCTGGACATCTTCA), the reverse primer (TGCAAAGATTCTGCTGGTTTTC) and the FAM-TAMRA probe (TCCCGCCTC-TACAACATACGGGACC) revealed that mA3 RNA was below the level of detection in 3T3 cells (data not shown). Plasmid DNA encoding mA3 was transfected into 3T3 cells using lipofectamine (Life Technologies) according to the manufacturer's instructions and selected in media containing 1 mg/ml G418. Single colonies of cells were transferred to new dishes and tested for the expression of the C-terminal HA-tagged mA3 construct after ten passages. Cells stably expressing the C-Terminal HA-tagged mA3 construct (3T3mA3) were maintained in selection media. $CasFr^{KP}$ is a MuLV derived from the wild mouse ecotropic virus CasBrE and contains a short sequence of the F-MuLV, FB29 (106).

2.2.2. Immunoblotting

Cellular mA3 levels were determined in lysates prepared using the CelLytic M Cell Lysis Reagent (Sigma). Cell lysates were centrifuged at 20,000 x 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 1X 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. Western Blot analysis was performed using a monoclonal anti-HA horseradish peroxidase (HRP)-conjugated antibody (Roche).

Immunoblots to detect virion p30 were performed on virions sedimented from the virus stocks in a Beckman SW 50.1 rotor for 1 hr at 50000 rpm. The pelleted virions were resuspended in TSE (100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 7.5), adjusted to 1X 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 monoclonal antibody R187 to p30 (17) 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 (122).

2.2.3. Specific infectivity and RT activity

The infectivity of virions released during an 8 hr interval from mA3 expressing cells as well as from cells not expressing mA3 was assessed by a focal immunofluorescence assay (FIA) (128). The specific infectivity's of virions in the samples were compared by normalizing the infectivity to the levels of the p30 capsid (CA) protein, the major structural protein of the virion. A portion of each sample was also assessed for RT activity using the Reverse Transcriptase Assay, colorometric (Roche Applied Science) according to the manufacturer's instructions and normalized to the quantity of virions in the samples assessed by the level of p30.

2.2.4. 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 3T3 cells or from 3T3 cells expressing mA3 (3T3mA3). We derived clonal cell lines after infection of 3T3 or 3T3mA3 cells with the exogenous ecotropic MuLV CasFr^{KP} at a low multiplicity of infection as previously described (88). This served to minimize the initial genetic heterogeneity of the virus as well as to obtain infected clonal cell lines expressing varying amounts of mA3. Mutation rates were assessed after infection of 3T3 cells with virus harvested from 3T3 cell lines or from 3T3/mA3 cell lines expressing varying levels of mA3. Eight hours after infection cellular DNA was isolated using the Aqua-Genomic DNA isolation kit (Aquaplasmid) 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 specific for different regions of the CasFr^{KP} genome included primers near the *gag* gene (CasFrKP0234, GTCCATTGTCCTGTGTCTTT-GATTGATTTTA as the forward primer and CasFrKP1193RC, ATTGGGGAGGGATCCGGTG as the reverse primer): within the *pol* gene (CasFrKP3592, GTCCAGACCAGCAAAAAGCCTT as the forward primer and CasFrKP4687RC, AAGTGGCTCCTAGTTTGGTCAAATC as the reverse primer) and within the *env* gene (CasFrKP6805, TTGAGAGAGTACACTAGTC as the forward primer and CasFrKP7886RC, TCTGTTCCTGACCTTGATC 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. Their DNA was purified with an Extract-N-Amp™ Tissue PCR Kit (Sigma) according to the manufacturer's instructions. DNA from individual cultures were screened by PCR to confirm the presence of the appropriately sized insert and the PCR products from 96 confirmed clones were purified with a QIAquick 96 PCR Purification Kit (Qiagen) and sequenced using the primers used to generate the amplicons.

In experiments comparing the mutation rates in different regions of the genome, the DNAs isolated from cells 8 hours after infection were amplified using GE Healthcare Ready-to-go beads (Cat # 27-9559-01) according to the manufacturer's protocol. Amplicons were then cloned into the PCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Transformed colonies were isolated on selective LB agar plates contain 250 µg/ml carbenicillin, their DNAs purified, screened and sequenced as described above. Mutation rates for the different regions of the genome were corrected for background levels of mutation by the Taq PuRe polymerase. Background levels were determined by sequencing amplicons of plasmid DNA encoding the $CasFr^{KP}$ provirus which exhibits minimal genetic heterogeneity.

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.

2.2.5. Statistical analysis

The significance of differences between mutation rates was calculated by one way ANOVA. Probability values <0.05 were considered significant. The significance of the sequence preference of bases near mutation sites was calculated by the two-tailed Binomial Test with probability values of <0.05% considered significant. All statistical tests were computed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

2.3. Results

2.3.1. Incorporation of mA3 into CasFr^{KP} virions reflects the level of mA3 expression in cells

Previous studies examining the effect of virion incorporation of mA3 have utilized co-transfection of 293T cells with plasmids encoding MuLVs and plasmids encoding mA3 (11,13,18,46,73,118). In a recent report we did not observe an influence of virion-associated mA3 on the infectivity of CasFr^{KP} (68). However, is was not clear if the level of expression and incorporation into virions was similar in our analyses using mouse cells expressing mA3 to those using co-transfection of human cells. In this regard, it has been reported that the level of mA3 correlates with the inhibition of infectivitiy (119). In order to examine the effects of different levels of mA3 incorporated into MuLV virions we derived clonal cell lines infected with CasFr^{KP} at a low multiplicity of infection such that only 1 to 5% of the clonal lines were infected (88). This procedure served to minimize virion genomic heterogeneity as well as yield clones expressing different levels of mA3 (Fig. 7). CasFr^{KP}-infected clonal lines selected for study included an NIH 3T3 cell line which does not express mA3, 3T3/CasFr^{KP}, as well as two infected clonal cell lines expressing different levels of HA-tagged mA3 designated 3T3mA3/CasFrKP-1 and 3T3mA3/CasFr^{KP}-2 (Fig. 7). The level of virion incorporation of mA3 into the clones infected with CasFr^{KP} was lower in the virions released from 3T3mA3/CasFr^{KP}-1 which expresses a lower level of mA3 (Fig. 7). These results are consistent with previous studies in which the level of transfected plasmid encoding mA3 corresponded to the level of virion incorporation (13,118,119).

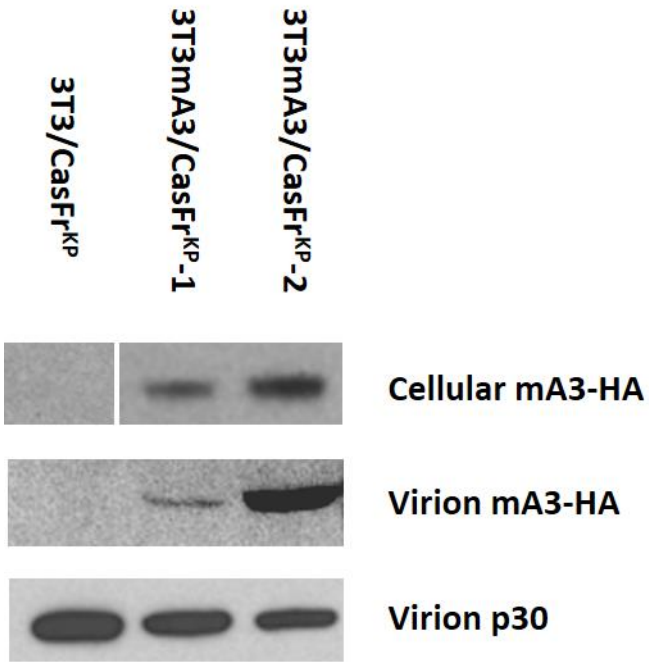


Figure 7. Incorporation of mA3 into virions released from clonal cell lines. The expression of mA3-HA in the clonal cell line, 3T3mA3/CasFr^{KP} was determined from an immunoblot of a cellular extract containing 15 µg protein and developed using an anti-HA monoclonal antibody. Virion mA3-HA released from 3T3mA3/CasFr^{KP} cells was determined from an immunoblot of a gel of virion proteins contained in 1.5 mls of culture supernatant using the anti-HA antibody while virion p30 was determined from immunoblots of gels of virion proteins contained in 0.075 mls of culture supernatant developed with a monoclonal antibody to p30. Exposure times for the immunoblots of virion p30 were 1 hour while immunoblots of virion mA3-HA were exposed for 5 minutes. Parallel analyses of 3T3/CasFr^{KP} cells and virions, devoid of mA3-HA were included as a controls

