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INTERACTIONS BETWEEN THE GLYCOSYLATED GAG PROTEIN OF A MURINE LEUKEMIA VIRUS AND MURINE APOBEC3: NOVEL INSIGHTS INTO HOW A MURINE LEUKEMIA VIRUS COUNTERACTS A RESTRICTION FACTOR

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Interactions between the glycosylated Gag protein of MuLV and murine APOBEC3: Novel insight into how MuLVs counteract restriction factors

Chairperson: Leonard Evans, Ph.D.

APOBEC proteins have evolved in mice and humans as potent innate defences against retroviral infections. APOBEC3G (hA3G) in humans and mouse APOBEC3 (mA3) deaminate cytidine in single-stranded DNA which ultimately results in hypermutation of newly synthesized proviral DNA. Other deaminase-independent mechanisms of inhibition have been identified, such as directly inhibiting reverse transcription. Both HIV and murine leukemia viruses (MuLVs) have evolved mechanisms to evade the action of the APOBEC proteins. HIV encodes the Vif protein which binds to hA3G and facilitates its rapid degradation through the proteasome. The mechanism(s) by which exogenous MuLVs evade mA3 inhibitory activity is unknown.

Exogenous MuLVs encode a glycosylated gag protein (gGag) originating from an alternate CUG start site upstream of the AUG start site of the Gag structural polyproteins. gGag is synthesized to similar amounts as the structural Gag polyprotein in MuLV infected cells but is glycosylated in the endoplasmic reticulum and undergoes distinct proteolytic processing. The function(s) of gGag remain unclear, but eliminating its synthesis through mutation markedly impedes *in vivo* replication of the virus with very little affect on *in vitro* replication. Endogenous retroviruses have not been found to express gGag and are tightly controlled by mA3. APOBEC3 proteins are expressed in many tissues in the mouse but are not expressed in most *in vitro* cell lines. These observations are consistent with a link between gGag expression and the evasion of mA3 by MuLVs.

Studies described herein demonstrate that gGag is protective against both cellular and virion-associated mA3 *in vitro* and is protective against mA3 *in vivo*. While there was no direct interaction between mA3 and gGag in an infected cell, gGag and mA3 are localized in the same compartment in the virion and are able to be coprecipitated together from lysed virions. G-to-A hypermutation is not a mechanism used by mA3 to inhibit gGag-negative MuLV replication. Through an affect on reverse transcription, cellular and virion-associated mA3 reduce viral transcripts in MuLV infected cells in a gGag-dependent manner.

Chapter 1- Introduction

1.1 Retroviruses

Retroviruses are RNA viruses that replicate through DNA intermediates and ultimately are incorporated into the genome of the target cell. If this occurs in a germline cell the retrovirus becomes a permanent component of the host genome that is passed down to subsequent generations[177]. 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[177]. The majority of these sequences are no longer full length and do not encode a functional retrovirus. However, several endogenous retroviral elements function in important physiological roles such as transcriptional regulation of host genes[11,41,46,106] and the induction of placenta-trophoblast fusion during embryogenesis[45,61,160].

Retroviruses are positive sense (+) single stranded (ss) RNA viruses that replicate through DNA intermediates generated by the viral polymerase which is termed reverse transcriptase[177]. The viral RNA is comprised of two identical RNA genomes that are incorporated into the virion and resemble a large polycistronic messenger RNA[98]. 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[177]. Once integrated, the provirus can use the cell's transcriptional machinery to transcribe its genome into messenger RNA[177]. From this RNA, the retroviral proteins are translated. The translated proteins are assembled to create new retroviruses that are subsequently released from the cell through budding, allowing for an infection of a new cell.

1.2 Murine Leukemia viruses

Murine leukemia viruses (MuLVs) are classified in the Baltimore classification of viruses as Type IV (+) ssRNA, DNA intermediate retroviruses that are part of the Retroviridae family and the gammaretroviral genus[98]. MuLV virions are also classified as type C[10], with a centrally located spherical inner core composed of the nucleocapsid (NC) and capsid (CA) proteins[10,98]. The genetic structure of a typical MuLV RNA genome and the location within a virion of the gene products it encodes is schematically depicted below (Figure 1.1). Within the core are: the RNA genome, reverse transcriptase (RT), integrase (IN), protease (PR), and the $tRNA^{Pro}$ primer[98]. The inner core is surrounded by an outer envelope composed of a lipid bilayer that contains the transmembrane (TM) and surface (SU) subunits of the envelope (Env) glycoprotein[98]. In contrast to other types of retroviruses, type C particles appear to be assembled largely at the plasma membrane during budding[10]. The approximate diameter of a mature MuLV particle is 120nm[10]. MuLVs can be either endogenous (integrated into the host genome) or exogenous (passed from one host to another)[98]. Further classification of MuLVs has been based on host specificity or tropism. Ecotropic[115] MuLVs infect only mice, and utilize the mCAT-1 receptor. Amphotropic[81] MuLVs infect mice and cells of many other species using the Pit-2 receptor. Xenotropic[115] MuLVs, although endogenous to mice, can infect cells of many species but do not infect mouse cells, and utilize the Xpr-1 receptor. Polytropic[82] viruses can infect many species including mice and humans and also use Xpr-1 as a receptor. Interestingly, the Xpr-1 receptor in most mouse strains is functional for polytropic viruses but not for xenotropic MuLVs[102]. All of the receptors thus far identified are multiple membrane spanning proteins[102].

Murine leukemia viruses (MuLVs) were originally identified and named for their

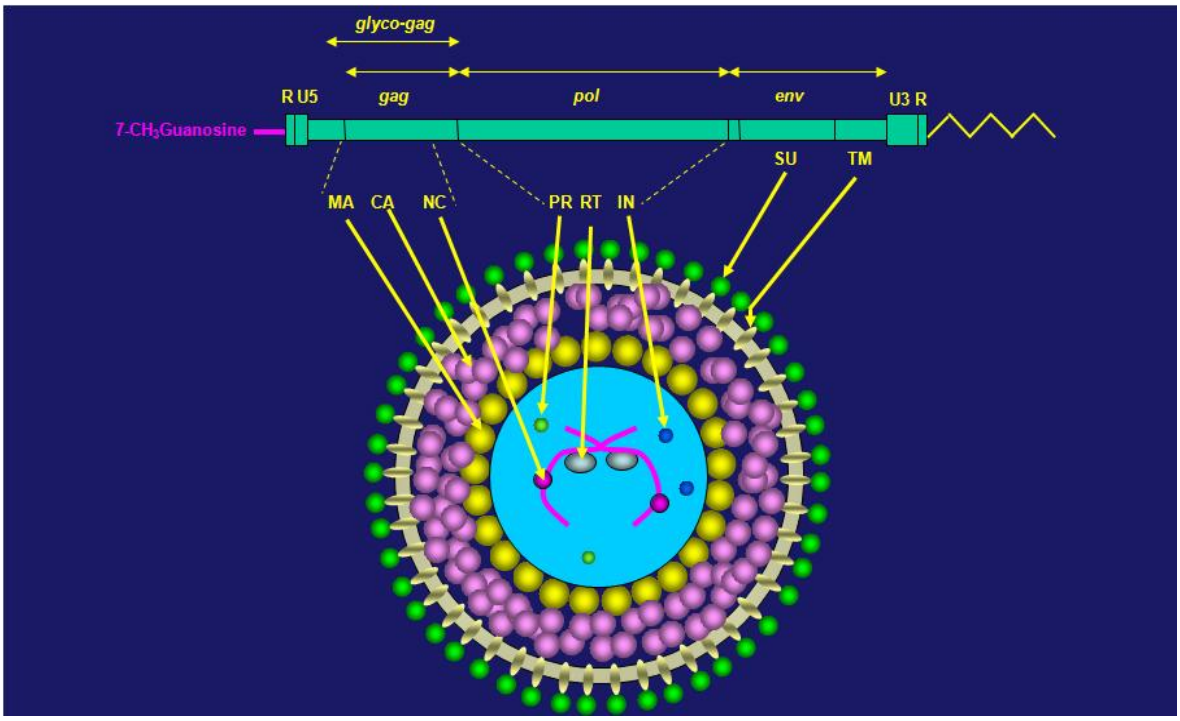

Figure 1.1. Structure of a MuLV virion. The most inner part of a virion is known as the core, which contains the RNA genome, NC, IN and PR surrounded by the CA. The CA is surrounded by the MA protein, which helps anchor the lipid membrane of the virus which contains the envelope proteins TM and SU. Figure credit: Dr. Leonard Evans.

ability to cause leukemias in mice[1,47,62,70,75,97,156]. The term has since been adopted to represent a large group of mouse gammaretroviruses. Some MuLVs that cause proliferative diseases in mice are named after their discoverers: Gross MuLV by Dr. Ludwig Gross (1951)[75] (lymphocytic leukemia); Graffi MuLV by Dr. Arnold Graffi (1955)[70] (erythroleukemia); Friend MuLV (F-MuLV) by Dr. Charlotte Friend (1957)[62] (erythroleukemia); Moloney (M-MuLV) by Dr. John B. Moloney (1960)[47] (lymphocytic leukemia); Rauscher MuLV (R-MuLV) by Dr. Frank J. Rauscher (1962)[156] (erythroleukemia); Kirsten MuLV (Ki-MuLV) by Dr. Warner H. Kirsten (1967)[97] (lymphocytic leukemia); and Abelson MuLV (A-MuLV) by Dr. Herbert T. Abelson (1970)[1] (B-cell leukemia).

1.3 The RNA genome

MuLV virions include a dimeric RNA genome[125,198] which is contained within an inner core[98,135]. The structure and organization the MuLV genome is depicted (Figure 1.2). The viral RNA resembles a large mRNA (~8.3 kb) containing a 5' cap[158] and is polyadenylated at the 3' end[73]. The RNA genome begins with a 5' short terminal repeat, R[84,167], followed by a short region which is termed U5[83]. Immediately downstream of the

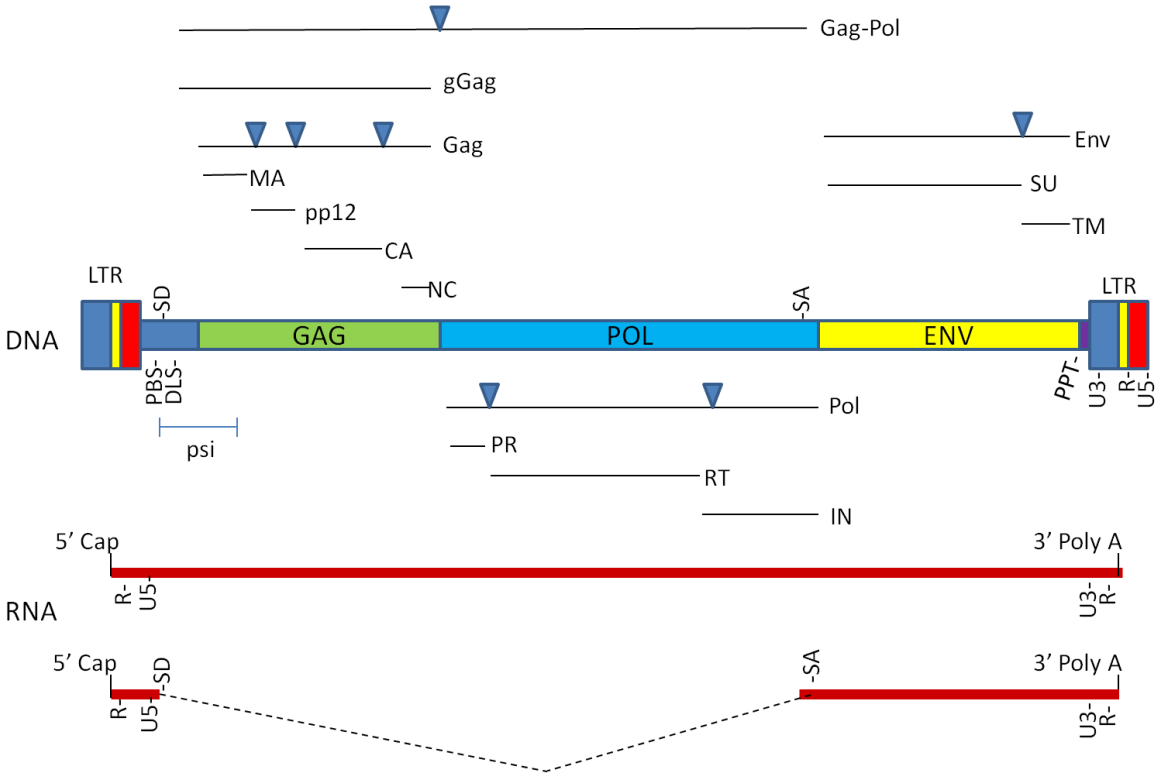

Figure 1.2. MuLV genomic structure and organization. The proviral DNA is flanked by two LTRs, both of which contain a U3, R and U5 region. Following the 5' LTR are signals that function in primer binding (PBS), splicing (SD), dimerization of the RNA genome (DLS), and packaging (psi). Next are the genes 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 (see text).

U5 is the primer binding site (PBS), which is complementary to the cellular $tRNA^{Pro}$ [80] and is the start site for reverse transcription[80]. The splice donor (SD)[170] site is after the PBS but before the packaging signal (psi)[119] and thereby avoids virion incorporation of spliced *env* gene mRNA. The psi region provides the packaging signal for the virion RNA and also contains the dimer linkage site (DLS) that is required for RNA dimerization[131,161]. The next portion of the genome consists of the *gag* gene[6] so named because the major gene product was initially identified as a group specific antigen. This gene, which partially overlaps with psi, encodes a polyprotein that is proteolytically cleaved to yield the structural proteins: matrix (MA, hydrophobic $p15^{gag}$)[7], phosphorylated protein (acidic pp12)[143], capsid (CA, p30)[142], and nucleocapsid (NC, basic p10)[60]. Upstream of the *gag* methionine start site is an alternative leucine start site that encodes another polyprotein, termed glycosylated gag (gGag, $gp80^{gag}$)[54]. gGag is translated in the same reading frame as *gag* but contains an additional eighty-eight amino acids on its N-terminus and appears to undergo distinct post-translational processing compared to the Gag polyprotein[54]. The *gag* gene is followed by the *pol* gene[6] which encodes the viral protease (PR, p14)[146] reverse transcriptase (RT, p80)[5], and integrase (IN, p46)[72]. After the *pol* gene is the splice acceptor (SA) which, with the SD, generates the spliced *env* gene mRNA. The *env* gene encodes the surface glycoprotein (SU, $gp80^{env}$)[93], and transmembrane protein (TM, $p15^{env}$)[87]. Following the *env* gene is the polypurine tract (PPT)[178] which is the initiation site for positive strand DNA synthesis. After the PPT is the 3' (U3)[31] region which contains the transcriptional regulatory elements of the virus. The U3 is followed by the 3' short terminal repeat (R)[84,167]. 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.

1.4 Replication Cycle

1.4.1 Binding and entry

The outer subunit (SU, gp70) of the MuLV envelope protein is responsible for recognition of cellular receptors[85] and the initiation of infection. The viruses are classified into four groups originally based on the infectious host range of the MuLVs and later shown to correspond to the utilization of distinct cell-surface receptors[81,82,114]. After SU/receptor binding, the virus enters the cell through a pH-dependent endocytic

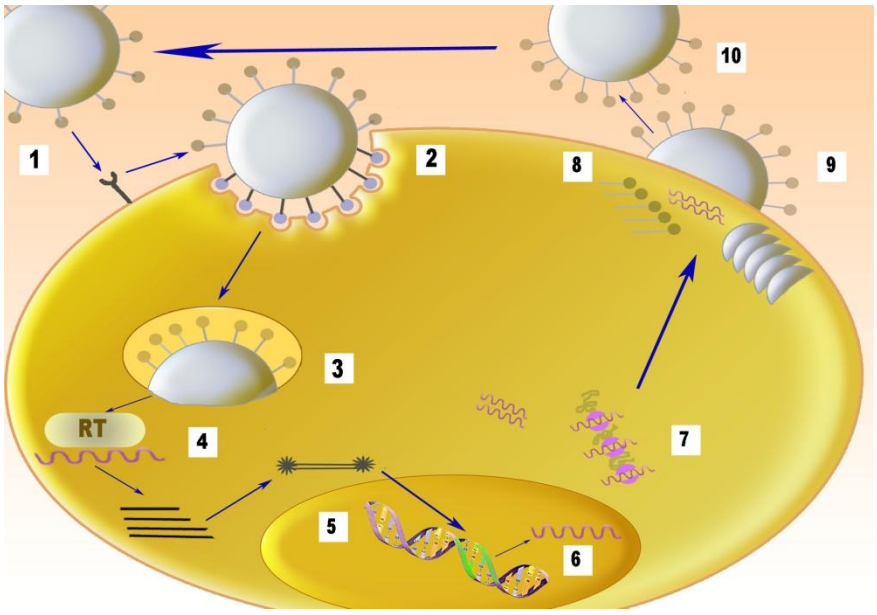

Figure 1.3. Replication cycle of MuLV. **(1)** Binding of viral envelope to cell surface receptor. **(2)** Fusion and entry into the cell. **(3)** Uncoating of virion **(4)** Reverse transcription yielding proviral DNA. **(5)** Preintegration complex enters nucleus, integrates proviral DNA into host 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. Figure credit: Jennifer Kolokithas.

pathway[92] and fuses with the membrane, a process that is dependent on the insertion of a fusion peptide from the transmembrane (TM, $p15^{Env}$) portion of the viral envelope into the cellular membrane[86]. Upon fusion, the virus uncoats (viral core components separate from outer membrane of the virion) and reverse transcription of the RNA genome proceeds in the cytoplasm[98,135].

1.4.2 Reverse transcription

The RNA genome serves as the template for the synthesis of the DNA provirus mediated by the viral RNA-dependent DNA polymerase (RT). As illustrated in Figure 1.2, the RNA genome which contains a 5' cap and is polyadenylated at the 3' end, consists of the R, U5 and PBS at the 5' end of the molecule, followed by the *gag*, *pol* and *env* genes, with the PPT, U3 and R on the 3' end. The steps of reverse transcription are depicted (Intermolecular model, Figure 1.4). First, the $tRNA^{pro}$ binds to the PBS of the genome[84]. From the PBS, using the $tRNA^{Pro}$ as a primer, RT transcribes minus (-) strand DNA in the 3' to 5' direction ending at the R region[5,84]. The RNase H activity degrades the RNA portion of the RNA/DNA hybrid[71]. The newly synthesized DNA (called the minus strand strong stop DNA) coupled to the tRNA primer dissociates from the 5' end of the RNA genome and hybridizes to the complementary 3' R region of the genome[32,33]. Minus strand DNA synthesis continues to the PBS at the 5' end of the molecule while the RNase H activity of RT degrades the RNA genome, except for the PPT region[44,178]. RT uses the PPT RNA as a primer to synthesize (in the 5' to 3' direction) the plus (+) strand of DNA to the PBS carried by the strong stop (-) DNA, using the minus strand as a

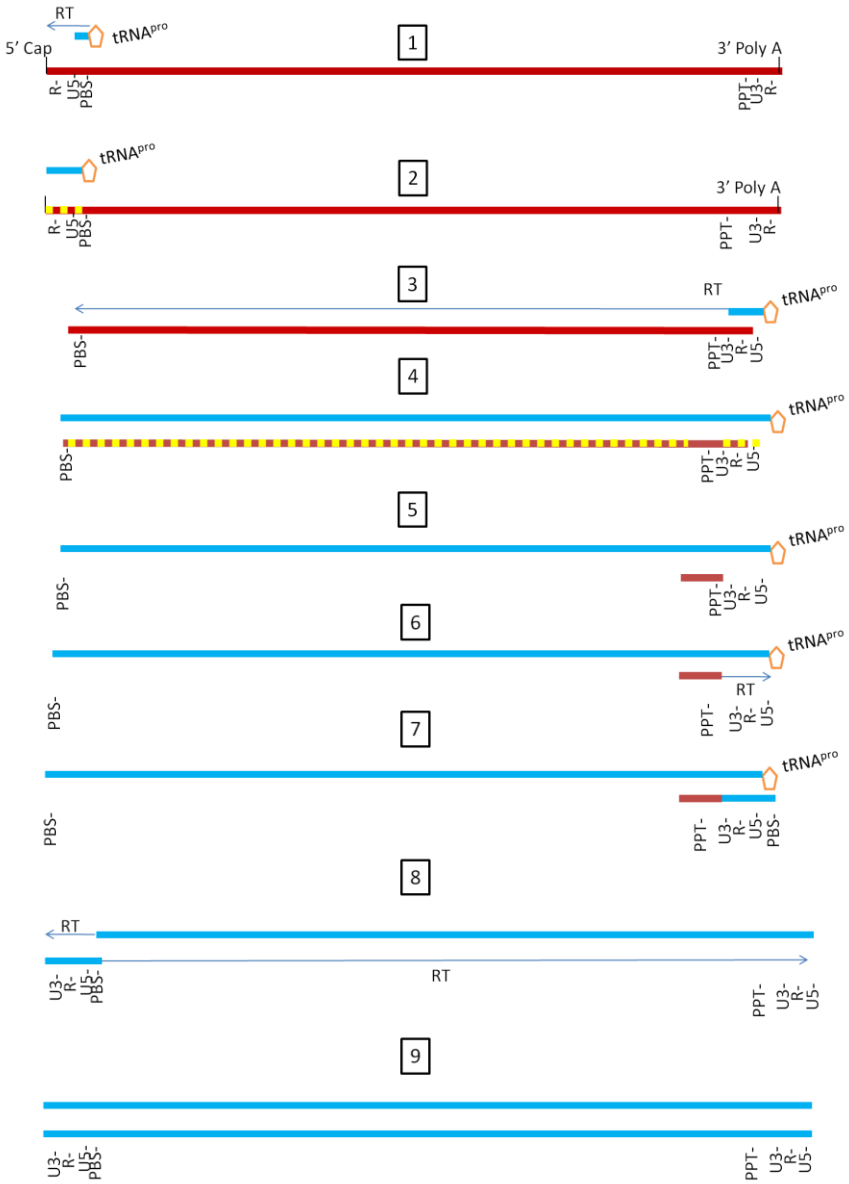

Figure 1.4. Reverse transcription of the viral genome. **1).** The $tRNA^{Pro}$ binds to the PBS of the RNA genome (▬) and RT transcribes minus (-) strand DNA (▬) in the 3' to 5' direction ending at the R region. **2).** RT's RNase H activity (▪ ▪ ▪) degrades the RNA portion of the RNA/DNA hybrid. **3).** 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. Minus strand DNA synthesis continues to the PBS at the 5' end of the molecule while the **4).** RNase H activity of RT degrades the RNA genome, except for the PPT region. **5).** RT uses the PPT RNA as a primer to **6).** 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. **7).** The $tRNA^{Pro}$ primer, PPT RNA and all remaining RNA is removed. **8).** 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.

template[178]. The resultant strand is called strong stop plus (+) strand DNA. The $tRNA^{Pro}$ primer, PPT RNA and all remaining RNA is removed, leaving complementary PBS single stranded DNA at the ends of the newly formed DNA genome [104,195]. The PBS of the (+) strand anneals to the PBS of the minus strand and serves as a primer for RT to further synthesize DNA producing the proviral DNA genome flanked by two long terminal repeats (LTRs) consisting of the U3, R, and U5 regions[104,195].

1.4.3 Integration

The proviral cDNA is incorporated in a nucleoprotein complex termed the pre-integration complex (PIC). The PIC contains the viral proteins IN, CA and pp12[15,72]. The function of the CA and pp12 are unclear, however the IN plays a direct role in integration. The

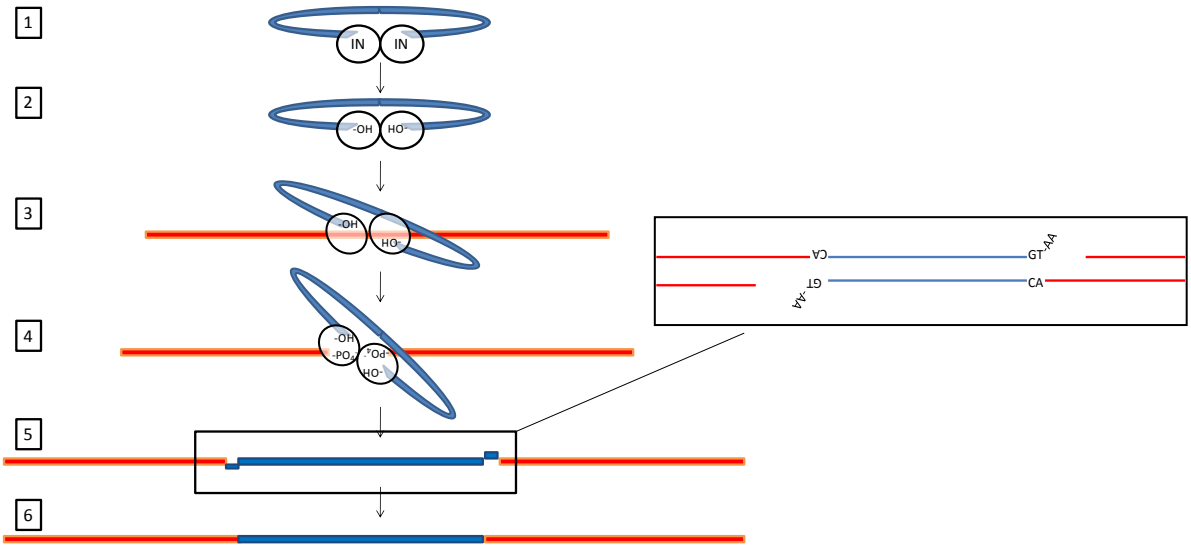

Figure 1.5. Integration of proviral DNA. 1). The proviral cDNA becomes part of the pre-integration complex (PIC). **2).** The 3' ends of the DNA are nicked by IN to produce 3' hydroxyl ends. **3).** The PIC is then shuttled into the nucleus, which in the case of MuLVs only occurs in actively dividing cells during dissolution of the nuclear membrane. **4).** The IN produces a 4-6 bp staggered cut in the host genome which acts as a target for the proviral genomes free hydroxyl groups to integrate into. **5).** The gaps left behind and the non-complementary ends of the proviral genome are **6).** repaired and removed, respectively.

process of integration is depicted (Figure 1.5). At the ends of the MuLV proviral DNA are conserved sequences: 5'-AATG and CATT-3'[16,66]. The 3' ends of the CA are nicked by IN to produce 3' hydroxyl ends[16,66]. The PIC is then shuttled into the nucleus where the IN produces a 6 bp staggered cut in the host genome generating free phosphates which act as nucleophilic targets for the proviral genome's free hydroxyl groups[16,66]. The remaining gaps in the host genome are repaired and the overhanging sequences of the proviral DNA produced by the IN are removed[16,66].

1.4.4 Transcription of the provirus

The proviral sequence from the host genome is transcribed as a cellular gene by RNA polymerase II and mediated by transcriptional control elements contained within the proviral LTR[42,76]. The 5' LTR (specifically U3)[39,40] encodes promoter sequences (TATA box) that are recognized by cellular transcription factors leading to the synthesis of the RNA viral genome[76], which is 5' capped and 3' polyadenylated. The start of transcription is the first nucleotide of the 5' R and the polyadenylation signal is near the end of the 3' R[76]. Some of the full-length RNA genome is spliced to produce *env* mRNA[57]. Both the spliced and unspliced mRNAs serve as templates for translation of viral proteins. Unspliced RNA is also transported to the plasma membrane where it is incorporated into budding virions.

1.4.5 Translation and maturation

The full-length unspliced mRNA is translated to the gGag, Gag as well as the Gag-Pol polyproteins while the spliced mRNA is translated to the Env polyproteins[57]. The Gag polyprotein is targeted to the inner cell membrane by myristoylation of the amino terminus of the polyprotein. The Env polyprotein is targeted to the cell

endoplasmic reticulum and ultimately to the cellular membrane through glycosylation. The polyproteins are further processed during virion assembly by the viral protease, which exists as a zymogen on the Gag-Pol precursor[21]. The protease is activated by dimerization of the Gag-Pol precursor and the cleavage of the protease from the Gag-Pol precursors occurs in *trans*. The Gag polyprotein (NH_2-p15-pp12-p30-p10-COOH)[8] is cleaved to produce the 4 major components of the internal structure of the virion. p15 (or MA) has a neutral charge and is the most hydrophobic protein of the virus[7]. It is located between the lipid bilayer and the core of the virion and retains the myristoyl group acquired before cleavage of the Gag polyprotein. In the context of the polyprotein MA functions to align Gag at the plasma membrane during maturation[157]. The only phosphorylated protein in the virion is the acidic pp12. It is thought to be localized to the core, binds with low affinity to the RNA genome and may serve some regulatory role[143]. The icosahedral shell of the viral core is composed of the neutral p30 (or CA)[142] which is the major structural protein of the virus. Bound to the genomic RNA is the basic p10 (or NC), which is a major component of the ribonuclear protein complex[60]. It has very high affinity to the packaging signal and the DLS of the RNA genome[60]. The Pol polyprotein is generated by cleavage of the Gag-Pol polyprotein and consists of NH_2-p14-p80-p46-COOH. The protease (p14, PR) is responsible for cleavage of the polyproteins into their final, mature forms during budding from the cell[146]. Reverse transcriptase (RT, p80) is packaged into the core of the virion and is responsible for reverse transcription of the RNA genome upon infection of a target cell[8]. Integrase (IN, p46) is also incorporated in the core of the virion and is responsible for integrating the proviral DNA that results from reverse transcription of the viral RNA genome into the host DNA[72]. The Env polyprotein

is produced from a spliced viral mRNA and is organized as NH_2-gp70-p15^{Env}-COOH[6]. The glycosylated gp70 is the surface subunit (SU) of the envelope protein and is localized on the lipid membrane of the virion[93]. It is responsible for the virus's host-range and binding to the cellular receptor[93]. The transmembrane (TM) portion of the envelope is responsible for anchoring the SU to the virion by disulfide linkage and also contains a fusion peptide that is required for the virus to fuse to the membrane of a target cell[87]. The viral RNA is packaged as a dimer along with the tRNA^{pro} [98]. Immature virions start to bud from the cell while the protease is continuing to cleave the Gag polyprotein.[146].

1.5 Pathology and the 5 prime leader sequence

Many retroviruses have been discovered because of their ability to cause disease in animals[1,47,62,70,75,97,156]. Various strains of MuLV were discovered because of their pathogenicity *in vivo*[1,47,62,70,75,97,156]. Many studies of these MuLVs using mutational analyses or chimeric viruses have been aimed at determining the viral components responsible for pathogenesis and *in vivo* dissemination. Figure 1.6 lists some of the functions of the 5' leader sequence discerned from such studies. Among these are studies of chimeric viruses between F-MuLV and M-MuLV. F-MuLV induces early hemolytic anemia followed by erythroleukemia[176] and M-MuLV causes lymphocytic leukemia[116]. Molecular clones between M- and F-MuLV in which the LTRs of F-MuLV and M-MuLV were exchanged resulted in an alteration in pathology. M-MuLV carrying the LTR of F-MuLV induced erythroleukemia while F-MuLV carrying the LTR of M-MuLV induced lymphocytic leukemia[24]. Another study revealed that the 5' U5-Gag-Pol region was important for viral dissemination and disease *in vivo,* but not for replication *in vitro*[174]. Later studies specifically mapped attenuation of disease using mutant F-MuLV

constructs. A mutation abrogating the expression of gGag attenuated disease *in vivo* but the mutant was still able to replicate similarly to the wild-type virus in cell culture[36]. It was also found that 6-7 weeks after infection the majority of the gGag mutants isolated had reverted back to expressing gGag, indicating that the gGag mutations were under strong selection *in vivo*[36]. CasBrE-MuLV is another ecotropic MuLV that was isolated from mice and has been extensively studied because it causes a severe neurodegenerative disease[63-65,127,150-153]. Constructs replacing the 5' leader (U5-Gag) sequence of CasBrE with F-MuLV (CasFrE) showed a rapid acceleration of disease[150]. The sequences responsible for this acceleration mapped to the N-terminus of gGag (construct

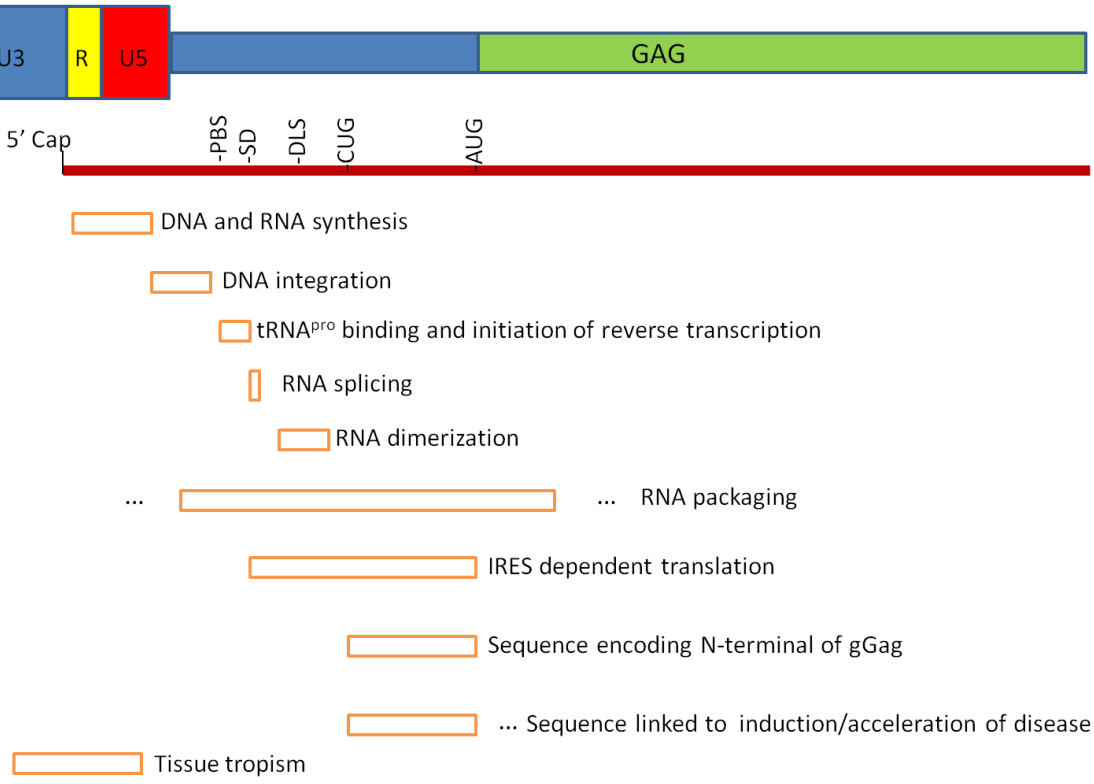

Figure 1.6. The 5' leader sequence of MuLV. The 5' leader sequence of MuLV has been linked to many functions that are essential for retroviral replication and disease induction. The boxes (▭) represent the span of the sequence that has been identified to have a certain function in the retroviral life cycle.