2.3.2. Virion associated mA3 suppresses MuLV infectivity

The infectivity's of CasFr^{KP} harvested from the two mA3-expressing clonal cell lines, 3T3mA3/CasFr^{KP}-1 and 3T3mA3/CasFr^{KP}-2, was compared to CasFr^{KP} harvested from NIH 3T3 cells not expressing mA3, 3T3/CasFr^{KP}. Reverse transcriptase (RT) activity has frequently been used to normalize infectivity to virion particles (2,11, 46,102,118,119,120). However, several studies have implicated RT as a possible target of APOBEC3 proteins (9,83,132,143) suggesting that RT activity may be misleading with regard to MuLV specific infectivity. In these studies, we have normalized the infectivity of the viruses using the level of the capsid protein (CA, p30) which is the major structural protein of MuLVs and easily detected by immunoblotting procedures. We found that the specific infectivity of virions released from cells expressing mA3 was significantly diminished compared to virions released from cells devoid of mA3 (Fig. 8). Furthermore, the degree of inhibition correlated with the level of mA3 in the virions (Figs 7 and 8).

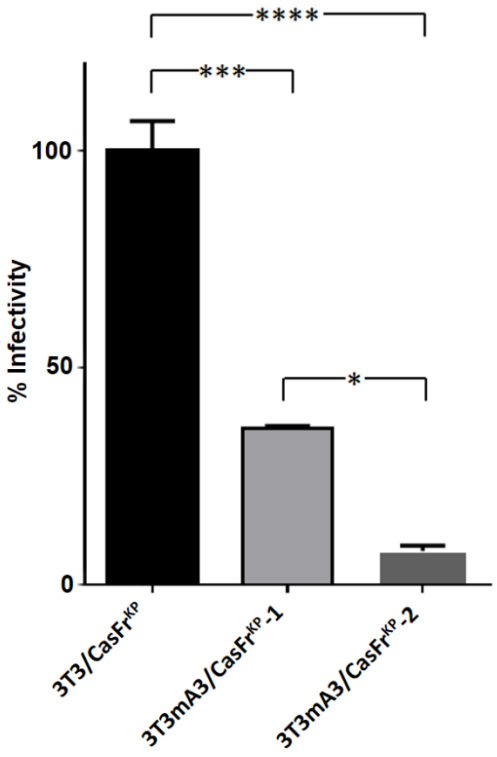


Figure 8. Suppression of infectivity by virion-incorporated mA3. The infectivity's of virions released from cells during an 8 hour interval were determined by the FIA and normalized to the levels of p30 present in the samples. The determinations are expressed as a percentage of the mean specific infectivity exhibited by virions released from cells not expressing mA3 (3T3/CasFr^{KP}). Error bars represent the standard error of 3 to 6 determinations. Significant differences determined by one way ANOVA are indicated (**** P<0.0001, *** P>0.0001 to 0.001, *P>0.001 to 0.01). Parallel analyses of 3T3/CasFr^{KP} cells and virions, devoid of mA3-HA were included as a controls.

2.3.3. Decrease in RT activity by virion-incorporated mA3

A change in the efficiency of reverse transcription may reflect an interaction of mA3 with RT and contribute to the loss of infectivity observed in virions that have incorporated mA3. To examine this possibility we determined the RT activity in virions containing different levels of mA3 and normalized the activity to the virion p30 (Fig. 9). As was the case with infectivity, the RT activity was significantly lower in 3T3mA3/CasFr^{KP}-1 and 3T3mA3/CasFr^{KP}-2 than in 3T3/CasFr^{KP} and correlated with the level of virion mA3.

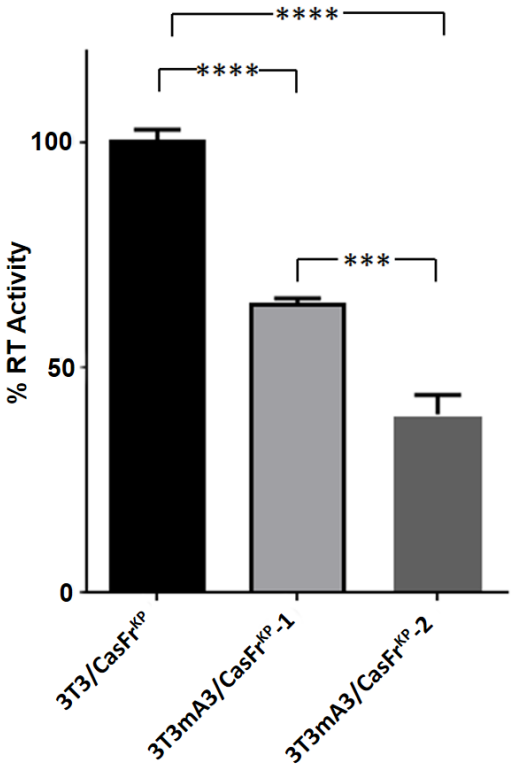


Figure 9. Supression of RT activity by virion-incorporated mA3. The RT activity of virions released from cells during an 8 hour interval were determined and normalized to the levels of p30 present in the samples. RT activity was determined for virions contained in 2.5 mls of supernatant media of the samples assayed for infectivity in Fig. 1. 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 error of 3 to 7 determinations. Significant differences determined by one way ANOVA are indicated (**** P<0.0001, *** P>0.001 to 0.01).

2.3.4. Incorporation of mA3 into virions results in a decrease of RT fidelity

One of the mechanisms of retroviral inhibition by APOBEC3 proteins, including mA3 and human APOBEC3G (hA3G), is through deamination of C→U in newly transcribed single stranded DNA resulting in G→A hypermutation upon subsequent DNA replication. Although most studies examining the effect of mA3 on MuLVs have not revealed hypermutation, one MuLV (AKV) has been reported to be susceptible to hypermutation by mA3 (68). Single stranded DNA containing U can be degraded through the action of enzymes such as uracil DNA glycosylase and apurinic endonuclease (70,123,147) and considerable degradation may occur before integration of the provirus (81,82) masking the extent of hypermutation. Therefore we isolated and examined RT transcripts after only 8 hours of infection to determine the rates of mutation.

NIH 3T3 cells were infected with viruses harvested from each of the three clonal cell lines (3T3/CasFr^{KP}, 3T3mA3/CasFr^{KP}-1 or 3T3mA3/CasFr^{KP}-2), and total cellular DNA was isolated. Newly synthesized transcripts were amplified using virus-specific primers from the *env* gene. The amplicons were gel purified, subsequently cloned and transformed into E-coli. Approximately 90 bacterial colonies encoding individual amplicons were selected from each of the viral infections and their nucleotide sequences determined.

In agreement with other reports (2,31,46,73,118,119,120), analyses of the mutations incurred in the transcripts did not reveal a significant increase in G→A mutations attributed to mA3 after infection by either 3T3mA3/CasFr^{KP}-1 or 3T3mA3/CasFr^{KP}-2 compared to 3T3/CasFr^{KP} (Fig. 10). 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 one of the mA3-containing viruses. C→T and T→C mutations appear elevated but the differences between the rates observed in mA3-containing viruses and 3T3/CasFr^{KP} did not reach significance ($P < 0.05$).

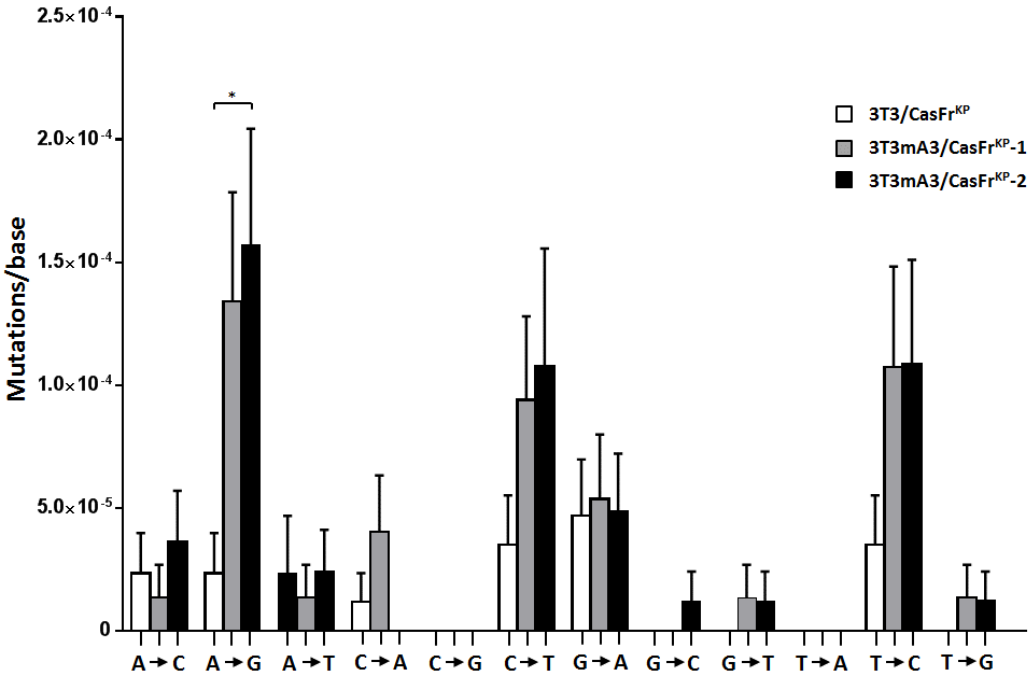


Figure 10. Influence of mA3 on individual mutations. 3T3 cells were infected with viruses released from each of the clonal cell lines and the nucleotide sequences of env gene transcripts synthesized during the first 8 hours were determined. The rates of all transition and transversion mutations are shown. Error bars represent the standard error of detected mutations on approximately 90 transcripts from each of the infections totaling about 85,000 bases analyzed for each virus. Significant differences determined by one way ANOVA are indicated (* P>0.01 to 0.05).

Although only the A→G mutations were significantly different between the mA3-containing viruses and 3T3/CasFr^{KP}, the inclusion of all of the mutations to calculate overall mutation rates yielded very significant differences (Fig. 11). Interestingly, viruses released from the two mA3-expressing clones did not exhibit significant differences between overall or individual mutation rates after infection.

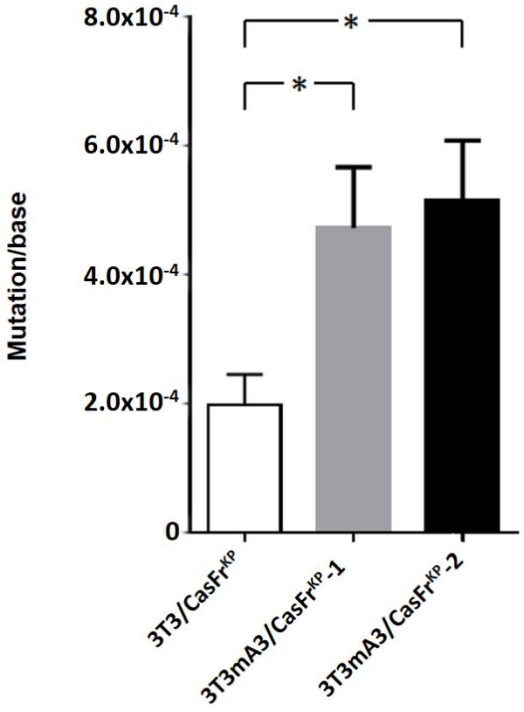


Figure 11. Influence of mA3 on the overall mutation rate. The rates of all transition and transversion mutations shown in Fig. 10 were combined to calculate the overall mutation rates. Error bars represent the standard error of detected mutations on approximately 90 transcripts from each of the infections totaling about 85,000 bases for each virus. Significant differences determined by one way ANOVA are indicated (*P>0.001 to 0.01).

A bias in the mutation rate in retroviruses has been reported in which the frequency of incurred mutations differs in different regions of the viral genome (16,107,153). In addition to the *env* gene amplicons, we examined the mutation rates in amplicons generated by primers specific to the *pol* gene as well as the *gag*-5'LTR region for mA3-containing virions. A significant differential effect of mA3 in different regions of the genome was not observed (Fig. 12).

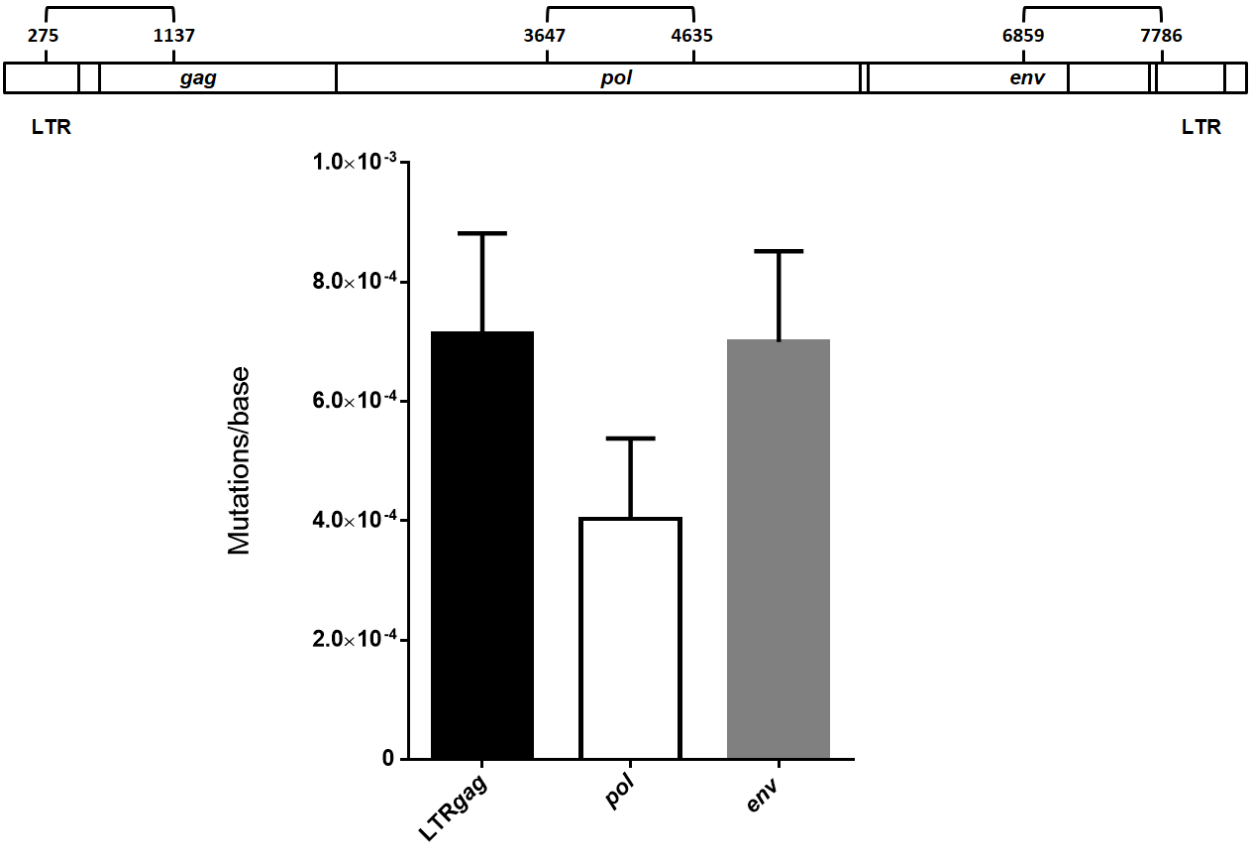


Figure 12. Effect of mA3 on mutation rates of different regions of the viral genome. Mutation rates were determined for transcripts from different regions of the viral genome obtained 8 hours after infection of cells with viruses from the 3T3mA3/CasFrKP-2 clonal cell line. The schematic depicts the viral genome and coordinates of the sequences included in the analysis. Bars in the graph represent mutation rate +/- the SEM detected on approximately 90 transcripts totaling about 85,000 bases from each region.