CasFrKP)[153]. A mutant that abrogated the expression of gGag (CasFr-3+4) was unable to induce neurodegenerative disease and showed reduced dissemination *in vivo*[153]. The mutant virus also showed revertants after infection, suggesting again a strong selective pressure for gGag expression[153]. It is noteworthy that the revertants did not restore the original gGag but rather were second site reversions which introduced new initiation codons downstream of the original gGag start site. Nonetheless, both the replicative and pathogenic functions of the mutant viruses were restored. When mice were inoculated with revertants the viruses replicated and induced rapid disease similar to the wild-type virus[151].

1.5.1 Glycosylated Gag

All exogenous MuLVs encode an alternate form of the Gag polyprotein that is glycosylated (gGag)[54,112,154]. gGag was first recognized on AKR MuLV-induced tumor cells as an antigen[112]. The glycosylated protein is translated from the same viral mRNA as Gag but uses a CUG start codon that is upstream of the AUG start codon for Gag[54,65,133]. The coding region of gGag also overlaps with those required for RNA packaging, ribosome binding, IRES-dependent translation and pathological determinants. The alternative start codon for gGag results in an additional 88 N-terminal amino acids on the Gag polyprotein. These additional amino acids contain a signal peptide that targets gGag to the rough endoplasmic reticulum for glycosylation where it is then exported to the cell membrane[65,147]. The proposed glycosylation sites exist in the p15 and p30 regions of the polyprotein. gGag has been reported to be cleaved near the end of the CA region, producing two products that migrate between 55kD and 45 kD on an SDS-PAGE gel. Some of the C-terminal cleavage product of gGag is secreted while a pool of the N-

terminal portion adopts a type II integral membrane configuration(N_{cyto}, C_{exo})[65,147]. Some gGag is incorporated into the virion, but the specific amount has yet to be determined[63]. The levels of Gag and gGag protein expressed in an infected cell are similar[65]. Mutant viruses that lack gGag are able to replicate efficiently *in vitro*, however, *in vivo* gGag mutants are severely limited in their ability to replicate[63,65,121]. Reversion is strongly selected for *in vivo* suggesting an important replicative role for gGag. The role of the gGag protein is unknown in viral replication, though it has been suggested that it is required for efficient budding and release *in vitro*[121].

1.6 Restriction Factors

All eukaryotes carry mobile genetic elements in their genomes[177]. Retroelements are in the family of mobile DNA sequences that use RNA intermediates and reverse transcriptase to insert into a new point in the genome. In many species these elements make up a large amount of the total amount of genetic content (10% in humans)[177] and represent insertion events into host genomes caused by retroviral infections over the course of evolution[177]. In some instances these mobile elements can insert themselves into regions of the genome that affect the control of cellular growth[177] which obviously could be detrimental to the survival of the host. Exogenous retroviruses can also infect and insert themselves into the genome of their host, in many cases causing proliferative diseases[37,38,117,171,194]. Over evolutionary time, many species have evolved mechanisms to limit or restrict active retroviral replication[9,69]. In many cases retroviral expression is suppressed by methylation of promoter sequences[9,69], however, there are also distinct factors that function to restrict retroviral replication. These 'restriction factors' operate on many different aspects of the retroviral life cycle ranging from viral receptor blockade

to viral RNA degradation[9,69].

1.6.1 Fv1 restriction

One of the first restriction factors identified was Fv1 (Friend virus susceptibility 1) in mice[118]. It was found to control susceptibility to F-MuLV disease, but has since been shown to affect other MuLVs. There are two different alleles for the Fv1 gene, $Fv1^b$ (in Balb/c mice) and $Fv1^n$ (in NIH/swiss mice). N-tropic viruses replicate efficiently $Fv1^n$ mice but inefficiently in $Fv1^b$ mice. Conversely, B-tropic viruses replicate efficiently in $Fv1^b$ mice but not in $Fv1^n$ mice. Crosses between $Fv1^n$ and $Fv1^b$ mice are resistant to infection by N- or B-tropic viruses, while some viruses are NB-tropic and cannot be blocked by either Fv1 allele[148]. Viral tropism is determined by one amino acid (residue 110) in the capsid (CA)[103]. The restriction mechanism of Fv1, which is thought to be related to an endogenous retroviral gag protein, is thought to involve prevention of the preintegration complex (PIC) from entering the nucleus, however the exact mechanism has yet to be fully elucidated[155]. Several human cell lines infected with N-tropic MuLVs encapsulated by the vesicular stomatitis virus-G envelope protein are also resistant to infection, but do not carry $Fv1^b$. Interestingly, the viral tropism in these human cells was determined by the same residue in the CA (residue #110) of MuLVs. This resistance was initially referred to as Ref1 (Restriction Factor 1; human antiretroviral activity related to Fv1)[190,191] and was observed in cells of many different species. Through cDNA expression library screening, the activity of Ref1 was connected to a protein, TRIM5α[184]. The mechanism of this restriction is currently under investigation but is thought to be similar to that of Fv1.

1.6.2 Fv4 restriction

After the identification of Fv1, another gene, Fv4, was found to restrict certain retroviruses in Japanese wild mice[91,137]. The ecotropic MuLVs (able to infect only mice) use the murine cationic amino acid transporter (mCAT) as their cell-surface receptor for infection. Mice with mCAT and Fv4, however, are resistant to infection by ecotropic MuLVs. Fv4 was found to be a defective envelope (*env*) gene of a provirus expressed at the cell surface[88,89]. The Fv4 gene product is able to bind directly to mCAT, preventing ecotropic MuLV infection by receptor blockade[89].

1.6.3 HIV-1, Vif, and the discovery of APOBEC3

Human Immunodeficiency virus-1 (HIV-1) has also been shown to be restricted to certain cell types. Evidence of this first emerged with the study of an accessary protein of HIV-1, virion infectivity factor (Vif). Viruses lacking Vif (HIV-1 ΔVif) were unable to spread in $CD4^+$ T cells or macrophages as well as some transformed T-cell lines (nonpermissive cells). However, these same viruses were able to spread in other T-cell lines (SupT1 and Jurkat) and in permissive, nonhematopoietic cells (COS, HeLa, and 293T)[180,197]. One important observation made in these experiments was that nonpermissive cells were able to produce HIV-1 ΔVif virions, but progeny viruses from these cells were unable to replicate in any target cell line regardless of whether it was permissive or nonpermissive. One possible explanation for this phenomenon was the existence of a host factor able to inhibit viral replication in the absence of Vif but abrogated when Vif was expressed. Such a factor was eventually identified by using subtractive hybridization techniques with two cell lines: CEM-S (permissive) and CEM-SS (nonpermissive). A gene was cloned that could convert permissive into nonpermissive cells upon transfection and expression. This clone was CEM15[172], which

was later found to be a member of the apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like family termed human APOBEC3G (hA3G). The expression of hA3G correlated with the permissiveness of cell lines. Furthermore, virions from nonpermissive cells infected with Vif-deficient HIV-1 were shown to incorporate hA3G accounting for their inability to replicate efficiently in either permissive or non-permissive target cell lines.

1.6.4 Human APOBEC3G

hA3G is a member of a family of cytidine deaminases that is restricted to certain tissues that exhibit both RNA and DNA editing activity[189]. Members of this family include activation induced deaminase (AID) and APOBEC1-4, with APOBEC3 having several isoforms designated A through H[35]. These enzymes catalyze the deamination of the C4 position of the cytidine base. APOBEC1 has been found to regulate cholesterol and lipid metabolism[129]. Another family member, AID, is required for somatic hypermutation and class switch recombination in germinal center B cells[130].

A member of the APOBEC3 family, hA3G, has been identified as an inhibitor of retroviral replication. A principal mechanism by which this occurs is through cytidine deamination of single stranded (ss) DNA transcripts during reverse transcription. The inhibitory activity of hA3G is dependent on two catalytic domains (CDs). CD1 mediates RNA[23] binding and incorporation into the virion and CD2 functions as the deaminase active site[134]. Encapsidation of hA3G requires the nucleocapsid (NC) region of HIV-1 Gag[186], 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[200]. The deamination-dependent mechanism for hA3G is depicted (Figure 1.7). In the target cell, virion-associated hA3G

introduces dC to dU mutations in the minus strand viral DNA formed during reverse transcription[185]. These DNA strands may be degraded by apurinic-apyrimidinic endonuclease or uracil DNA glycosylase[201]. 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.

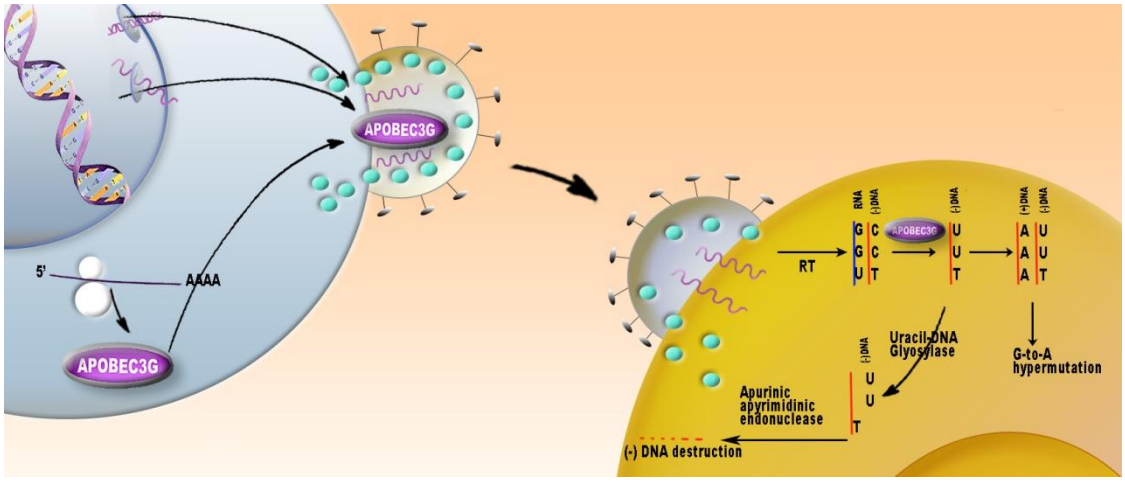

Figure 1.7. APOBEC3 and G-to-A hypermutation activity. During assembly at the cellular inner membrane, APOBEC3 is incorporated into budding virions. Upon a subsequent infection of a target cell, APOBEC3 exerts its deaminase activity during reverse transcription. The newly formed transcripts are deaminated. This can lead to either integration of the mutated transcripts or recognition of the transcripts by cellular enzymes that lead to transcript degradation. Figure credit: Jennifer Kolokithas.

Furthermore, hA3G appears to inhibit viral replication through deaminase-independent mechanisms. One mechanism appears to operate through binding RNA via CD1, sterically blocking the $tRNA^{Lys3}$ primer binding during reverse transcription initiation[77]. Another mechanism has been reported to be independent of the catalytic domains and is mediated by binding of hA3G to the HIV integrase, further inhibiting the virus's ability to replicate[124]. The promiscuity of the deaminase activity of hA3G requires it to be

sequestered to the cytoplasm[199]. Cellular localization, however, is not the only mechanism involved in controlling hA3G's activity. It associates with a high molecular mass (HMM) ribonucleoprotein (RNP) complex in resting $CD4^+$ T cells in the thymus[29], where hA3G bound to RNA is inactive[29]. In contrast, in circulating $CD4^+$ T cells hA3G associates with a low molecular mass (LMM) RNP complex where it is active. Mitogens control the switch between HMM and LMM complexes[183], though HMM complexes can be artificially changed to LMM complexes by treatment with RNase A[29]. LMM hA3G is not recognized by Vif, therefore LMM hA3G can restrict entering viruses whether or not they encode Vif. The identification of HMM and LMM complexes was the first evidence that hA3G can affect HIV-1 replication without being incorporated into the virion[29] though the authors claiming this recently retracted their study. Unlike virion-associated hA3G, the antiviral activity of LMM hA3G does not involve DNA editing, but rather inhibits the production of reverse transcription product (Figure 1.8). Neither LMM- nor HMM-associated hA3G is incorporated into virions. Only newly synthesized hA3G is

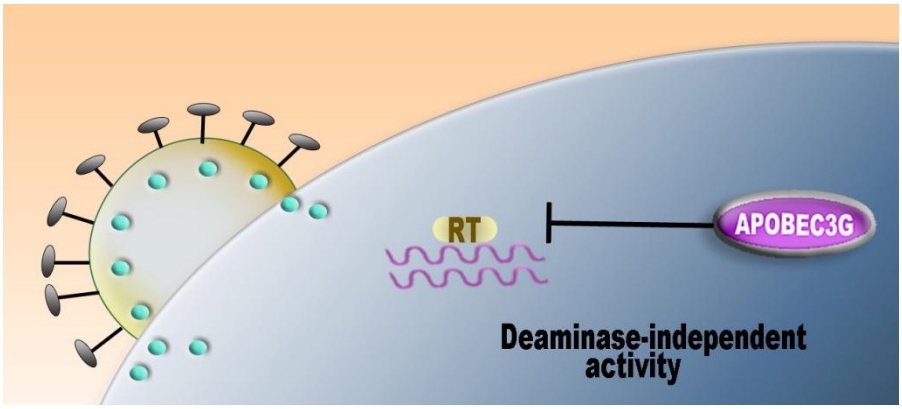

Figure 1.8. Deaminase-independent activity. APOBEC3 proteins have been found also to inhibit retroviral replication through deaminase-independent activity, such as blocking reverse transcription or integration. Figure credit: Jennifer Kolokithas.

incorporated into virions, but is enzymatically inactive[179] because of interactions with the HIV RNA genome and Gag proteins, similar to what is observed in HMM complexes[179]. As noted above, the binding of hA3G to the genome impairs the initiation of reverse transcription[179]. Once reverse transcription begins, RNase H degradation of the RNA releases hA3G allowing cytidine deamination of the minus-strand DNA substrate produced from reverse transcription[179].

1.6.5 Murine APOBEC3 and Murine Leukemia viruses

HIV-1 encodes a gene product, Vif, which counteracts the antiviral effects of hA3G. Vif binds to the N-terminus of hA3G and recruits an ubiquitin ligase complex that marks hA3G for destruction in the proteasome[34] (Figure 1.9). Vif has also been shown to impair the translation of hA3G mRNA[128] and prevent encapsidation by binding to

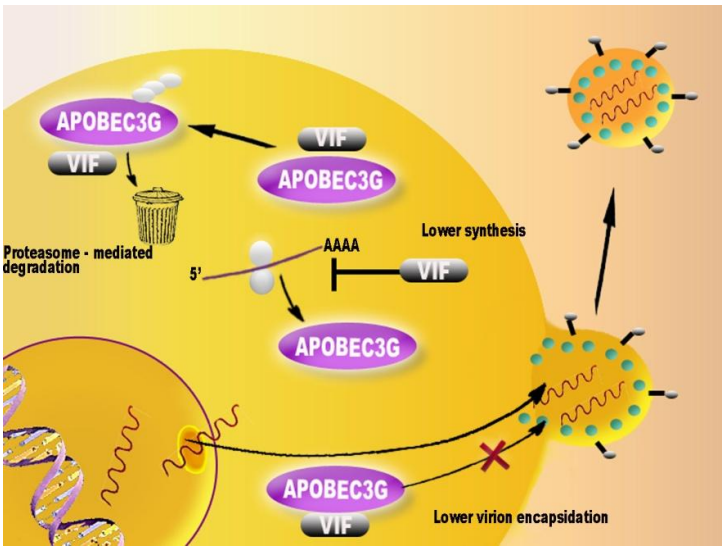

Figure 1.9. APOBEC3 and Vif. HIV encodes an accessory protein known as Vif, which can bind to APOBEC3 during retroviral assembly and lead to its proteasome-dependent degradation. This allows the HIV virions to prevent encapsidation and continue to replicate. Figure credit: Jennifer Kolokithas.

hA3G[141]. Similar to HIV and hA3G, MuLVs have evolved a mechanism(s) to evade the action of mA3 and are largely insensitive to its actions[13,18,43,100,110,145,162], however, MuLVs do not encode a Vif like accessory protein. While there are 7 family members of

A3 in humans, mice contain only one[26]. mA3 exhibits a number of differences from hA3G. The deaminase activity of mA3 resides in the CD1 while the RNA binding and encapsulation into the virion resides in CD2[78]. As noted above the converse is true of hA3G. Unlike hA3G, two splice variants (delta-exon 2 and delta-exon 5) have been identified for mA3[159,164]. Different mouse strains express different amounts of each variant as well as the full length protein[159,164]. Different inhibitory effects of these variants on the *in vivo* replication of certain MuLVs have been reported but remain a subject of debate[159,164]. Recent studies have identified mA3 as Rfv3[164]; a gene involved 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)[164]. The mechanism(s) by which mA3 affects replication of the Friend virus complex remain unclear and warrant further investigation. Deaminase-dependent and -independent mechanisms have both been associated with mA3, though the exact nature of these mechanisms remains unclear[26,27,164].