2.3.5. Sequence context of mutations effected by mA3 in CasFr^{KP}

G→A mutations induced by mA3 exhibit a consensus of two 3'-flanking A residues corresponding to a preferred target sequence of 5' TTC on newly synthesized minus strand transcripts for the cytidine deaminase activity (11,62,73,102). In the present study, very significant increases in transition mutations other than G to A transitions were observed. It was of interest to examine the flanking sequences of these mutations to determine if any sequence consensus was evident. The mutation rates of the transcripts of viruses from the two mA3-expressing clones were not found to be significantly different, thus mutations from both were included in these evaluations. The analyses revealed only four instances of flanking sequences that were significantly different from the expected frequency of bases (Fig. 13). A→G transitions had significantly elevated A residues at positions +1 and +2, G→A transitions exhibited a significantly elevated incidence of G at the +1 position compared to the expected frequency, while T→C transitions exhibited a significantly elevated incidence of C at the -1 position.

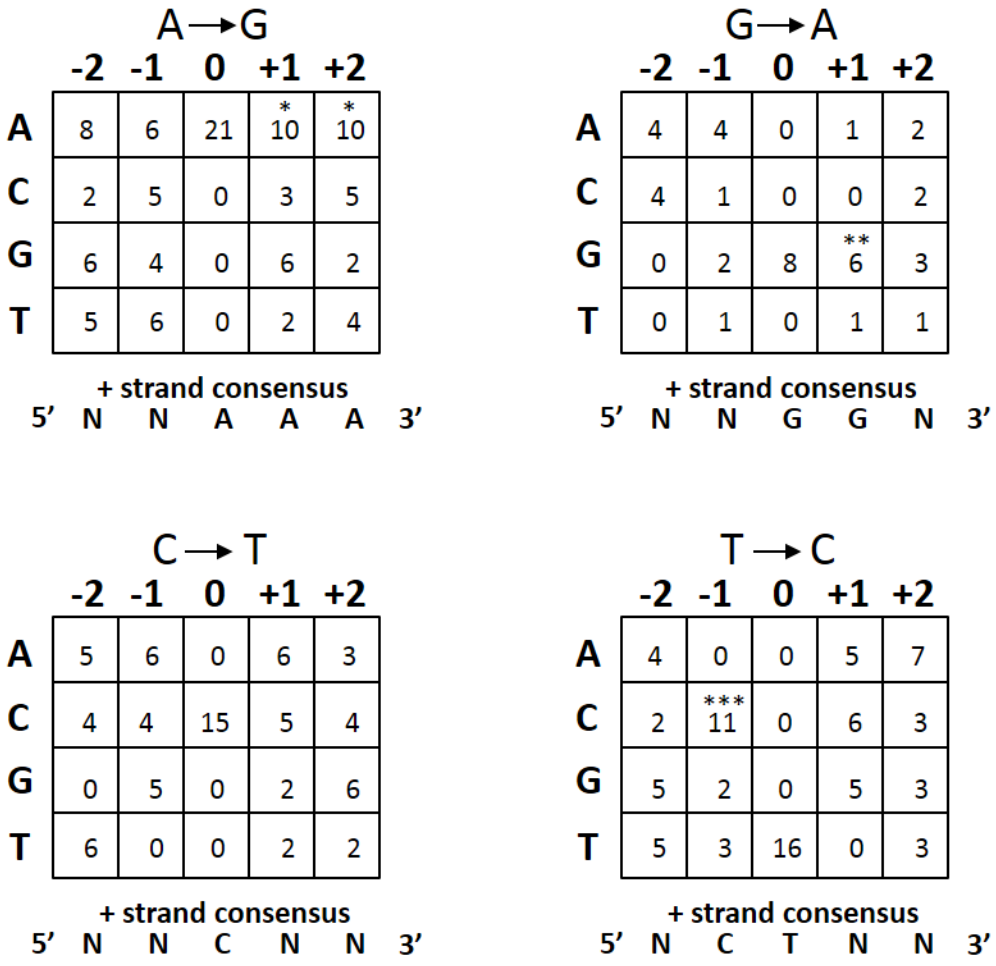


Figure 13. Consensus 3' and 5' sequences of mA3-induced transition mutations. The incidence of flanking bases for each of the transition mutations observed in transcripts obtained 8 hours after infection by mA3-containing viruses is shown. Analyses include mutations detected in approximately 180 transcripts totaling about 170,000 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>0.0001 to 0.001, **P>0.001 to 0.01, *P>0.01 to 0.05).

2.4. Discussion

MuLVs have evolved mechanism(s) to evade the action of mA3 however numerous studies have reported partial inhibition of infectivity by the restriction factor ranging from 10 to ~90%. In an earlier study we reported that CasFr^{KP} virions released from cells expressing mA3 did not exhibit a loss of infectivity (68). In that study infectivity was normalized to the level of RT activity in the infectious virus stocks; a frequently employed criterion for calculations of specific virion infectivity (2,11,46,102,118,119,120). In this study we normalized both infectivity and RT activity to the level of virions by quantifying p30, the major structural protein of the virus. We found that both the specific infectivity and the RT activity per virion were significantly decreased in the presence of mA3. Thus, normalization to RT activity in our earlier study may have resulted in an underestimation of the number of virions and an overestimation of the specific infectivity. Further, previous reports of suppression of infectivity by mA3 have utilized virions released from human 293T cells after co-transfection with plasmids encoding mA3 and plasmids encoding MuLV proviruses (2,11,46,102,118,119,120). It is possible that co-transfection procedures yield higher levels of virion-incorporated mA3 than was achieved in our earlier study. In the present report we selected clones of CasFr^{KP}-infected cells expressing higher levels of mA3.

A decrease in the specific activity of RT in virions after incorporation of mA3 was observed. Several studies have implicated RT as a possible target of APOBEC3 proteins (9,83,132,146), however few of these studies have investigated if virion-incorporated mA3 influences the efficiency of reverse transcription in cell-free reactions. One recent study reported a decrease in the strong-stop DNA, the initial product of reverse transcription, synthesized by mA3-containing virions in endogenous RT reactions (132). A decrease in strong-stop DNA may reflect an inhibition of an early step in the replication of the genome rather than an actual decrease in the tran-

scriptional activity of the enzyme. Similarly, another report proposed an effect of hA3G on the elongation of HIV-1/Δ*vif* transcripts in reactions utilizing cell-free virions (9). It was suggested that this activity may contribute to the inhibition of HIV-1 infection and may be the result of steric hindrance of the HIV RT by binding of the hA3G to HIV RNA rather than a direct interaction with the RT. In the present study the CasFr^{KP} RT activity was assessed using exogenous polyA/oligo dT as the substrate/primer. It seems more likely that the inhibition observed resulted from an interaction of mA3 with the RT and a decrease in the inherent transcriptional activity rather than steric inhibition of mA3 bound to the virion RNA.

Significant differences in G→A mutation rates were not observed in mA3-containing CasFr^{KP} compared to CasFr^{KP} devoid of mA3, in agreement with other reports of mA3-inhibition of MuLVs (2,31,46,73,118,119,120). However, very significant increases in overall mutation rates were observed, largely as a result of other transition mutations. G→A mutations mediated by mA3 have been reported to exhibit a strong consensus of two 3' flanking A residues corresponding to a 5' TTC consensus in the reverse transcribed single stranded DNA; the preferred target of mA3 cytidine deaminase activity (11,62,73,102). A paucity of target sequences in CasFr^{KP} could reflect the low G→A mutation rate. In contrast to other MuLVs, G→A hypermutation mediated by mA3 has been reported for the ecotropic AKV from AKR mice. A comparison 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 (Table 1).

It has been reported that different regions of the genome undergo different rates of G to A hypermutation, notably in a 5' to 3' gradient along the transcripts (16,62,140,153). The gradient of G to A mutations presumably reflects the exposure of single stranded DNA negative strand transcript to the action of the cytidine deaminase activity of mA3. Analyses of the overall mutation

rates of different regions of the viral genome including the LTR/gag region the pol region and the env region revealed no region that was significantly elevated indicating the absence of hypermutation in those regions.

Although the level of mA3 in the virions released from the two mA3-expressing cell lines were dissimilar (Fig. 7), the mutation rates observed in the transcripts from the viruses were not significant different (Figs. 10 and 11). If mA3 interacts with RT in a stoichiometric manner, the mA3 level necessary to affect the fidelity of the polymerase may be low and only a small portion of the virion-incorporated mA3 may be pertinent to this activity. Indeed, it has been estimated that as little as 4 to 7 molecules per virion is sufficient to affect inhibition of infectivity by hA3G (147).

Analysis of the sequence context of the mA3-induced transition mutations considered the two adjacent bases both 3' and 5' of the mutation. Context analysis of all of the transition mutations revealed residues at only four positions whose occurrence was significantly higher than the expected frequency (Fig. 7). A→G transitions exhibited 3' adjacent A residues at positions +1 and +2, G→A transitions exhibited a 3' adjacent G at the +1 position while T→C transitions exhibited a 5' adjacent C at the -1 position. The relevance of these differences to the mutagenic activity of mA3 is unknown, however it is clear that the context of the G to A mutations of CasFr^{KP} (GGN) is distinct from that observed with mA3 induced G to A hypermutation (GAA) (11,62,73,102) and is unlikely to involve cytidine deaminase activity.

The results of this study revealed a direct effect of virion-incorporated mA3 on the RT of the virus which affects the rate of the transcription process as well as the fidelity of the enzyme. These results suggest a direct interaction of mA3 with the RT that facilitates a marked loss of infectivity. 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.

TABLE 1. Putative mA3 target sequences of CasFr^{KP} and AKV[1]

MuLV	C (%)	TC (%)	TTC (%)
CasFr^{KP}	2050 (24.9%) [2]	593 (7.2%)	149 (1.7%)
AKV	2055 (25.5%)	585 (7.0%)	148 (1.8%)

[1]Target sequences for mA3 cytidine deaminase sites were compiled from the complementary sequences of the MuLVs.

[2]The sequence of CasFr^{KP} was deduced from the sequences of the two parental MuLVs, CasBrE and FB29 (accession nos. X57540and Z11128, resp.). The AKV sequence is from (accession no. J01998).

Chapter 3: The ecotropic AKV MuLV undergoes G→A hypermutation independent of augmented APOBEC3

3.1. Introduction

Mouse APOBEC3 (mA3) is a cytidine deaminase that acts on single-stranded DNA during reverse transcription by murine retroviruses resulting in G→A hypermutation of newly synthesized proviral DNA (18,73). Although mA3 inhibits endogenous retroviruses (35), exogenous murine leukemia viruses (MuLVs) such as Friend (Fr-) MuLV and Moloney (Mo-) do not undergo G→A hypermutation upon infection in the presence of mA3 (73,2,10,13,31,68). AKV is an endogenous ecotropic MuLV isolated from thymoma-prone AKR mice and can be propagated as an exogenous ecotropic MuLV (115,116). In contrast to other MuLVs, AKV has been reported to undergo G→A hypermutation upon infection in the presence of mA3 (73). The mutations exhibited a strong consensus of two 3' flanking A residues, GAA, corresponding to TTC in the cDNA transcript sequence which is the target of cytidine deamination. In a recent study, we found that an MuLV that has incorporated an HA-tagged mA3 (mA3-HA) does indeed exhibit a significantly higher level of mutations. However, the mutations incurred in the presence of mA3-HA corresponded to transition mutations other than G→A. The mutations did not exhibit a strong consensus of flanking bases suggesting a loss of fidelity of the reverse transcriptase induced by mA3-HA rather than recognition of a nucleotide target sequence. In this study we have examined the mutations incurred by AKV in the presence or absence of mA3-HA. Surprisingly, we observed G→A hypermutations in transcripts generated after infection by viruses released from cells that did not express mA3-HA. The mutations exhibited a strong consensus of flanking bases similar to that reported for mA3. Infection by AKV released from cells expressing mA3-

HA exhibited a nearly identical G→A mutation rate and consensus of flanking bases. Furthermore, an unequal distribution of mutations was observed such that many of the transcripts exhibited multiple mutations while the majority of transcripts exhibited none. Our studies suggest that AKV is very susceptible to an restriction factor endogenous to 3T3 cells that exhibits cytidine deaminase activity that is active in only a portion of virions released from cells The observation that the incorporation of mA3 did not alter the extent of distribution of the transcripts suggests that the restriction factor may be distinct from mA3.

3.2. Materials and Methods

3.2.1. Plasmids, cells and viruses

The maintenance of cell lines, the derivation of cells expressing mA3 by transfection of plasmids and the use of viruses in this study were as described in chapter 2.2.1. In addition AKV was derived by transfection of NIH 3T3 cells with the molecular clone 623 (80).

3.2.2. Immunoblotting

Immunoblotting procedures were performed as described in chapter 2.2.2.

3.2.3. 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 3T3 cells or from 3T3 cells expressing mA3 (3T3mA3). We derived clonal cell lines after infection of 3T3 or 3T3mA3 cells with the exogenous ecotropic MuLV CasFr^{KP} and AKV at a low multiplicity of infection as previously described (88). This served to minimize the initial genetic heterogeneity of the virus as well as to obtain infected clonal cell lines expressing varying amounts of mA3. Mutation rates were assessed after infection of 3T3 cells with virus harvested from 3T3 cell lines or from 3T3/mA3 cell lines. Eight hours after infection cellular DNA was isolated using the AquaGenomic DNA isolation kit (Aquaplasmid) 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} (CasFrKP6805, TTGAGAGAGTACACTAGTC as the forward primer and CasFrKP7886RC, TCTGTTCCTGACCTTGATC as the reverse primer). Viral DNAs from AKV-infected cells were amplified using AKV *env*-specific primers (AKV 6899 CACAAATTGACCTTGTCCG as the forward primer, 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 1ml Kanamycin-containing medium. Their DNA was purified with an Extract-N-Amp™ Tissue PCR Kit (Sigma) according to the manufacturer's instructions. DNA from individual cultures were screened by PCR to confirm the presence of the appropriately sized insert and the PCR products from 96 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.

3.2.4. Statistical analysis

Statistical analysis were performed as described in chapter 2.2.5.

3.3. Results

3.3.1. AKV undergoes G→A hypermutation in NIH 3T3 cells in the absence of mA3-HA expression.

AKV has been reported to undergo G→A hypermutation upon incorporation of mA3 into virions. We have recently observed that another MuLV ($CasFr^{KP}$) does not undergo G→A hypermutation upon incorporation of mA3 but rather, undergoes a loss of RT activity as well as a loss of fidelity of the enzyme as evidenced by a significant increase in transition mutations other than G→A. In order to study the differences between the two MuLVs with regard to 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 and viruses released from the lines were used to infect new NIH 3T3 cell lines. Viral DNA transcripts synthesized during the intitial 8 hours after infection were amplified by PCR, the amplicons cloned and subsequently sequenced. These analyses revealed a marked difference in the patterns of mutations observed between the two MuLVs (Fig. 14A). The G→A mutation rate of AKV was much higher than the G→A mutation rate of $CasFr^{KP}$ resulting in a significantly higher overall mutation rate for AKV (Fig. 14B).