It is unknown how exogenous MuLVs largely resist mA3 activity. It is of note that APOBEC3 family members from both humans and mice inhibit endogenous LTR retrotransposons/retroviruses. This includes Human Endogenous Retroviruses (HERVs), murine intracisternal A-particle (IAP), and MusD sequences[12,49,50]. Interestingly, one major difference between endogenous and exogenous (MuLVs) retroviruses in the mouse is the absence of an alternate initiation sequence in endogenous retroviruses for gGag.

1.7 Significance

Mice as well as other mammals contain a very large number of endogenous retroviruses comprising about 10% of the genome[177]. Transcripts of these viruses are expressed in a very controlled fashion throughout life and likely are involved in a number of physiological processes[49,50]. Endogenous retrovirus replication may lead to insertional mutagenesis as well as inappropriate expression of retroviral proteins, thus it is likely that the host has developed mechanisms to control such replication. The evolution of several restriction factors that act post-transcriptionally, such as the APOBECs, may reflect this necessity. In contrast to replication competent exogenous MuLVs, all of which encode gGag, no endogenous proviruses encode this protein and are controlled in part by mA3[49-51,90,113]. Mutant viruses that lack gGag are able to replicate efficiently *in vitro* but are severely restricted *in vivo*[63,65,121]. These observations are consistent with the possibility that gGag may serve to evade the action of mA3. Some reports in the literature suggest a direct interaction of mA3 with gag encoded proteins[110,123,162,188], however, a direct interaction between mA3 and gGag has not been investigated.

Elucidation of the mechanism(s) by which MuLVs evade the action of mA3 may provide additional insight into the means by which mammals evade the action of retroviruses. Furthermore, it is has recently been reported that exogenous MuLVs that are resistant to mA3, pseudotype endogenous viruses and allow their transfer to cells of other species[52,53,55,56]. This may be of particularly interest in light of recent reports indicating cross-species retroviral infections from mouse to humans[120,166,193].

Chapter 2- Experimental rationale and pilot experiments.

The effects of hA3G on HIV replication have been tested by many experimental methods, each with unique advantages and disadvantages. Many methods to examine the effect of mA3 on MuLV replication have been adopted from studies with HIV[2,14,18,30,43,90,100,101,110,149,162,203]. In addition, other approaches examining the effects of mA3 and gGag on MuLV replication have been developed. This chapter discusses the rationale for the methods used in experiments presented throughout this dissertation.

2.1 Selection and generation of cell types

At the onset of these studies nearly all reports dealing with the effects of mA3 on MuLV replication examined the effects of transfection of MuLV and mA3 expression vectors in the 293T human embryonic kidney stem cell line transformed with the SV40 large T antigen[2,18,43,110,162,188,203]. There are a number of potential difficulties with this approach. First, it is unclear if protein processing in this human cell line parallels that observed in mouse cells[202]. Further, because of variation in transfection efficiency, the expression of these vectors may vary from cell to cell and from experiment to experiment. In addition, this approach is limited to examining virion-associated mA3 rather than the effect of cellular mA3 on virus infection. In this regard, primary bone marrow-derived dendritic cells from mA3 knockout (KO) mice are more susceptible to infection by MuLVs than their mA3-expressing wild-type (wt) counterparts[123] . To circumvent some of these difficulties stable mouse cell lines expressing mA3 were developed. The lines chosen were NIH3T3 (3T3) and *Mus dunni* cells which do not constitutively express mA3 as determined by quantitative PCR (qPCR) analyses (data not shown).

To generate stable cell lines expressing mA3, a pcDNA3 plasmid encoding the full length mA3 derived from the Balb/c mouse strain and tagged at the C-terminus with hemagglutinin (HA)[18] was used to transfect the cell lines which were subsequently selected in geneticin (G418). As no suitable antibody to mA3 was available, experiments involving immunoblotting and immunoprecipitation required the use of a tagged version of mA3.

2.2 Selection of MuLVs

To study the affects of gGag on viral replication in mA3 expressing cells, the CasFrKP and CasFr-3+4 MuLVs were chosen. CasFrKP ($gGag^+$) is a MuLV derived from the wild mouse ecotropic virus CasBrE and contains a short sequence of the F-MuLV, FB29, which includes the initiation site of the gGag protein[152]. CasFr-3+4 ($gGag^-$) is a derivative of CasFrKP in which two mutations have been introduced to disrupt the initiation site of gGag at the -3 and +4 positions of the Kozak consensus sequence[153]. These MuLVs have been studied extensively to determine the role of gGag in pathogenesis as well as in *in vivo* and *in vitro* replication[30,58,63-65,151,153]. It has been established using gGag-specific peptide antibodies that gGag is expressed in infected cells and that an N-terminal cleavage product of gGag is incorporated into the virion[63].

2.3 *In vitro* infectivity assays

Most studies that have examined the effects of human APOBEC3G (hA3G) on replication have focused on the action of virion-associated hA3G upon infection of naïve target cells[17,20,22,23,34,77,96,100,124,126,128,132,179]. However, the results of one study suggest that cellular hA3g can inhibit infection by HIV virions early after entry[29]. It was of interest to study the effects of both cellular mA3 and virion-associated mA3 on MuLV

replication and determine if either exerted its effect in a gGag-dependent manner. In order to examine the effects of cellular mA3 on viral replication, $gGag^+$ or $gGag^-$ viruses harvested from cell cultures that did not express mA3 were used to infect both cells that express and cells that do not express mA3. Infectivity was quantified using either a focal immunofluorescence assay (FIA) or assays in which a reporter gene was expressed. To analyze the effects of virion-associated mA3 on replication, $gGag^+$ or $gGag^-$ viruses were harvested from cell cultures expressing mA3. The mA3-containing viruses were compared to viruses harvested from cells that did not express mA3 by their infectivity of *Mus dunni* cells. Infectivity was quantified using either the FIA or reporter assays and the data was normalized by RT activity of the virions in the supernatant as well as p30 quantification in order to deduce the specific infectivities of the viruses.

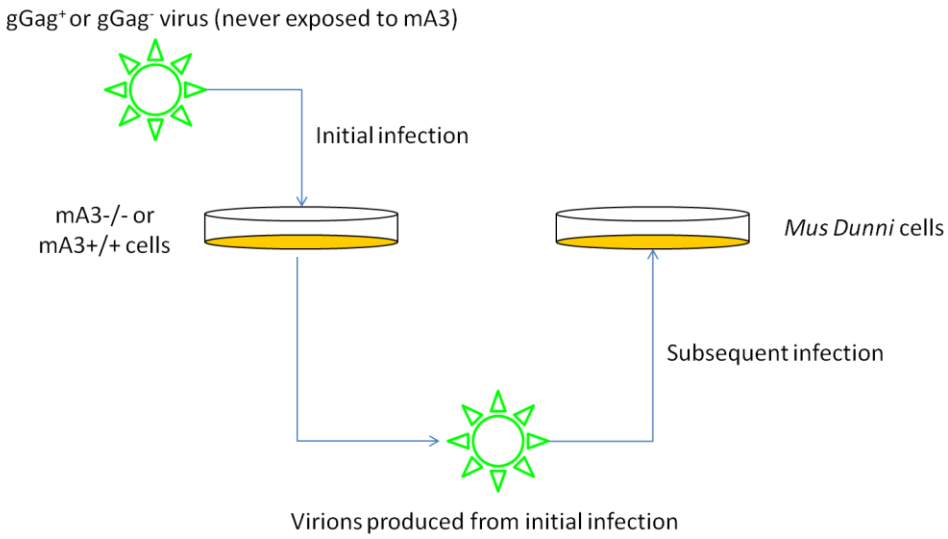

Figure 2.1. *In vitro* assay system. To test the effect of cellular mA3 on MuLV replication, $gGag^+$ or gGag- MuLVs were used to infect cells expressing or not expressing mA3 (Initial infection). To test the effect of virion-associated mA3 on MuLV replication, $gGag^+$ or gGag- MuLVs produced from cells expressing or not expressing mA3 (Initial infection) were used to infect *Mus dunni* cells (subsequent infection).

2.3.1 Focal immunofluorescence assay

This assay[175] exploits the display of virally encoded proteins on the surface of infected cells. Using antibodies specifically reactive to the viral proteins, infected cells decorated with the antibody can be detected either by direct conjugation of a fluorescent dye to the antibody or, more commonly, by detection using a fluorescently conjugated second antibody directed at immunoglobulin (Ig). Sparsely seeded cells are exposed to the virus and allowed to grow to confluence. An infectious event can be quantified by counting focal areas of infected cells that arise either from virus spread or by replication of an initially infected cell.

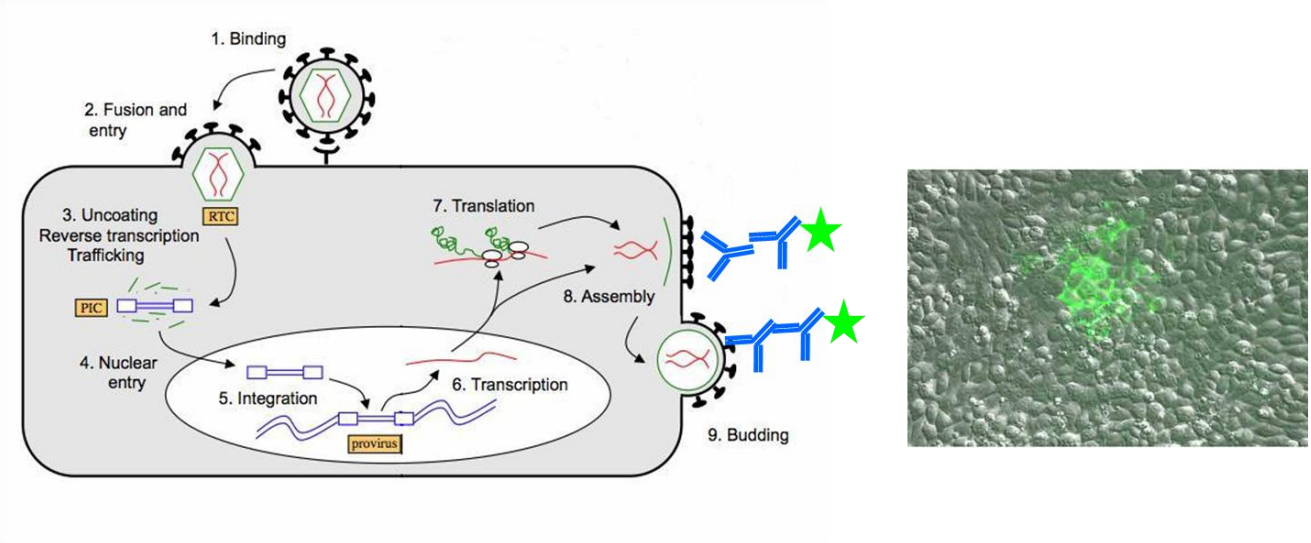

Figure 2.2. Focal immunofluorescence assay. The FIA methodology takes advantage of late stages in the viral replication cycle. During assembly of virions at the cell surface, infected cells can be detected by labeling viral specific antigens with antibodies. This can be done with directly conjugated antibodies specific for viral antigens or with labeled secondary antibodies specific to the Ig that was used to bind to the viral antigen. Inset: A focus of infected cells. Figure adapted from http://www.nimr.mrc.ac.uk/research/kate-bishop by Kate Bishop, used with permission.

Initial studies using the FIA examined the effects of cellular mA3 on infectivity of MuLVs and on cell-free passage of viruses on cell lines. The effects of cellular mA3 were performed by comparing virus titers in normal cell lines and in cell lines expressing mA3. Both 3T3 cells and *Mus dunni* cells were examined and in both cells lines a significant reduction in infectivity was observed with the gGag-deficient virus in cells expressing mA3 (Figure 2.3).

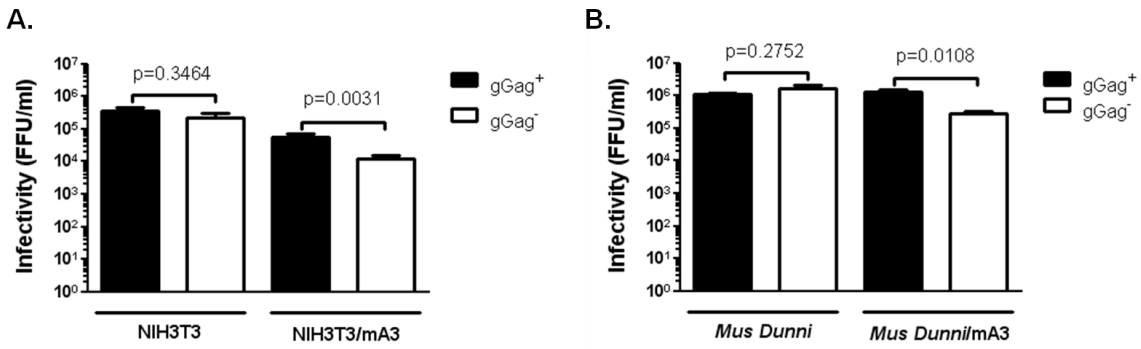

Figure 2.3. Cellular mA3 and infectivity. **A)**. 3T3 or 3T3/mA3 cells were infected with gGag$^+$ or gGag-. When the cells reached confluence (~4 days), infectivity was quantified using the FIA with mAb 667 (envelope specific) and a fluorescently labeled secondary antibody. **B).** *Mus dunni* or *Mus dunni*/mA3 cells were infected with gGag$^+$ or gGag- MuLVs and assayed as described in **A**).

2.3.2 Passaging of viruses on mA3 cells

In vivo studies of CasFr-3+4 as well as other gGag-negative MuLVs revealed a marked selection for second site revertants that expressed gGag[121,151]. Considering that the initial experiments suggested that gGag facilitates replication in the presence of mA3, it is possible that mA3 is an important component of the *in vivo* selection for gGag and that similar revertants might be generated in the *in vitro* system. If revertants were generated *in vitro*, one might expect them to persist in the mA3-expressing lines after

extended passage. To examine this possibility, serial cell-free passages of the gGag$^+$ and gGag$^-$ viruses were performed on normal and on mA3-expressing cell lines.

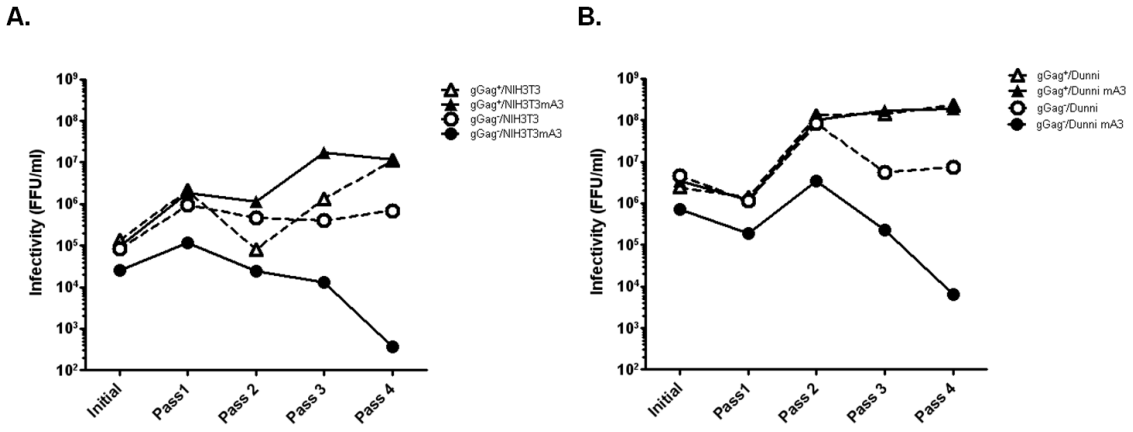

Figure 2.4. Cell free serial passage of gGag$^+$ and gGag$^-$ on mA3 -/- and mA3 +/+ cells. **A)**. 3T3 and 3T3/mA3 cells were infected with each virus. 56 hours post infection the supernatants were transferred onto uninfected 3T3 and 3T3/mA3 cells. At the time of each transfer (pass) the supernatants were also transferred to *Mus Dunni* cells and assayed for infectivity. **B).** *Mus dunni* (Dunni) *Mus dunni*/mA3 (Dunni mA3) cells were infected with each virus. 56 hours post infection the supernatants were transferred onto uninfected *Mus Dunni* and *Mus dunni*/mA3 cells. At the time of each transfer (pass) the supernatants were also transferred to *Mus dunni* cells and assayed for infectivity. Each experiment was repeated twice with three replicates.