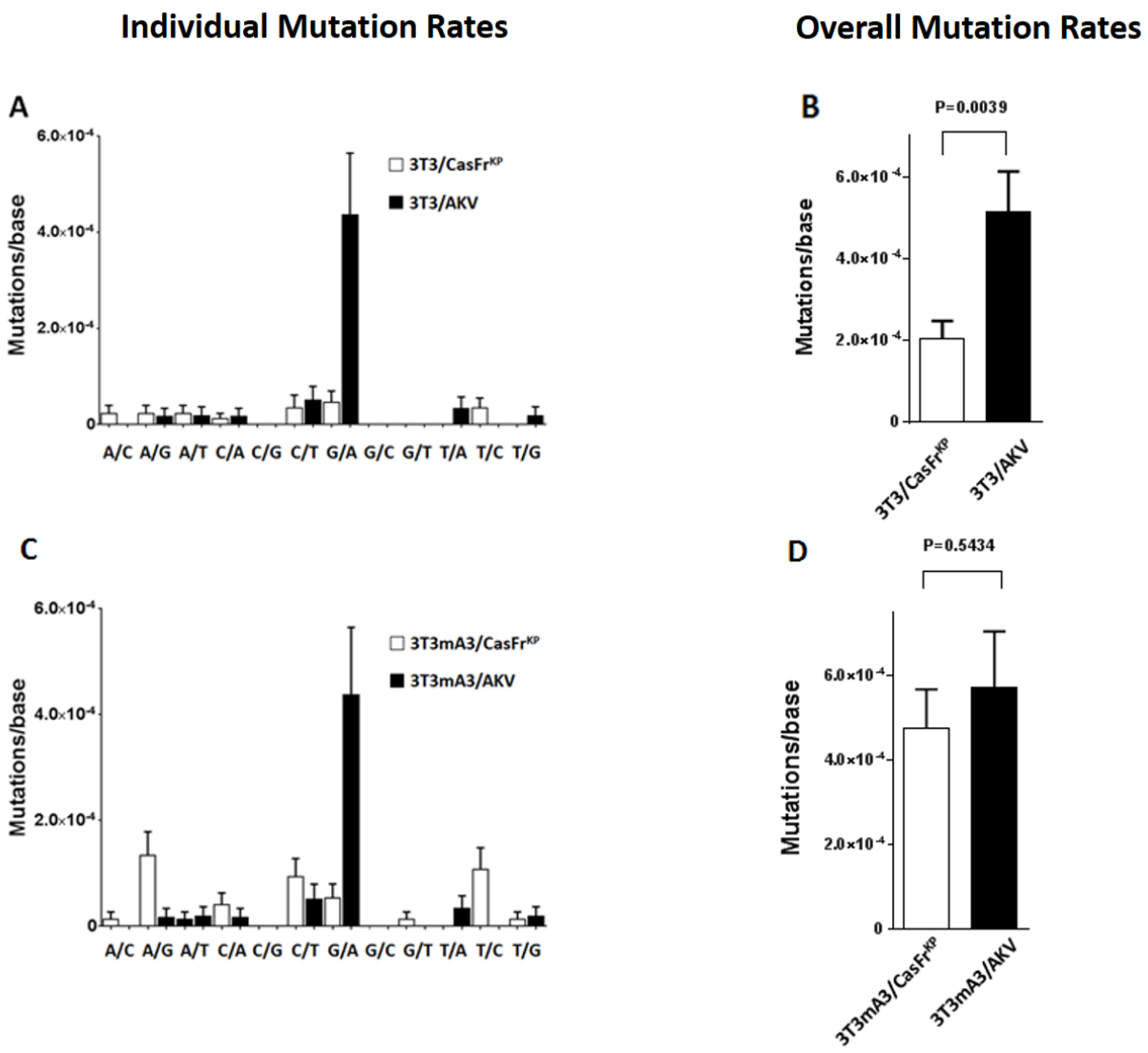


Figure 14. Mutation rates of AKV and CasFrKP MuLVs in the presence or absence of mA3-HA. Mutations incurred in transcripts of AKV or CasFrKP at 8 hr post-infection were determined. A) CasFrKP and AKV individual mutation rates in the absence of mA3 for each of the possible point mutations. B) Overall (total) mutation rates for CasFrKP and AKV in the absence of mA3. C) CasFrKP and AKV individual mutation rates in the presence of mA3 for each of the possible point mutations. D) Overall (total) mutation rates for CasFrKP and AKV in the presence of mA3. The bars show the mean and SEM for each rate. P values were calculated by the students t test with $P<0.05$ considered significant.

3.3.2. G→A hypermutation of AKV is not augmented in the presence of mA3

Cell lines expressing mA3-HA were infected with AKV or CasFr^{KP} at a low MOI and clonal lines were established. Both of the clonal cell lines expressed mA3-HA and both released virions that had incorporated mA3-HA (Fig. 15). The level of mA3-HA in the AKV virions appears to be slightly higher than in the CasFr^{KP} virions even though the expression of mA3 in the cells infected with CasFr^{KP} was higher than that expressed by cells infected with AKV.

The mutation frequencies of mutations upon infection of 3T3 cells by the viruses released from each of the mA3-HA clonal cell lines was determined (Fig. 14C). In agreement with our earlier observations (submitted for publication) the mutation rates for transition mutations other than G→A transitions were elevated resulting in a significant change (P=0.0072) in the overall mutation rate of CasFr^{KP} (Fig. 14B and 14D). In contrast, AKV did not exhibit significant changes in mutation rates upon incorporation of mA3-HA. Indeed, the overall mutation rates of AKV and CasFr^{KP} were not significantly different in the presence of incorporated mA3 (Fig. 14D).

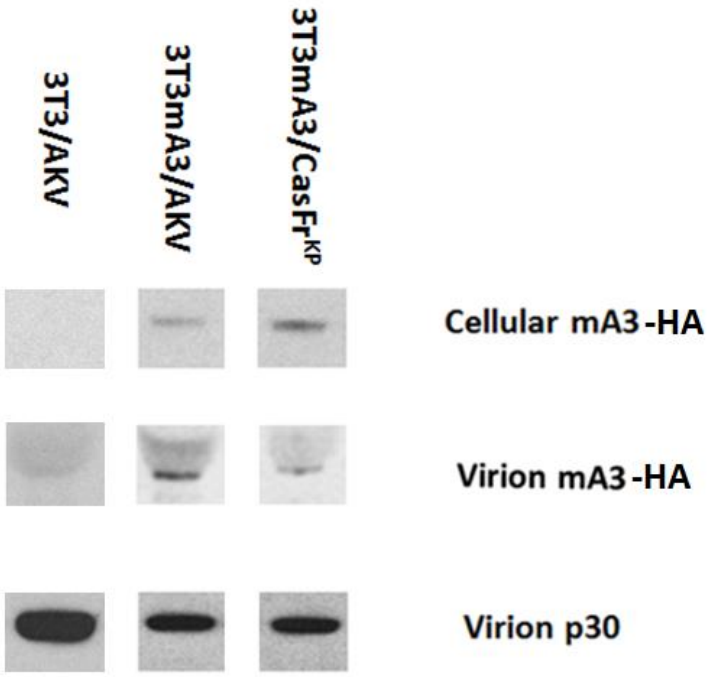


Figure 15. Immunoblots of 3T3, 3T3/AKV and 3T3mA3 cell and virions. The expression of mA3-HA in the clonal cell line, 3T3mA3/CasFr^{KP}, 3T3/AKV and 3T3mA3/AKV was determined from an immunoblot of a cellular extract containing 15 µg protein and developed using an anti-HA monoclonal antibody. Virion mA3-HA released from 3T3mA3 cells was determined from an immunoblot of a gel of virion proteins contained in 1 ml of culture supernatant using the anti-HA antibody while virion p30 was determined from immunoblots of gels of virion proteins contained in 0.1 ml of culture supernatant developed with a monoclonal antibody to p30. Exposure times for the immunoblots of virion p30 were 5 minutes while immunoblots of virion mA3-HA were exposed for 1 hour.

3.3.3. 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 TT**C** in the negative sense transcript with **C** the base that becomes deaminated (73,10,62,102). Determination of the the sequence context of G→A mutations in AKV transcripts observed in the presence and absence of mA3-HA revealed a strong consensus target sequence consistent with the cytidine deaminase target of mA3 (Fig. 16). Indeed, a very conserved consensus sequence flanking the 5' and 3' ends of the cytidine target was observed corresponding to TT**C**AA 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.

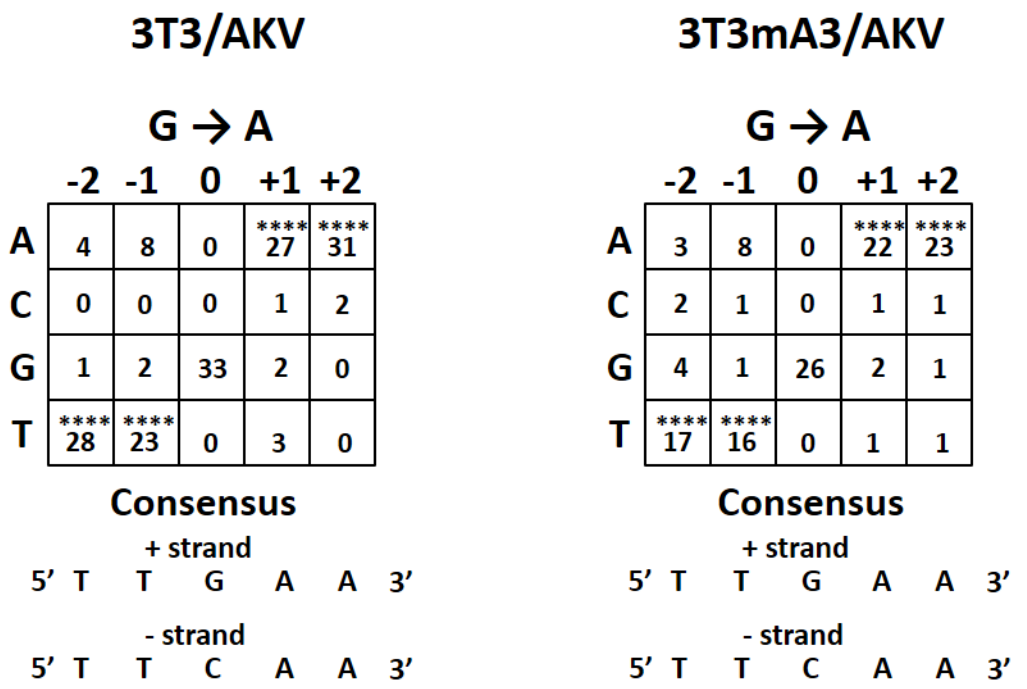


Figure 16. Consensus target for cytidine deamination of AKV transcripts. The sequence context of G→A mutations in transcripts generated after an 8 hr infection were determined for the two bases flanking the 3' and 5' side of the mutation site. The consensus sequences of the observed G→A mutation as well as the consensus of the (–)strand DNA flanking the cytidine deaminase target C residue are shown. Significance of the frequency of flanking based was calculated by the two-tailed Binomial Test (**** P<0.0001).

3.3.4. G→A mutations are disproportionate among AKV transcripts

When compiling the results of the AKV mutations it was noted in both the analyses (in the presence or absence of mA3) that the majority of the transcripts were devoid of mutations while some of the transcripts exhibited multiple mutations (Tables 3 and 4). In both cases nearly 100% of the mutations were present in less than 25% of the transcripts.

The results of these 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.

TABLE 2. Distribution of G→A mutations in 3T3/AKV

Mutations Per Transcript (#)	% Mutations	%* Transcripts
0 (64)	0	74
1 (16)	49	19
2 (2)	12	2
3 (3)	27	3
4 (1)	12	1

*The distribution of G→A mutations in AKV transcripts in the absence of mA3 after 8 hrs. of infection are tabulated. The percent of transcripts were rounded down resulting in less than 100%. The data are not consistent with a random distribution of mutations using a chi-square test with $p<0.0001$.

TABLE 3. Distribution of G→A mutations in 3T3mA3/AKV

Mutations Per Transcript (#)	% Mutations	%* Transcripts
0 (54)	0	81
1 (6)	23	9
2 (3)	23	4
3 (2)	23	3
4 (2)	31	3

*The distribution of G→A mutations in AKV transcripts in the presence of mA3 after 8 hrs. of infection are tabulated. The data are not consistent with a random distribution of mutations using a chi-square test with $p<0.0001$.

3.4. Discussion

This investigation was initiated with the aim of comparing the effect of mA3 on two MuLVs that have been reported to be inhibited by two distinct mechanisms. AKV has been reported to undergo G→A hypermutation (73) while our studies have established that the MuLV, CasFr^{KP} does not undergo G→A hypermutation but rather undergoes a significant inhibition of RT activity and an increase in overall mutation rate reflecting a loss of RT fidelity. These findings correlate with a significant loss of infectivity. We unexpectedly found that AKV exhibited G→A hypermutation in the absence of detectable mA3 incorporation into virions. Furthermore, the incorporation of mA3 into AKV virions did not augment hypermutation of the AKV transcripts. In contrast, Langlois et al., (73) reported that incorporation of mA3 into AKV virions resulted in G→A hypermutation. Although the mutation rate of AKV in the absence of incorporated mA3 was not examined in that study, G→A hypermutation was not observed after incorporation of human APOBEC2 suggesting that mA3 was necessary for G→A hypermutation of AKV.

The discrepancy between the results reported here and those of Langlois et al., (73) may be the result of the levels of mA3 incorporated into the virions as well as the source of the AKV stock. In that study AKV stocks were obtained after co-transfection of human cells with a plasmid encoding mA3 and a separate plasmid encoding AKV. A much higher level of mA3 incorporation could account for their observation that virion incorporated mA3 altered the extent of hypermutation. Our AKV stocks were obtained from infected mouse NIH 3T3 cells or from NIH 3T3 cells that were expressing HA-tagged mA3. The incorporation of a restriction factor endogenous to mouse cells but not to human cells could explain why hypermutation occurred in the absence of augmented mA3-HA. A strong sequence consensus for the cytidine deaminase targets was ob-

served in the minus strand reverse transcript (TTCAA) suggesting mA3-mediated cytidine deamination in agreement with previous studies (18,73,35,2). However a consensus on both the 3' and 5' flanking sequences has not been previously observed with either mA3 or mouse APOBEC1 which has also been reported to restrict MuLVs (2).

Another pertinent observation was that the distribution of G→A hypermutations was skewed, with a small percentage of the transcripts exhibiting all of the mutations. This result is reminiscent of the results of Pathak and Temin (99) who noted G→A hypermutation in a small proportion of transcripts of retroviral vectors under investigation. In their studies it was speculated that hypermutation was the result of aberrant polymerases in a small proportion of the virions. In the present analysis an aberrant polymerase is very unlikely. The AKV virus stocks were obtained from clonal cell lines infected at a low multiplicity of infection and should have only one or at most a few proviral copies. Moreover, the results were obtained with progeny viruses from two independent clonal lines, yet both populations yielded nearly identical distribution patterns. It seems more likely that only a portion of the AKV virions released from NIH 3T3 cells have encapsulated a cytidine deaminase restriction factor similar or identical to mA3.

Although we have previously examined NIH 3T3 cells by quantitative PCR and found the level of mA3 mRNA to be undetectable, it is possible that a low level of mA3 is expressed and is responsible for the hypermutation of AKV transcripts we have observed. To investigate this possibility we utilized a newly available antibody to mA3 in immunoblotting procedures. Immunoblots of AKV-infected and uninfected 3T3 cells, as well as 3T3 cells expressing mA3-HA were developed with an anti-HA antibody and also with the commercial anti-mA3 antibody (Fig. 17).