The passage experiments revealed no evidence of selection for effective replication in mA3 expressing cells. Indeed, in cells expressing mA3 the gGag$^-$ MuLV was virtually undetectable by passage four. In contrast, in passage experiments using cells not expressing mA3, the gGag$^-$ virus persisted albeit at somewhat lower levels than the wild-type virus.

2.3.3 Reporter assays

The FIA requires amplification of the initially infected cells, either by virus spread or cell replication, for efficient detection of focal infections[175]. The foci of the gGag$^-$ virus on mA3 cells were smaller and more difficult to quantify than those observed

on cells devoid of mA3. Thus the difference in the number of foci scored in the FIA may have been due to differences in the initial infection of the cells or, alternatively, inhibition of virus spread occurring subsequent to infection. Infectivities of retroviruses are frequently quantified using retroviral vectors encoding a gene, such as alkaline phosphatase or β-galactosidase, whose expression is detectable in the infected target cells (reporter vectors). Reporter vectors generally include retroviral LTRs, promoters, and a retroviral packaging signal in addition to the reporter gene. They resemble a viral genome but do not encode viral structural proteins, thus infection by virions containing a reporter vector does not result in a productive infection capable of spread subsequent to the initial infection. Foci scored using these viruses are developed by cell division and reflect the number of initial infectious events.

There are a number of procedures to generate retroviruses that have packaged reporter vectors. The viruses can be generated by co-transfection of plasmids encoding virus structural proteins with plasmids encoding a reporter vector. Alternatively, cell lines harboring a reporter genome can be infected with a MuLV resulting in the release of virions which have incorporated the reporter genome as well as the wild-type viral genome. Two reporter genomes were utilized in these studies: LAPSN (Clontech Laboratories, Inc.) which encodes alkaline phosphatase and is expressed in the cytoplasm of infected cells and G1nβgSVNa[92] which encodes β-galactosidase expressed in the cell nucleus. Assays using either of these vectors yielded results similar to the FIA experiments suggesting inhibition of infectivity by cellular mA3 in a gGag-dependent manner.

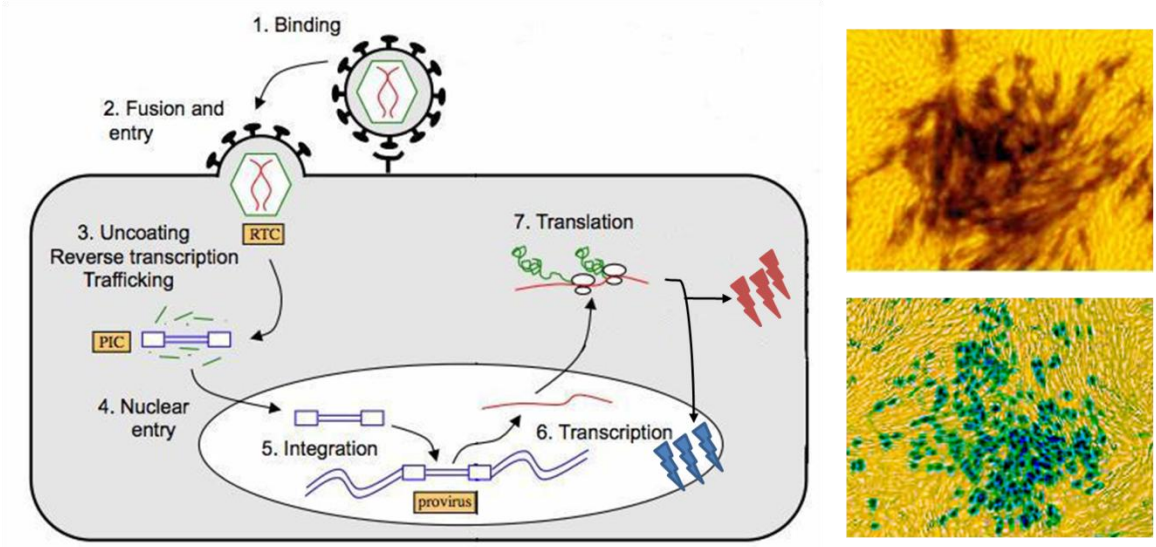

Figure 2.5. Reporter gene assay. The reporter gene assay allows for the detection of infected cells through a non productive infection. The reporter genome is transcribed and integrated into the host genome, but does not encode viral structural proteins, and therefore expresses only the reporter enzymes. In the case of the LAPSN reporter genome, alkaline phosphatase is expressed and activity is detected in the cytoplasm. G1nβgSVNa encodes β-galactosidase that is expressed in the cell nucleus. Inset: Top: A focus of cells infected with MuLVs harboring the LAPSN reporter genome. Bottom: A focus of cells infected with MuLVs harboring the G1nβgSVNa reporter genome. Figure adapted from http://www.nimr.mrc.ac.uk/research/kate-bishop by Kate Bishop, used with permission.

The quantification of infectivity of different viruses in single cycle reporter assays can be further refined by mixing viruses with different reporter genomes together and using the mixture to infect cells. This allows for a comparison of two viruses in precisely the same infection and effectively eliminates variables inherent in assays utilizing parallel cell cultures. In these experiments mixtures of $gGag^{+}$ and $gGag^{-}$ virus stocks carrying distinct retroviral reporter genes were adjusted to give equal focus forming units (FFUs) and used to simultaneously assess both viruses in the same infection on 3T3 cells or 3T3/mA3 cells. The results of these experiments corroborated the earlier findings regarding inhibition by cellular mA3 and are described in Chapter 3.

2.4 Determination of virion-specific infectivity

Many studies have shown that hA3g is incorporated into HIV-1 ΔVif virions[23,100,126,128,141,173,179,182]. Some studies have also shown that mA3 is incorporated into the virions of MuLVs[100,100,188,188,203,203]. Incorporated A3 proteins affect the infectivity of the virions on target cells irrespective of whether they express mA3 or not[23,100,126,128,141,173,179,182]. Inhibition of infectivity by virion-associated mA3 would be reflected in a decrease in the specific infectivity of the virions. In order to measure the affect of virion-associated mA3 on MuLV infectivity, virions were produced from mA3 expressing cells as well as from cells not expressing mA3 and normalized to the number of virion particles by the quantification of reverse transcriptase (RT) activity. This has been done in other studies[100,123,192,204] using the enzymatic activity of the virion RT. It is possible, however that the presence of mA3 in the virion may affect the RT activity rendering it an unreliable measure of virion quantity. For this reason, the level of the major structural protein, p30, was estimated through immunoblotting procedures to provide an independent measure. In all of the virus stocks generated in these studies, the RT activity reflected the level of p30.

The virions used for these studies were produced from mA3 expressing cells transduced with the LAPSN reporter genome and subsequently infected with the $gGag^{+}$ or $gGag^{-}$ virus. Stocks collected from these cells consist of virions that have packaged the reporter LAPSN genome or alternatively, the MuLV genome, enabling the assay of released viruses by either the reporter assay or the FIA. Released virions were assayed for infectivity on *Mus dunni* cells. The choice of *Mus dunni* cells was based on their permissiveness to infection with high efficiency by MuLVs. In these experiments, the

titers of the viruses measured by the FIA were similar to titers measured by the alkaline phosphatase assay and the data from the two assays were combined. These experiments, described in chapter 3, indicated that virion-associated mA3 affected MuLV specific infectivity in a gGag-dependent manner.

2.5 Determination of *in vivo* replication

Both cellular and virion-associated mA3 were shown to restrict infectivity of MuLVs in a gGag-dependent manner[101]. It was unknown, however, if mA3 exerted a similar effect *in vivo*. In order to determine if there was a gGag-dependent effect of mA3 on MuLV replication *in vivo,* knock-out mouse strains in which the mA3 gene had been inactivated ($mA3^{-/-}$) and their wild-type counterparts ($mA3^{+/+}$) were utilized[164]. Two mouse strains were examined in these studies. The 129/Ola strain predominately expresses the full-length mA3[164] similar to the isoform utilized in the *in vitro* studies while the C57BL/6 strain predominantly expresses the delta exon 5 splice variant[164]. The C57BL/6 strains were included in the study because of the ease with which mouse colonies could be maintained compared to the 129/Ola strains. Furthermore, analyses in C57BL/6 mice provide an assessment of the *in vivo* activity of a distinct mA3 isoform.

The absence of an antibody specifically reactive with mA3 makes the assessment of *in vivo* levels of the protein difficult. mRNA measurements suggest that the expression of the protein increases during development and that mA3 expression is largely tissue-specific[159,164]. With these considerations, the assessment of MuLV replication was accomplished at various times after infection by measuring viremia in the mice. Viremia was measured with the expectation that it would more closely reflect total virus production in the animal than assays of particular tissues. Viremia data for the

knock out and wild-type mouse strains supporting an *in vivo* effect of gGag on the action of mA3 is presented in Chapter 3.

2.6 Protein analysis of infected cells and virions

To determine expression of mA3 and viral proteins in the cell and virions, immunoblots of proteins resolved by electrophoresis on polyacrylamide gels (PAGE) were utilized. The rabbit gGag-antibody was initially provided in the form of antiserum[63]. Use of the antiserum presented background problems in the immunoblot procedure that was not corrected by increased incubation times in blocking reagents or by extensive washing. Further, the antiserum reacted poorly with HRP conjugated anti-rabbit antibodies tested. In attempts to alleviate these problems, IgG from the antiserum was purified using protein G and the IgG fraction directly conjugated with HRP. This procedure coupled with the addition of 5% rabbit serum to the standard blocking buffer markedly reduced the background seen with the anti-gGag antiserum. Other antibodies used in these studies were also directly conjugated to HRP, which significantly reduced background.

Immunoprecipitation of mA3 and viral proteins from lysates of cells or virions for immunoblot analysis was achieved with purified, unconjugated antibodies. In order to eliminate non-specific absorption of proteins to the protein G Dynal beads used in the immunoprecipitation experiments, beads were first bound to the antibody under saturating conditions. The bead/antibody complexes were then incubated in the presence of 10% BSA to minimize non-specific protein binding. In addition, the lysates were pre-absorbed with unconjugated protein G Dynal beads to reduce the level of non-specific

binding proteins. Immunoprecipitated complexes subjected to PAGE were analyzed by immunoblot procedures.

The localization of mA3 and gGag in infected cells was determined by confocal microscopy. During the course of these analyses it was found that sequential incubation of antibodies specific for HA-mA3 (rat) and gGag (rabbit) with the cells rather than incubation with a mixture of the antibodies yielded far superior images. These primary antibodies were then detected by fluorescent goat anti-rat or anti-rabbit antibodies. These analyses are presented in chapter 4.

2.7 Localization of virion-incorporated mA3 and gGag

For determination of the localization of mA3 and gGag in the virion, virions purified by isopycnic centrifugation[54] on sucrose gradients were applied to a 10-30% sucrose step gradient, with the 10% layer containing detergent to separate the outer membrane of the virion from the virion core. This procedure was adapted from previously reported methods to differentiate membrane components from the core components of various retroviruses including human, avian and murine retroviruses[3,136,140,162]. The level of detergent was adjusted to eliminate the outer envelope protein from the sedimented core. It is important to note that these conditions also resulted in the loss of substantial levels of the major structural core protein, in agreement with previously reported studies[3,136,140,162]. These experiments revealed the presence of both gGag and mA3 in the virion core and are presented in chapter 4.

2.8 Determination of G-to-A hypermutation

mA3 and hA3G both exhibit cytidine deaminase activity at the level of the single stranded transcripts produced during reverse transcription. This process which results in

G-to-A mutations is thought to be a primary mechanism for retrovirus restriction[4,12,13]. It was of interest to determine if this mechanism is operative in the restriction of MuLVs and if it is affected by the presence of gGag. To determine the rate of G-to-A mutation in the presence of cellular mA3, cells expressing or not expressing mA3 were infected with either the $gGag^{+}$ or $gGag^{-}$ MuLV. A 24 hour time point after infection was chosen to avoid additional replicative cycles which would complicate the mutational analyses. After 24 hours, total DNA was harvested from the cell and the proviral DNA was amplified by PCR, cloned and sequenced to detect point mutations. A similar methodology was used to determine the effects of virion-associated mA3. In these determinations, virions produced from cells expressing or not expressing mA3 were used to infect *Mus dunni* cells. It is known that cells have evolved mechanisms to degrade uracil-containing DNA[18]. It is possible that mutated transcripts might be eliminated by such a mechanism, yielding an underestimate of the actual incidence of mutations. This possibility was addressed by determining the mutations in transcripts produced by viruses isolated from cells expressing or not expressing mA3. Transcripts were produced in cell-free RT reactions with lysed virions, amplified by PCR, cloned and sequenced. This procedure eliminated any possible effect of transcript elimination by cellular machinery; however, the analysis was limited to the action of virion-associated mA3. In contrast to many other reported studies, the viral genome, rather than an artificial transcript[18,99,110,145], was used to determine the mutation rate. To make sequence analysis comparable among all experiments, sequences of the PCR amplicons were trimmed to 755 bp to minimize ambiguous sequence data and to ensure uniformity. Although steps, such as biological cloning of the viruses, were taken to minimize pre-existing sequence

heterogeneity this is difficult to achieve with retroviruses due to the unedited replication of RNA viral genomes, Furthermore, amplification of a limited number of proviral transcripts could result in multiple clones of the same transcript which would contain the same mutations. The probability of a base change occurring randomly in multiple clones at the same position is quite small. Therefore, only those mutations found to be unique were considered in these analyses. Mutation rates were calculated as the number of mutations per nucleotide sequenced and are presented in chapter 4.

2.9 Determination of transcript levels

While deamination seems to be the main mechanism of action used by APOBEC3 proteins to inhibit retroviral replication, other mechanisms have been observed. In HIV infection, cellular hA3G was shown to inhibit replication by inhibiting the initiation of reverse transcription[29]. Similarly, no deamination activity was observed following infection by M-MuLV, however, the small inhibition of replication associated with mA3 was attributed to a reduction of viral transcripts in the cell[18]. As described in chapter 4, there was no significant difference in cytidine deaminase activity in the presence or absence of mA3 or gGag. It is possible that mA3 affects viral replication by restricting the level of proviral synthesis in infected cells in a gGag-dependent manner. To examine this possibility viral transcript levels in cells infected by either $gGag^{+}$ or $gGag^{-}$ MuLVs were quantified at 8 and 24 hours after infection by PCR. Primers were employed that would specifically amplify the infecting viral sequences rather than endogenous retroviral sequences present in the mouse genome. The results of these analyses indicate a gGag-dependent reduction in viral transcript levels in the contexts of both cellular and virion-associated mA3, and are described in chapter 4.

Chapter 3- The glycosylated Gag protein of a murine leukemia virus inhibits the anti-retroviral function of APOBEC3

3.1 Abstract

APOBEC proteins have evolved in both humans and mice as innate defenses against retroviral infections. To counteract the effects of human APOBEC3G, HIV has evolved the Vif protein. Murine leukemia viruses (MuLVs) that infect and replicate in mice do not encode a Vif homologue and it has not been understood how they evade mouse APOBEC3 (mA3). We report here a MuLV that utilizes a glycosylated form of its gag protein (gGag) to counteract mA3. gGag is critical for infection of mA3-expressing cell lines and for the infectivity of released viruses which have encapsulated mA3. Finally, a gGag-deficient virus that is restricted for replication in wild-type mice replicates efficiently in mA3 knockout mice implying a novel role of gGag in circumventing the action of mA3 *in vivo*.

Studies described in this chapter were published in Virology:

Kolokithas, A., K. Rosenke, F. Malik, D. Hendrick, L. Swanson, M. L. Santiago, J. L. Portis, K. J. Hasenkrug, and L. H. Evans. 2010. The glycosylated Gag protein of a murine leukemia virus inhibits the antiretroviral function of APOBEC3. J Virol **84**:10933-10936.

3.2 Introduction

APOBEC3G (hA3G) in humans and its mouse ortholog, APOBEC3 (mA3), act as potent innate defenses against retroviral infection. Both proteins deaminate cytidine in single-stranded DNA ultimately resulting in hypermutation of newly synthesized proviral

DNA[28,110], although additional deaminase-independent mechanisms of inhibition have been identified[4]. Infectious exogenous retroviruses, including HIV and murine leukemia viruses (MuLVs), have evolved mechanisms to circumvent the action of the APOBEC proteins[12,28]. HIV encodes the Vif protein which facilitates the rapid proteolysis of hA3G, while the mechanism by which exogenous MuLVs evade the action of mA3 is unknown[28].

Exogenous MuLVs, as well as some other gamma retroviruses, encode a glycosylated gag protein (gGag) originating from an alternate translation start site upstream of the methionine start site of the gag structural polyproteins[54,112,154]. gGag is synthesized at similar rates and levels as the structural gag polyprotein in MuLV infected cells but is glycosylated and undergoes distinct proteolytic processing[65,133]. A carboxyl fragment of gGag is released from the cell while an amino fragment is incorporated into the plasma membrane as a type 2 transmembrane protein[65,147]. The functions of gGag remain unclear, but mutations that eliminate its synthesis severely impede *in vivo* replication of the virus with little, if any, effect on replication in fibroblastic cell lines[36,121,153]. APOBEC3 proteins are expressed in many tissues *in vivo* but are poorly expressed in many *in vitro* cell lines[28] suggesting a possible link between gGag expression and the evasion of mA3 by MuLVs. These studies were undertaken to determine if the expression of the gGag protein facilitated MuLV replication in the presence of mA3 *in vitro* and *in vivo*.

3.3 Materials and methods

3.3.1 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[18]. It was

provided in the pcDNA3 vector and was tagged at the C-termini with hemagglutinin (HA)[18]. NIH3T3 (3T3) and *Mus dunni* cells 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 (TCCCGCCTCTACAACATACGGGACC) revealed that mA3 RNA was below the level of detection in both 3T3 and *Mus dunni* cells (data not shown). Plasmid DNA was transfected into 3T3 or *Mus dunni* 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 their ability to stably express the C-terminal HA-tagged mA3 construct after ten passages. 3T3 and *Mus dunni* cells stably expressing the C-Terminal HA-tagged mA3 construct (3T3/mA3 and *Mus dunni*/mA3 cells, respectively) were maintained in selection media. $CasFr^{KP}$ ($gGag^{+}$) is an MuLV derived from the wild mouse ecotropic virus CasBrE and contains a short sequence of the F-MuLV, FB29, which includes the initiation site of the gGag protein [152]. CasFr--3+4 ($gGag^{-}$) is a derivative of $CasFr^{KP}$ in which mutations have been introduced to disrupt the expression of gGag[153]. Mice were infected by intraperitoneal injection with approximately 2×10^5 infectious units of virus within hours of birth.

3.3.2 Virus isolation. Virus was harvested from 3T3/mA3 cells infected with either $gGag^{+}$ or $gGag^{-}$ viruses and purified by isopycnic gradient centrifugation as previously described[54]. Briefly, supernatants were collected from infected cells and cleared of cellular debris by low speed centrifugation. Cleared supernatants were subjected to ultracentrifugation to pellet the virions. Pellets were resuspended in buffer

and placed on a 20 to 60% linear sucrose gradient for further ultracentrifugation. The fraction of the gradient containing virions was pelleted by ultracentrifugation, resuspended in a storage buffer and kept at -20C until used in further experimentation.

3.3.3 Mice. The derivation of 129P2/OlaHsd mA3$^{-/-}$ and C57BL/6 mA3$^{-/-}$ mice has been previously described[164]. C57BL/6 and 129P2/OlaHsd mice were obtained from Jackson Laboratories and Harlan Laboratories, respectively. In the interest of brevity, 129P2/OlaHsd will be referred to as 129/Ola mice throughout this article. Mice were treated in accordance with the regulations and guidelines of the Animal Care and Use Committee of the National Institutes of Health.

3.3.4 Assays of viral infectivity. The quantification of viruses in single cycle infectivity assays was accomplished using the retroviral vectors LAPSN (Clontech Laboratories, Inc.) and G1nβgSVNa[92]. Briefly, cells were plated in 60 mm tissue culture dishes, the cells infected the next day with virus and allowed to grow to confluence (3-4 days). Foci of cells expressing alkaline phosphatase or β-galactosidase were detected as previously described[92] and counted as focus-forming units (FFU). For mixed virus assays, vectors pseudotyped within gGag^{+} or gGag^{-} virions were harvested from 3T3 cells that had been transduced with LAPSN or G1nβgSVNa. Mixtures of gGag^{+} and gGag^{-} virus stocks carrying distinct retroviral vectors were adjusted to give equal FFUs and used to simultaneously assess both viruses in the same infection on 3T3 cells or 3T3/mA3 cells. For assays of progeny viruses released from 3T3 cells or 3T3/mA3 cells, the cell lines were transduced with the LAPSN vector and subsequently infected with a gGag^{+} or gGag^{-} MuLV. The levels of progeny virus were assessed on *Mus dunni* cells and were normalized for reverse transcriptase activity using a colormetric reverse

transcriptase kit (Roche) according to the manufacturer's instructions. Reverse transcriptase activity was expressed as the absorbance at 405nm per ml. The infectivity of progeny virus released from 3T3 or 3T3/mA3 cells was also quantified using a monoclonal antibody (mAb) specifically reactive to the envelope protein of the viruses, mAb 667[127], in focal immunofluorescence assays[175].

3.3.5 Immunoblotting. After virus isolation, samples were lysed in Protein Extraction reagent Type 4 (Sigma) and incubated for 10 minutes at room temperature. For cell lysate samples, monolayers were lysed by addition of lysis buffer (0.01M NaH_2PO_4 [pH 7.6], 0.001M EDTA, 1% Triton X-100, 0.1 % SDS). Cell lysates harvested from the plates were centrifuged at 13,000 x g for 10 minutes to separate the soluble and insoluble fractions. The soluble protein fraction was used to measure total protein concentration in the sample 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 by probing with monoclonal anti-HA horseradish peroxidase (HRP)-conjugated antibody (Roche), polyclonal rabbit anti-gGag HRP-conjugated antibody or an HRP-conjugated monoclonal anti-p30 antibody (mAb 18-7)[25]. Membranes were stripped using 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS for 30 minutes at 55^{o} C on a rotary shaker. The anti-gGag antibody and the monoclonal anti-p30 antibody were purified using a HiTrap protein G HP affinity column (GE Healthcare) according to the manufacturer's instructions.

Antibodies were conjugated to HRP using a Lightning-Link HRP conjugation kit (Innova Biosciences) according to the manufacturer's instructions.

3.4 Results

3.4.1 Efficient infection of mA3-expressing cells is dependent on gGag. Several reports of the effects of mA3 proteins on virus replication have examined the infectivity of virions released from cells transfected with cloned proviral DNA in the presence or absence of mA3[51,110,162]. Such analyses do not test effects of mA3 present in the cytoplasm of the cells on the infectivity of MuLVs that have not been previously exposed to mA3. To address this issue we developed a 3T3 cell line that expressed an HA-tagged mA3 protein (3T3/mA3). The infectivity of the $gGag^+$ and $gGag^-$ MuLVs was compared on 3T3 and 3T3/mA3 cells using mixtures of the viruses, each carrying a distinct retroviral vector encoding either alkaline phosphatase (LAPSN) or B-galactosidase (G1nBgSvNa). Utilizing mixtures of viruses with different readouts

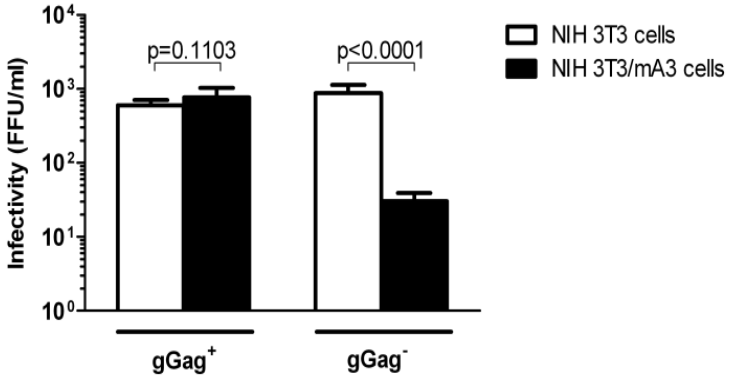

Figure 3.1. Effect of cellular mA3 on infection by $gGag^+$ or $gGag^-$ MuLVs.
3T3 and 3T3/mA3 cells were infected with mixtures of $gGag^+$ and $gGag^-$ viruses, each carrying a distinct retroviral vector encoding either alkaline phosphatase (LAPSN) or β-galactosidase (G1nβgSvNa) and assayed by scoring FFU of cells expressing the respective enzymes. The mixtures were adjusted to give equivalent titers of alkaline phosphatase and β-galactosidase on 3T3 cells. Statistical analysis was performed using the unpaired Student's *t* test.

for infectivity enabled the assessment of the effects of cellular mA3 on both viruses in precisely the same infection.

Experiments were performed using both MuLV/vector combinations (i.e., LAPSN(gGag$^+$ MuLV) plus G1nβgSvNa(gGag$^-$ MuLV) or alternatively, G1nβgSvNa(gGag$^+$ MuLV) plus LAPSN(gGag$^-$ MuLV)). These analyses indicated that cellular mA3 exerted a marked inhibitory effect on the infectivity of gGag$^-$ virus but not on the gGag$^+$ virus (Figure 3.1).

3.4.2 Both gGag$^+$ and gGag$^-$ MuLVs incorporate mA3 into progeny virions. A number of studies have reported partial inhibition of ecotropic MuLVs as a result of incorporation of mA3 into progeny virions[110,123,162,188]. Indeed, it has been suggested that MuLVs may evade the action of mA3 by exclusion of the protein from virions, although there are conflicting accounts regarding this matter[18,43,100,110,145,162]. To determine if the presence of gGag influenced the incorporation of mA3, virions were isolated from mA3 cells infected with gGag$^+$ or gGag$^-$ MuLVs and examined by immunoblot analyses. mA3 was readily detected in both virus preparations with no discernible differences in the levels of virion incorporation in gGag$^+$ or gGag$^-$ MuLVs (Figure 3. 2A). This result is in agreement with other reports that have shown incorporation of mA3 into virions [18,100,145,162]. Furthermore, gGag was also found to be incorporated into virions (Figure 3.2B) consistent with an earlier study[63].

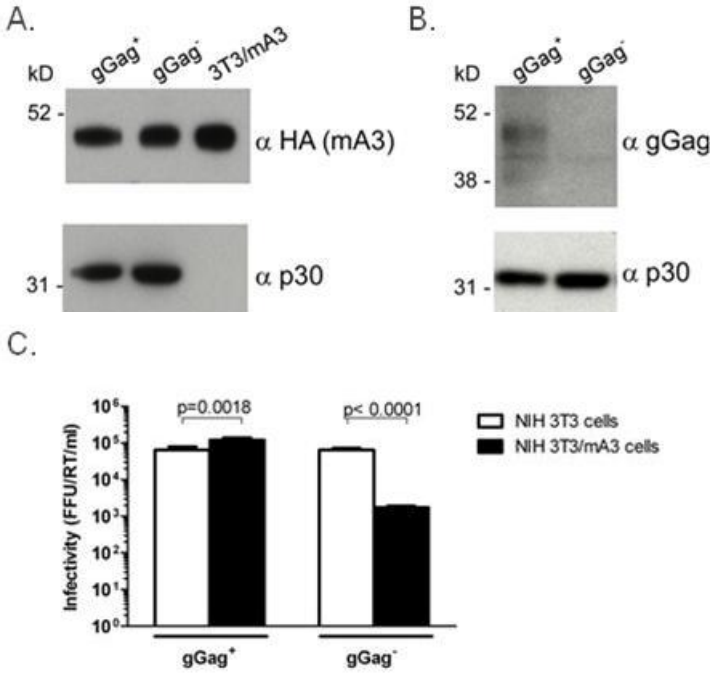

Figure 3.2. Infectivity of virions released from 3T3 or 3T3/mA3 cells infected with gGag$^+$ or gGag$^-$ MuLVs.
Virions released from 3T3/mA3 cells infected with gGag$^+$ or with gGag$^-$ were analyzed by immunoblotting for the presence of mA3 or gGag. **A).** Immunoblot analysis of gGag$^+$ virions, gGag$^-$ virions and a 3T3/mA3 cellular lysate for the presence of mA3 using a HRP-conjugated anti HA antibody. The 3T3/mA3 cellular lysate was included as a comparison of mA3 in the cells to that in the virions. The blot was also developed with a HRP-conjugated monoclonal antibody to p30 as a loading control. Exposure times for detecting mA3 were approximately 10-fold longer than for p30. **B).** Immunoblot analysis of gGag$^+$ or gGag$^-$ for the presence of gGag using a HRP-conjugated anti gGag antibody. The blot was subsequently stripped and developed with an HRP-conjugated monoclonal antibody to p30 as a loading control. Exposure times for detection of gGag were approximately 20-fold longer than for p30. **C).** 3T3 cells or 3T3/mA3 cells harboring the retroviral vector LAPSN were infected with gGag$^+$ or gGag$^-$ MuLVs. Infectivity of progeny virions was assessed on uninfected *M. dunni* cells by alkaline phosphatase assays and by FIA. Infectivity titers, expressed as FFUs were normalized to the number of virions by assessing reverse transcriptase activity (RT). RT was expressed as the absorbance per ml at 405nm. Statistical analysis was performed using the unpaired Student's *t* test.

3.4.3 Virion-associated mA3 selectively inhibits gGag$^-$ MuLV infectivity. To determine if virion-incorporated mA3 differentially influenced gGag$^+$ and gGag$^-$ MuLVs we examined the infectivity of viruses released from 3T3/mA3 cells as well as from 3T3 cells lacking mA3. Both cell lines were transduced with the retroviral vector, LAPSN,

which encodes alkaline phosphatase to enable the quantification of progeny virus infectivity in single cycle assays. Cells were infected with $gGag^+$ or $gGag^-$ MuLVs and released viruses were quantified in assays on *Mus dunni* cells. Infectivity was normalized to the relative number of virus particles by quantifying reverse transcriptase activity. Considering that the reverse transcriptase activity included virions carrying the retroviral vector as well as virions carrying the MuLV genome, the infectivity of progeny virus released from 3T3 or 3T3/mA3 cells was also quantified using a monoclonal antibody specifically reactive to the envelope protein of the viruses in focal immunofluorescence assays. The retroviral vector assays and the fluorescence assays closely paralleled one another and the results were combined (Figure 3.2C). These analyses revealed that the infectivity of $gGag^-$ virus released from 3T3/mA3 cells was markedly decreased compared to $gGag^-$ virus released from 3T3 cells. In contrast, no significant differences were observed in the infectivity of $gGag^+$ virus released from 3T3/mA3 and 3T3 cells (Figure 3.2C). Experiments using a *Mus dunni* cell line expressing the mA3 protein to assess the effects of cellular as well as virion-incorporated mA3 on the infectivity of $gGag^+$ and $gGag^-$ MuLVs yielded similar results (data not shown).

3.4.4 mA3-deficient mice support the replication of $gGag^+$ and $gGag^-$ MuLVs.

If mA3 restriction is a major factor influencing *in vivo* replication of MuLVs and its action is sufficiently repressed by gGag, it would be expected that mice lacking mA3 would be permissive to infection by both $gGag^+$ and $gGag^-$ MuLVs. To examine this possibility we determined the level of replication of $gGag^+$ and $gGag^-$ MuLVs in mA3

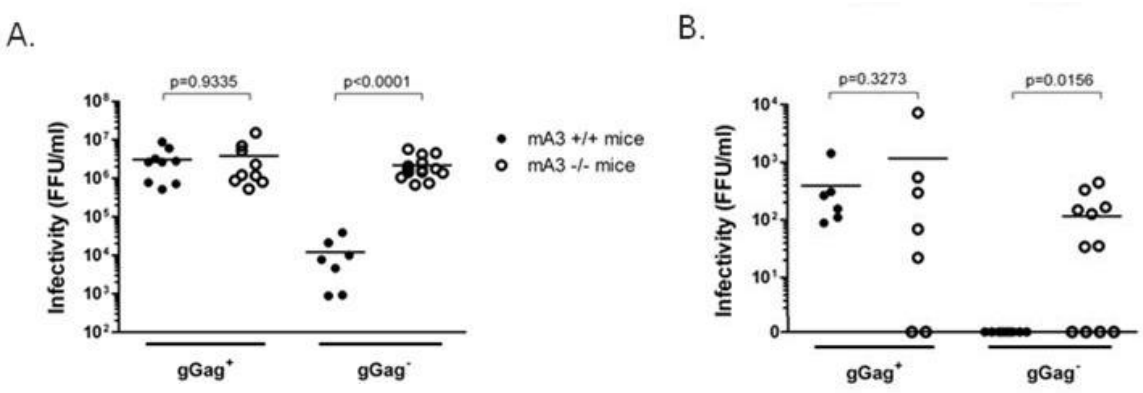

Figure 3.3. Replication of gGag$^+$ or gGag$^-$ MuLVs in wild type and mA3 knockout mice.