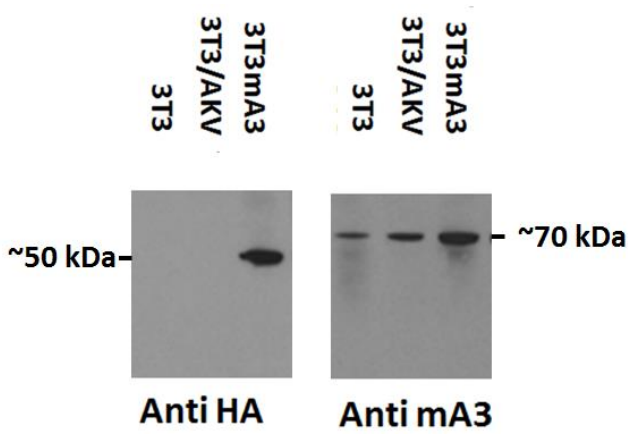


Figure 17. Immunoblots of 3T3, 3T3/AKV and 3T3mA3 cells. Protein extracts from uninfected 3T3 cells, 3T3 cells infected with AKV (3T3/AKV) and uninfected 3T3 cells expressing an HA-tagged mA3 (3T3mA3) were electrophoresed, blotted and developed with a commercially available antiserum to mA3. The membrane was stripped and redeveloped with an antibody to HA to detect the HA-labeled mA3 expressed in 3T3mA3 cells.

The anti-HA antibody detected the ~50 kDa protein in the 3T3mA3 sample, as expected, however the new commercially available mA3 antibody detected a protein of ~70 kDa in all three samples. Notably, the new antibody did not detect the mA3-HA present in the 3T3mA3 cells. These results suggested that the new antibody did not detect the HA-labeled mA3 even though the peptide used to elicit the antibody was encoded by the mA3-HA plasmid. It was conceivable that a previously undescribed form of mA3 was expressed in the 3T3 cells and that the epitope in the mA3-HA was masked. Alternatively, the new mA3 antibody may simply detect a cross-reactive protein present in the cells. To resolve this issue an immunoblot was performed with samples of cell extracts, samples of virion preparations and a mock virion preparation from uninfected 3T3 cells using the putative mA3 antibody (Fig. 18). The ~70 kDa protein was found in all samples including the mock virion preparation. Again, the antiserum failed to detect the mA3-HA

epitope. These results suggest that the antibody detects a cross-reactive ~70Da protein that is present in 3T3 cells and is released into the growth media, possibly as a vesicular element that pellets with virions.

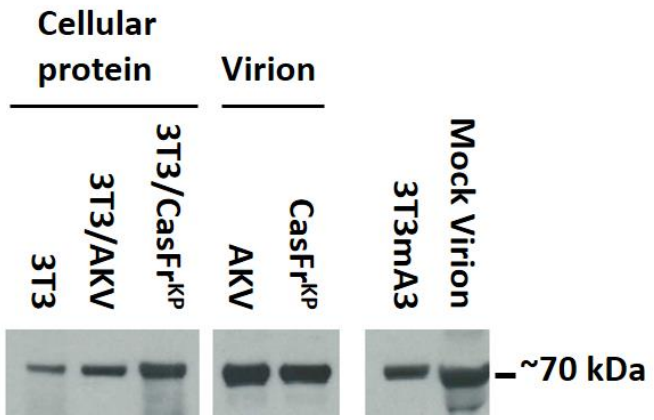


Figure 18. Immunoblots of cellular protein, virion preparations and mock virion preparations with a commercially available antibody to mA3. Protein extracts from 3T3 cells, 3T3 cells infected with AKV (3T3/AKV), 3T3 cells infected with CasFr^{KP} (3T3/ CasFr^{KP}) and uninfected 3T3 cells expressing mA3 (3T3mA3) as well as virion preparations of AKV, CasFr^{KP} and a mock virion preparation from uninfected 3T3 cells were electrophoresed, blotted and develped with a commercially available antiserum to mA3.

In the absence of a reliable reagent to detect endogenous mA3 we are unable to determine if it is the incorporation of endogenous mA3 or some other restriction factor into AKV virions that mediates the G→A hypermutation of AKV. However, if only trace amounts of mA3 are necessary for the hypermutation, it might be expected that even a small amount of mA3-HA incorporation would have effected a higher rate of hypermutation as well as elimination of the skew in the distribution of mutations. This was not observed suggesting that another restriction factor may be endogenous to mouse cells. In this regard, additional quantitative PCR experiments utilizing different sets of primers have not detected mA3 mRNA (data not shown). An approach to the reso-

lution of this problem may be the utilization of knock out mouse cells in which various restriction factors have been eliminated.

Chapter 4

4.1. Conclusions

APOBEC3 proteins are cytidine deaminases that are potent inhibitors of retrovirus replication. These restriction factors induce G→A hypermutation as a result of deamination of cytidine in the single stranded transcripts generated by reverse transcription during replication. Mouse APOBEC3 (mA3) effectively inhibits the replication of endogenous retroelements whereas most exogenous murine leukemia viruses (MuLV) are largely resistant to the action(s) of mA3. However, several studies have reported significant inhibition of infectivity by virion-associated mA3 that did not appear to be the result of G→A hypermutation. Recent studies have reported that MuLV glyco-Gag is involved it the resistance to mA3 during the retroviral replication. (68,92,132). It is not completely clear how mA3 is able to restrict MuLVs, and why in some cases, the inhibition proceeds by a deaminase-independent mechanism and in others with a deaminase-dependent mechanism (18,19,79,94,140,104).

In this work, we showed that CasFr^{KP} which encodes gGag does exhibit inhibition of infection, RT activity and an increase in the overall mutation rate if a sufficient level of mA3 is incorporated into the virion. However no G to A hypermutation was observed at any level of mA3. These results suggest a direct interaction of mA3 with the RT that facilitates a marked loss of infectivity. 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.

In contrast to CasFr^{KP}, AKV, an ecotropic MuLV isolated from AKR mice, has been reported to undergo hypermutation mediated by mA3 (73). In this report we confirm that AKV does undergo G→A hypermutation upon infection of 3T3 cells, however hypermutation is not augmented in

viruses that have incorporated HA-tagged mA3. Furthermore a strong sequence consensus was observed in the minus strand reverse transcript (TTCAA) suggesting mA3-mediated cytidine deamination in agreement with previous studies (11,62,73,102). However a consensus on both the 3' and 5' has not been previously observed with either mA3 or mouse APOBEC1 which has also been reported to restrict MuLVs (102). Hypermutations were not distributed randomly with 100% of the G to A mutations found on about 20 to 25% of the transcripts with some individual transcripts containing up to 15% of the total G to A mutations. These results suggest that AKV virions have incorporated an endogenous cytidine deaminase restriction factor upon release from 3T3 cells that exerts its activity on only a portion of the virions. This might be the result of incorporation of the factor in only a low proportion of virions. In this regard it has been reported that hA3G is able to inactivate target viruses with as little as 4 to 7 molecules per virion.

4.2. Future Directions

- The glycosylated gag proteins (gGag) of MuLVs have been implicated in the evasion of exogenous MuLVs from the action of mA3. We have shown that mA3 incorporation at high levels into virions encoding gGag can result in an inhibition of RT and a decrease in the fidelity of the enzyme. It is not known if a similar mechanism is responsible for the increased susceptibility of gGag-negative mutant MuLVs to mA3. Investigations of the effect of mA3 on the RT of gGag-negative mutants may elucidate this issue.
- MuLVs are also inhibited by cellular mA3 upon infection by gGag-negative viruses that have not encapsulated mA3. It would be of interest to determine if the mechanism of inhibition by cellular mA3 is the same as inhibition by virion-associated mA3. Mutation studies on these two manners of inhibition may be enlightening.

- mA3 appears to inhibit the infectivity of MuLVs by two different mechanisms. G→A hypermutations are observed in AKV while other MuLVs such as $CasFr^{KP}$ do not exhibit hypermutation. The construction of chimeric viruses between $CasFr^{KP}$ and AKV may define what regions of the viral genome are responsible for these differences. Chimeras involving the protective gGag encoding regions may be of particular interest.
- Our current studies suggest that G→A hypermutation of AKV may be mediated by an endogenous restriction factor of mouse cells. These studies will be extended to investigate the hypermutation of AKV originating from different cell types including cells of different species such as human 293T cells. In addition, hypermutation of AKV originating from KO mouse cells that do not express various restriction factors will be investigated.

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