A). 129/Ola wild type (mA3$^{+/+}$) or 129/Ola mA3 knockout (mA3 $^{-/-}$) mice were inoculated with gGag$^+$ or gGag$^-$ MuLVs. Three weeks after infection the mice were sacrificed, sera collected and viruses quantified by a focal immunofluorescence assay on *M. dunni* cells. n=9 for gGag$^+$ MuLV in mA3$^{+/+}$ mice, n=9 for gGag$^+$ MuLV in mA3$^{-/-}$mice, n=7 for gGag$^-$ MuLV in mA3$^{+/+}$ mice and n=14 for gGag$^-$ MuLV in mA3$^{-/-}$ mice. Statistical analysis was performed using the unpaired Student's *t* test**. B).** C57BL/6 wild type mice (mA3$^{+/+}$) or C57BL/6 mA3 knockout mice (mA3$^{-/-}$) were inoculated with gGag$^+$ or gGag$^-$ MuLVs. Three weeks after infection the mice were sacrificed, sera collected and viruses quantified by focal immunofluorescence assays on *M. dunni* cells. Each point represents the level of viremia from an individual animal with n=6 for the gGag$^+$ MuLV in mA3$^{+/+}$ mice, n=7 for the gGag$^+$ MuLV in mA3$^{-/-}$ mice, n=9 for the gGag$^-$ MuLV in mA3 $^{+/+}$ mice and n=11 for the gGag$^-$ MuLV in mA3$^{-/-}$ mice. Wilcoxon Signed rank test was used to differentiate whole number integers from zero values. The unpaired Student's *t* test was used to compare non-zero results.

knockout mice (mA3$^{-/-}$) and their wild type counterparts (mA3$^{+/+}$). Comparisons of the replication of gGag$^+$ and gGag$^-$ MuLVs revealed a clear influence of 129/Ola mA3 on their replication (Figure 3.3A). In agreement with previous studies on the replication of gGag-deficient mutants[36,123,153], the replication of the gGag$^-$ MuLV was severely restricted in normal 129/Ola mice. However, in 129/Ola mice lacking mA3, the gGag$^+$ and gGag$^-$ MuLVs replicated to equally high levels. These results indicate that the inability of the gGag$^-$ MuLV to replicate efficiently *in vivo* is the result of mA3 expression.

C57BL/6 mice contain the $Fv1^b$ allele[181]. The MuLVs used in this study are N-tropic, thus the levels of replication of the MuLVs in C57BL/6 mice were much lower than in the 129/Ola mice, which contain the $Fv1^n$ allele (Figure 3.3B)[181]. Nevertheless, replication in these mice was sufficient to observe mA3 inhibition in a gGag-dependent manner. Replication of the $gGag^+$ MuLV was easily detectable in both C57BL/6 $mA3^{+/+}$ and $mA3^{-/-}$ mice, while replication of the $gGag^-$ MuLV was below the level of detection in the wild-type C57BL/6 $mA3^{+/+}$ mice. However, in C57BL/6 $mA3^{-/-}$ mice, the $gGag^-$ MuLV replicated to the same level as the $gGag^+$ MuLV. Thus, the inability of the $gGag^-$ MuLV to replicate *in vivo* in C57BL/6 mice is the result of mA3 expression, similar to our findings using 129/Ola mice. It is noteworthy that C57BL/6 mice predominantly express a splice variant mA3 mRNA which lacks exon 5 while 129/Ola mice predominantly express a complete mA3 mRNA. Assuming that the mRNAs are translated with equal efficiencies, our results indicate that the MuLV gGag studied here is able to suppress the antiviral effect of both the full-length and exon 5-deleted proteins.

3.5 Discussion

Nearly all exogenous MuLVs contain sequences encoding gGag which is synthesized from an alternate initiation site upstream of the start site of the gag polyprotein[48,154]. Although studies have found gGag to be necessary for *in vivo* replication, the basis for this effect was unknown[36,121,153]. Moreover, the means by which exogenous MuLVs evade the action of mA3 was unclear[28,162]. Our results establish a novel role of gGag as an antagonist of mA3 thereby providing insight into both of these questions.

We observed gGag-dependent inhibition by mA3 in two distinct contexts: inhibition of infectivity by mA3 expressed in the cell as well as inhibition by virion-associated mA3. Interestingly, it was recently reported that infection by Moloney MuLV (M-MuLV) was partially inhibited by mA3 and that both virion and cellular mA3 contribute to the inhibition[123]. Inhibition of infection by both virion and cellular mA3 has also been observed with mouse mammary tumor virus[138]. Furthermore, HIV has also been reported to be inhibited by cytoplasmic hA3G[196].

It is somewhat surprising that cellular mA3 exerts a gGag-dependent effect on infecting MuLVs. The level of gGag incorporated into virions is low compared to the structural proteins but clearly detectable. Furthermore a previous study of the $gGag^+$ MuLV utilized here indicated that gGag is indeed a component of the virion[63]. Virion-associated gGag may directly influence the action of cellular mA3. Alternatively, the susceptibility of the $gGag^-$ MuLV to cellular mA3 may occur by an indirect mechanism. In this regard, it has been reported that gGag is involved in virion release and that $gGag^-$ M-MuLV exhibits an abnormal morphology during virion budding[121]. It is conceivable that mature virions may also be altered from an mA3-resistant to an mA3-susceptible phenotype.

We observed that progeny virions released from mA3 expressing cells were also inhibited in a gGag-dependent manner. While the level of gGag incorporated into virions is low, in infected cells the rate of synthesis and the level of gGag are comparable to that of the unglycosylated Gag polyprotein precursor[54]. It is possible that gGag counteracts the action of mA3 on progeny virions by a mechanism distinct from the effect of virion-associated gGag on cellular mA3 discussed above. Although both $gGag^+$ and $gGag^-$

MuLVs incorporate mA3 into virions at similar levels we have not determined if this incorporation is qualitatively the same with both MuLVs. It is possible that the virion-associated mA3 is altered in its location or association with virion components in the gGag$^+$ MuLV. Such an alteration could be influenced by an interaction of the newly synthesized gGag with mA3 in the cell. The mechanism(s) by which gGag influences the action of mA3 awaits further investigation.

A very recent report indicates that gGag exhibits some functional similarities to the Nef protein of HIV[149]. Nef is an accessory protein of HIV and SIV that exhibits an incredibly complex range of functions that include down regulation of CD4 and MHC proteins[67,169], induction of apoptosis[68] and increasing efficiency of reverse transcription[168]. Nef is incorporated into virions facilitating infectivity and *in vivo* replication[19,94,95,144], however an influence of Nef on the action of APOBEC3 proteins has not been described. It is quite possible that gGag, like Nef, has more than one function in the virus life cycle.

Our analyses indicated that mA3 did not inhibit the gGag$^+$ MuLV, however a number of studies have reported partial to marked inhibition of other MuLVs[110,123,162,188], all of which encode a gGag protein. A direct comparison of the inhibitory effects of mA3 on M-MuLV and the ecotropic AKV MuLV revealed that AKV was inhibited to a greater extent than M-MuLV[110]. Furthermore, inhibition of M-MuLV by mA3 was not accompanied by G-to-A mutations in newly synthesized DNA[110,162], whereas inhibition of AKV by mA3 exhibited extensive G-to-A mutations[110]. Differences in the susceptibility of MuLVs to inhibition by mA3 could reflect differences in the efficacy of their respective gGags to counteract mA3. In this regard, a comparison of the gGag

sequences of M-MuLV and AKV reveals extensive amino acid differences in their amino-terminal fragments (data not shown).

A xenotropic murine leukemia–like retrovirus (XMRV) has been found in human prostate cancer tissue as well as in cohorts of patients diagnosed with chronic fatigue syndrome [59,120,166,193]. Recent studies have reported that mA3 inhibits the replication of XMRV to a much greater extent than M-MuLV[74,145]. It is noteworthy that all XMRV isolates exhibit a termination codon in the coding sequences of gGag resulting in a truncation of the protein just before the transmembrane region[193]. Thus, the sensitivity of XMRV replication to mA3 may reflect the absence of a functional gGag.

In agreement with earlier studies, we observed a marked inhibition of *in vivo* replication of the $gGag^-$ MuLV compared to the $gGag^+$ MuLV. Levels of the $gGag^-$ MuLV were restored to the high levels observed with the $gGag^+$ MuLV in 129/Ola KO mice lacking mA3. These results further substantiated the role of gGag as an antagonist of the restriction factor. Similar results were observed with C57BL/6 mice with the level of replication of the $gGag^-$ MuLV reaching levels observed for the $gGag^+$ MuLV in $mA3^{-/-}$ mice. The level of replication of the $gGag^+$ MuLV in C57BL/6 $mA3^{+/+}$ mice was low due to Fv1 restriction and was not significantly increased in C57BL/6 $mA3^{-/-}$ mice. This result differs from studies of M-MuLV replication in C57BL/6 $mA3^{-/-}$ mice indicating enhanced MuLV replication compared to replication in C57BL/6 $mA3^{+/+}$ mice[123]. In contrast to M-MuLV, which is moderately inhibited by mA3[110,123,162], the $gGag^+$ MuLV utilized in this study was not inhibited. This difference would account for the increased replication of M-MuLV in the absence of mA3.

These studies provide at least partial answers to two difficult questions in retrovirology: those of the function of the gGag of MuLVs and the means by which MuLVs evade the action of APOBEC3. Although the gGag of exogenous MuLVs provides a similar function as the Vif protein of HIV, further studies are required to determine similarities and differences in their mode of action. Such studies are particularly relevant in light of recent reports indicating cross-species retroviral infections from mouse to humans[120,166,193].

Chapter 4-The Murine leukemia virus gGag protein facilitates reverse transcription in the presence of murine APOBEC3

4.1 Abstract

Humans and mice have evolved APOBEC proteins that act as innate defenses against retroviral infections. We have recently reported that a MuLV utilizes a glycosylated form of its Gag protein (gGag) to effectively inhibit the antiviral activity of murine APOBEC3 (mA3) *in vitro*. Our results indicated that gGag-dependent inhibition by mA3 occurs in two different contexts: cellular mA3, which inhibits initial infection of the cell by MuLVs not previously exposed to mA3; and virion-associated mA3, present in progeny MuLVs released from cells expressing mA3. Infection by MuLVs containing mA3 is inhibited irrespective of the expression of mA3 in the target cells. The mechanism by which gGag is protective against mA3 is unknown. To counteract the human APOBEC3G (hA3g), HIV has evolved the Vif protein, which depletes hA3g from infected cells by facilitating its degradation through the proteosome. We report here that a gGag-containing MuLV does not deplete mA3 from an infected cell. Further, immunoprecipitation and localization experiments suggest that mA3 does not interact with gGag directly in an infected cell. In contrast, both gGag and mA3 are associated with the virion core and are coprecipitated in the context of the mature virions. Both hA3g and mA3 have been reported to use deamination-dependent and -independent mechanisms to inhibit retroviral replication. Inhibition by mA3 on gGag-deficient virions correlates with a decrease in the level of transcripts upon infection of cells and appears largely independent of deamination activity.

4.2 Introduction

APOBEC3s (hA3G in humans, mA3 in mice) are cytidine deaminases that act on single-stranded DNA during reverse transcription resulting in hypermutation of newly synthesized proviral DNA[28,110]. HIV-1 has a gene product, Vif, to counteract hA3G antiviral effects. Vif binds to the N-terminal of hA3G and recruits a ubiquitin ligase complex that marks hA3G for destruction in the proteasome[34]. Vif has also been shown to impair the translation of hA3G mRNA[128] and prevent encapsulation by binding to hA3G[141]. MuLVs are largely insensitive[13] to the actions of murine APOBEC3 (mA3), although this has recently become a point of controversy[18,43,100,110,145,162]. It appears that MuLVs escape mA3 activity without a Vif-like accessory protein.

APOBEC3 family members from both humans and mice inhibit endogenous LTR retrotransposons and retroviruses[12,49,50]. A major difference between exogenous and most endogenous MuLVs in the mouse is the absence of a sequence for the expression of gGag. The gGag protein is translated from the same viral mRNA as Gag, but uses a CUG start codon that is upstream of the AUG start codon for Gag[54,65,133] resulting in an additional 88 amino acids on the N-terminus of Gag. The protein is then targeted to the rough endoplasmic reticulum for glycosylation and is subsequently exported to the cell surface[65,147] where it is cleaved in the CA domain. Some of the C-terminal portion of gGag is secreted whereas some of the N-terminal portion adopts a type II integral membrane configuration[65,147]. The amounts of Gag and gGag synthesized in infected cells are similar, however the amount of gGag incorporated into the virion is low compared to other Gag structural proteins[65]. MuLVs require gGag for efficient replication *in vivo* and it is a major determinant of virulence [65]. However, gGag is not required for efficient *in*

vitro replication, as the mutant viruses that lack gGag are able to replicate in cell lines with similar efficiency to their wild-type counterparts[63,65,121]. In a recent report we observed that mA3 suppresses the replication of a $gGag^-$ virus compared to a $gGag^+$ virus both *in vitro* and *in vivo*, however the mechanism by which gGag exerts this effect is unclear. The present studies were undertaken to investigate possible mechanisms by which gGag evade the action of mA3.

4.3 Materials and Methods

4.3.1 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 3.3.1.

4.3.2 Virion core isolation. Total virion isolation is described in chapter 3.3.2. For isolation of viral cores, a suspension of purified virions was sedimented through a 10-30% sucrose step gradient in PBS containing 5% IGEPAL, in the 10% sucrose layer of the gradient. The gradients were centrifuged at 30,000 rpm for 1.5 hours after which the pellets were resuspended in Laemmli sample buffer containing 5% β-mercaptoethanol and samples were incubated in a boiling water bath for 10 minutes.

4.3.3 Immunoblotting. Immunoblotting procedures were performed as described in chapter 3.3.5.

4.3.4 Determination of cellular mA3 levels. The effect of infection on the levels of cellular mA3 was examined in 3T3/mA3 cells infected with either $gGag^+$ or $gGag^-$ MuLVs. Forty-eight hours after infection, cells were harvested and analyzed by immunoblot analysis. The level of mA3 was normalized by comparing its signal intensity to GAPDH or b-Actin signal intensity using ImageQuant software (GE).

4.3.5 Immunoprecipitation. Purified virions were lysed in 50 mM Tris, 80 mM potassium chloride, 0.75 mM EDTA and 0.5% Triton X-100, pH 7.8, and incubated for 30 minutes at room temperature. Confluent monolayers of cells were lysed by addition of Cell lytic M cell lysis reagent (Sigma). Cell lysates harvested from the plates were centrifuged at 13,000 x g for 10 minutes to separate the soluble and insoluble fractions. The total protein concentration in the soluble fraction was determined using a BCA assay (Pierce) according to the manufacturer's protocol. Samples were normalized according to their protein concentrations. Protein G Dynal beads (Invitrogen) were incubated in the presence of monoclonal anti-HA antibody (Roche), polyclonal rabbit anti-gGag antibody or a monoclonal anti-p30 antibody (mAb 18-7). The bead/antibody mixtures were then incubated with BSA to block non-specific binding. The blocked bead/antibody mixtures were added to lysates which had been pre-cleared with unconjugated protein G beads and incubated on a rotating platform for 1hr at 4C. The incubated beads were washed with PBS five times and bound proteins were eluted by the addition of 1X Laemmli sample buffer containing 5% β-mercaptoethanol and incubating in a boiling water bath for 10 minutes. The samples were then subjected to SDS-PAGE (10% acrylamide, Life Technologies), followed by electrophoretic transfer onto a PVDF membrane.

4.3.6 Mutational Analyses. The effect of cellular mA3 on the mutation of viral transcripts was assessed in 3T3 or 3T3/mA3 cells infected with either $gGag^{+}$ or $gGag^{-}$ MuLVs that had been biologically cloned by endpoint dilution using the FIA (Chapter 3). Cells were infected with the respective viruses and twenty-four hours after infection the cells were lysed and newly synthesized viral sequences amplified using the Extract and Amp PCR kit (Sigma) according to the manufacturer's instructions. The primers used for

amplification were the *env* gene primers with 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 Zymo gel purification kit (Zymo) according to manufacturer's instructions. Purified PCR products were cloned into the PCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's instructions and sequenced using the *env* primers. For virion mutation analysis, virions were harvested from tissue culture supernatant of 3T3 or mA3/3T3 cells infected with the $gGag^+$ or $gGag^-$ viruses. The transcripts from lysed purified virions were generated using the Colormetric reverse transcription kit (Roche). Reaction conditions were followed according to the manufacturer's instructions; however, random hexamers and unlabeled nucleotides were used in place of the primers and nucleotides provided with the kit. Transcripts generated in this reaction originated from the virion RT with the virion genome as the template. The transcripts were amplified, purified, cloned and sequenced as described above. The influence of virion-associated mA3 on the mutation of viral transcripts was assessed after infection of naive *Mus dunni* cells with $gGag^+$ or $gGag^-$ viruses harvested from 3T3 or 3T3/mA3 cells. Twenty-four hours after infection the *Mus dunni* cells were lysed and viral sequences amplified, purified, cloned and sequenced as described.

Groups of sequences generated in these analyses were compiled and compared in the DNAstar Lasergene program, SeqMan. All sequences were trimmed from either end to minimize ambiguity and to yield a 755 bp sequence for uniform comparisons. Redundant mutations occurring at the same position in two or more clones likely

originated from pre-existing heterogeneity in the virus stock or by amplification of the same proviral transcript (see chapter 2). Therefore, coincident mutations were eliminated from the analysis. Mutation rates for each type of point mutation (e.g., G-to-A transitions) were calculated as the frequency per nucleotide of that change 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.

4.3.7 Accumulation of proviral transcripts in infected cells. To determine the effect of cellular mA3 on reverse transcription, 3T3 and 3T3/mA3 cells were infected with $gGag^+$ and $gGag^-$ viruses. Cellular DNA was isolated at eight and twenty-four hours post-infection using the AquaGenomic DNA isolation kit (Aquaplasmid) according to the manufacturer's instructions. Levels of viral- specific DNA were then determined by semi-quantitative PCR using primers specific for the viral *env* gene (CasFrKP6805 and CasFrKP7886RC). PCR products of the *gapdh* gene were amplified using Gapdh-152f AACGACCCCTTCATTGAC (forward) and Gapdh-342r TCCACGACATACTCAGCAC (reverse) and used as internal controls. Evaluation of the influence of virion-associated mA3 on the level of transcripts in infected cells was assessed by infection of naïve *Mus dunni* cells with $gGag^+$ and $gGag^-$ viruses harvested from 3T3 or 3T3/mA3 cells. DNA from infected cells was isolated and evaluated for viral transcript levels as described above. In these experiments *Mus dunni* cells were infected with virus stocks normalized

using the major structural virion protein, p30. A standard dilution curve was generated using amplification of a plasmid containing the sequence of the $gGag^+$ MuLV with the *env* gene primers. The standard dilution curve was used to quantify transcript levels by densitometry using the AlphaView software program (Cell Biosciences).

4.3.8 Localization of gGag and mA3 in infected cells. 3T3 or 3T3/mA3 were seeded on cover slips and infected with $gGag^+$, $gGag^-$ MuLVs, or mock-infected. Samples were collected at 6, 12, 24, and 48 hours post infection. Cells were fixed for 15 minutes in 3.7% formaldehyde, washed in PBS, and permeabilized in 0.5% Triton X-100 for 15 minutes. Samples were blocked with 10% BSA in PBS for 30 minutes, followed by incubation for 30 minutes with monoclonal rat anti-HA (1:100) and a 30 minute incubation with polyclonal rabbit anti-glycosylated gag (1:250) in 3% BSA in PBS. Samples were then washed in PBS and incubated with DylightFluor (Jackson Laboratories) 488-conjugated goat anti-rat IgG (1:250) and DylightFluor (Jackson Laboratories) 594-conjugated goat anti-rabbit IgG (1:250) with Hoechst 33342 (1:100,000) as a nuclear counterstain. Samples were washed in PBS followed by rinsing in ddH_2O. Processed coverslips were mounted onto slides with Mowiol (Polysciences) and fluorescence images were collected via sequential scanning on a Zeiss 510 Meta scanning laser confocal microscope.

4.4 Results

4.4.1 mA3 is not depleted from infected mA3 cells in a gGag-dependent manner

In a previous report we observed that mA3 inhibited the replication of a $gGag^-$ virus but not a $gGag^+$ virus[101]. The Vif protein of HIV blocks the action of hA3G by inducing its rapid proteolytic degradation through ubiquitination and transport to the proteosome[34]. It was therefore of interest to determine if MuLVs utilize a similar mechanism to counteract the antiviral activity of mA3. 3T3/mA3 cells were infected with either $gGag^+$ or $gGag^-$ MuLVs. Forty-eight hours after infection, cells were harvested and mA3 levels evaluated by immunoblot analysis. The levels of mA3 were quantified by comparing signal intensities of mA3 to GAPDH or β-Actin signal intensities using ImageQuant software (GE).

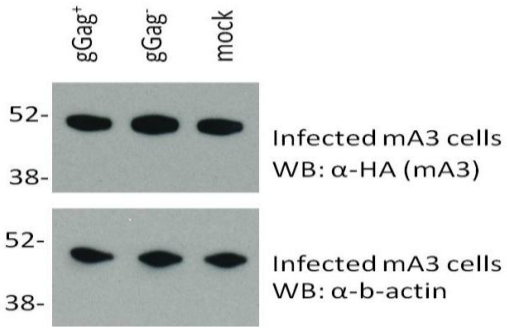

Figure 4.1. Determination of depletion in infected 3T3/mA3 cells. 3T3/mA3 cells were infected with either $gGag^+$ or $gGag^-$. Forty-eight hours after infection, cells were harvested and analyzed via immunoblot analysis. The amount of depletion was quantified by comparing signal intensity to β-Actin signal intensity using ImageQuant software (GE).

A reduction in the level of mA3 was not observed in $gGag^+$ MuLV infected 3T3/mA3 cells compared to cells infected with the $gGag^-$ virus or mock infected cells (Figure 4.1), indicating that mA3 is not depleted from infected cells in a gGag-dependent manner.

4.4.2 Interactions between gGag and mA3 are not detected in infected cells

The MuLV gGag protein is synthesized to levels equivalent to the structural Gag proteins in infected cells[54] and may serve to bind and deactivate mA3. To determine if there are binding interactions between mA3 and gGag in infected cells, 3T3/mA3 cells

were infected with either gGag$^+$ or gGag$^-$ MuLVs or mock-infected. Forty-eight hours after infection, cells were lysed and immunoprecipitated with anti-HA or anti-gGag antibodies. Immunoprecipitated complexes were analyzed by immunoblot analysis. Both of the antibodies precipitated their respective proteins, however, coprecipitation of mA3 and gGag was not observed with either antibody in lysates of infected 3T3/mA3 cells (Figure 4.2).

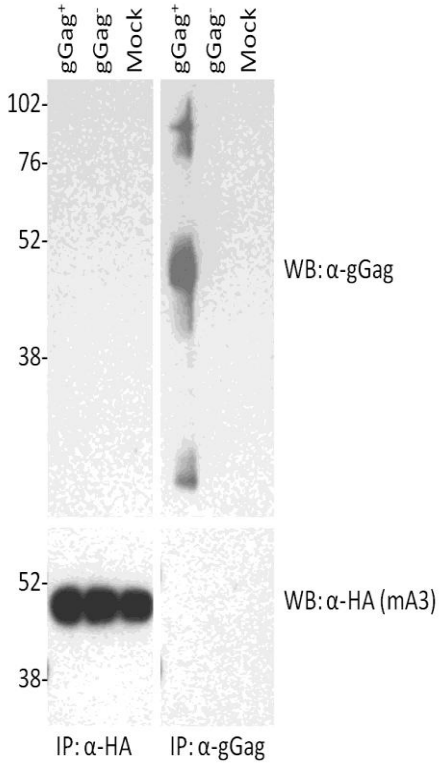

Figure 4.2. Immunoprecipitation of infected 3T3/mA3 cells. Infected 3T3/mA3 cell lysates were immunoprecipitated with a monoclonal anti-HA antibody or the polyclonal rabbit anti-glycosylated gag antibody. Immunoprecipitated samples were eluted by addition of running buffer and boiling for 10 minutes. The samples were then subjected to SDS-PAGE followed by transfer onto PVDF membranes. Membranes were analyzed by immunoblot technique.

4.4.3 Localization of mA3 and gGag in infected cells

It is possible the reaction conditions used in the immunoprecipitation reactions may disrupt weak interactions or that interactions between the mA3 and gGag proteins are transient. Human APOBEC3G has been suggested to associate with lipid rafts in cells[116]. An association of mA3 with lipid rafts has not been demonstrated, however

gGag has been reported to associate with lipid rafts during the process of virus budding from the cell [96,123] . It was of interest to determine where mA3 and gGag localize in an

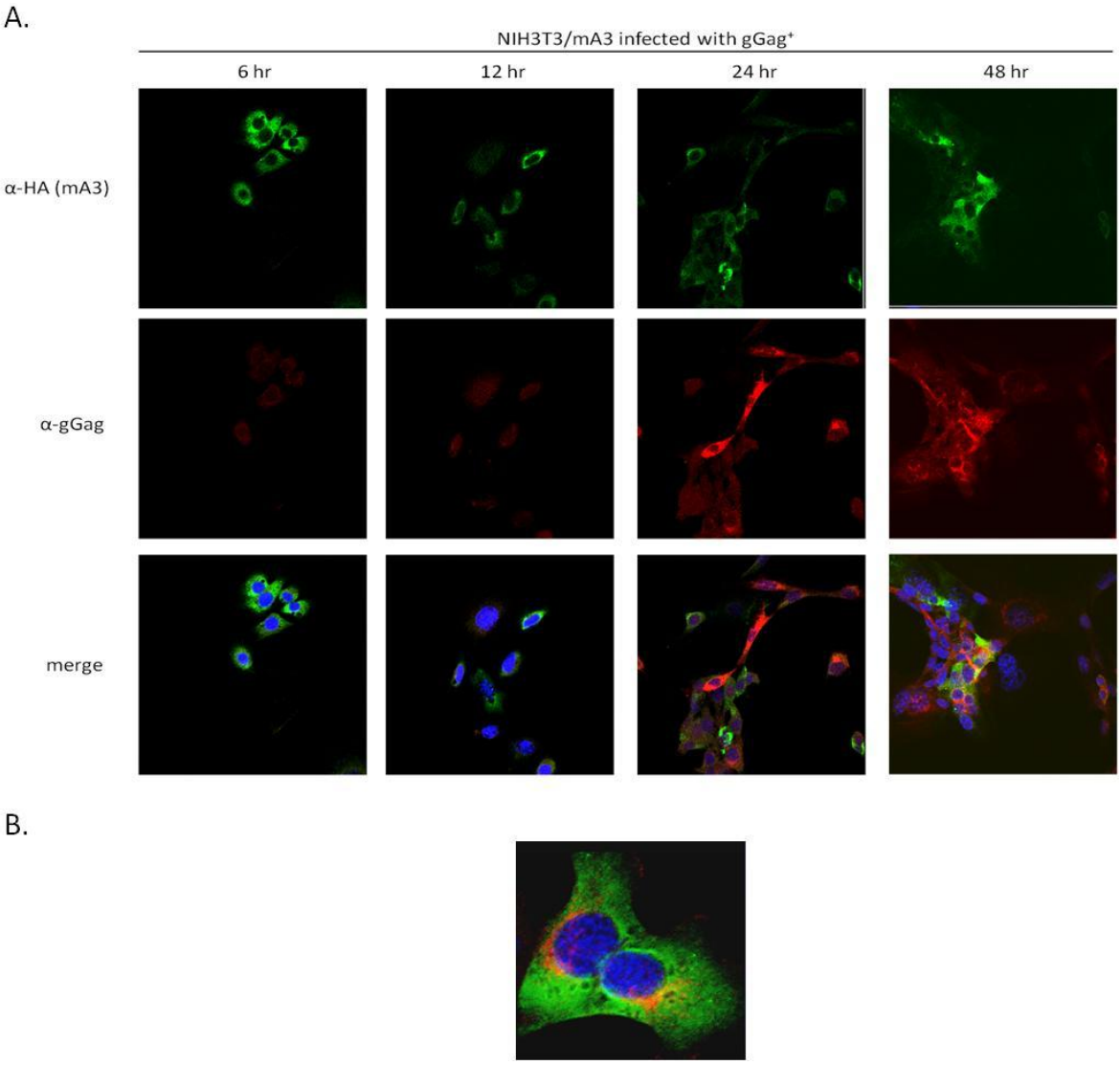

Figure 4.3. Localization of mA3 and gGag in an infected cell. 3T3/mA3 infected with the gGag^{+} MuLV. At 6, 12, 24, and 48 hours post infection cells were processed for colocalization analysis with monoclonal rat anti-HA and DylightFluor 488 conjugated Goat anti-rat IgG (green) or polyclonal rabbit anti-glycosylated gag IgG and DylightFluor 594 conjugated Goat anti-rabbit IgG (red) with Hoechst 33342 (blue) **A).** 3T3/mA3 cells infected with the gGag^{+} MuLV, processed for colocalization analysis 6, 12, 24 and 48 hours post infection (40 X). **B).** 3T3/mA3 infected with the gGag^{+} MuLV, processed for colocalization analysis 48 post infection (100X).

infected cell and determine if there is colocalization of the two proteins at different times after infection. 3T3 or 3T3/mA3 cells were grown on cover slips and infected with $gGag^+$, $gGag^-$ MuLVs or mock-infected. At different times after infection samples were processed for localization analysis. In agreement with other reports, mA3 was localized predominantly in the cytoplasm whereas gGag occupied a perinuclear location suggesting processing in the endoplasmic reticulum (Figure 4.3B). At no time after infection did gGag appear to be colocalized with mA3 in $gGag^+$ MuLV-infected cells (Figure 4.3A). Taken together with the immunoprecipitation studies, these findings suggest that gGag and mA3 do not directly interact in an infected cell.

4.4.4 Localization of gGag and mA3 in virions

In an earlier report, both mA3 and gGag were identified as virion components in $gGag^+$ virions released from mA3-expressing cells[101]. Differences in localization or association in the virion with gGag may have an influence on how virion-associated mA3 exerts its activity in an infection. It is unknown where gGag exists in the virion and the location of mA3 in the virion is unclear, though one report suggested that some mA3 exists in the viral core[162]. If gGag is a type II transmembrane protein as reported[65,147], it might be expected to be located on the surface of the virion as a component of the envelope and thus be unavailable to interact with mA3. To determine the localization of gGag and mA3 in the virion, 3T3/mA3 cells were infected with $gGag^+$ viruses. Purified virion samples were pelleted through a 10-30% sucrose step gradient in which the 10% sucrose layer contained the non-ionic detergent, IGEPAL. Non-ionic detergents such as IGEPAL or NP-40 have been used in similar gradients in several reports to separate viral cores from the membrane bound virion components[3,136,140,162].Virion pellets were

analyzed via immunoblot techniques. Under conditions in which the virion membrane envelope protein gp70 (SU) is completely stripped from the virion, both mA3 and gGag proteins persist in the sedimented material suggesting that both proteins are components of the viral core. Thus, their virion localization does not preclude an interaction between them (Figure 4.4). It is noteworthy that conditions that result in the loss of the SU protein from virions also results in the loss of substantial levels of the major virion core protein, p30 (CA) and may reflect a similar loss of mA3 and gGag from the virion core during their fractionation[162].

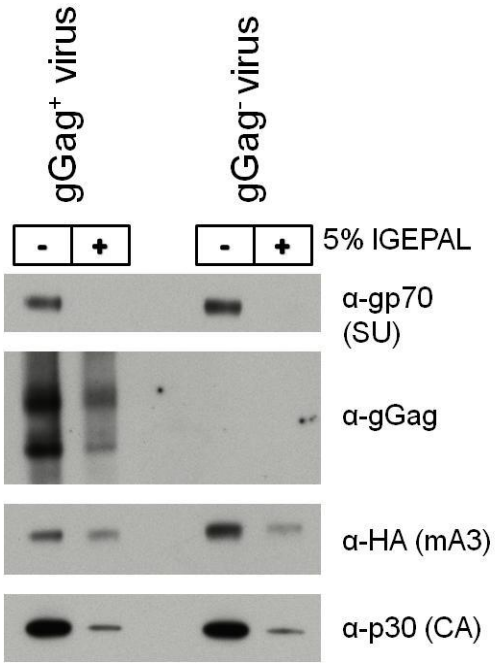

Figure 4.4. Isolation of viral cores from virions produced in mA3 cells. $gGag^+$ or $gGag^-$ MuLVs from mA3/3T3 cells were purified and sedimented through a 10-30% sucrose step gradient in PBS with IGEPAL in the 10% layer of the sucrose gradient at a concentration of 5%. Resulting pellets were analyzed by immunoblot technique with monoclonal anti-HA HRP-conjugated antibody, polyclonal rabbit anti-gGag HRP-conjugated antibody, polyclonal goat anti-gp-70 antibody or an HRP-conjugated monoclonal anti-capsid (p30) antibody (mAb 18-7).

4.4.5 Interactions between gGag and mA3 in virions

In our earlier studies, we observed that virions that have incorporated mA3 exhibit a lower infectivity and that this inhibition is gGag-dependent[101]. The finding that both mA3 and gGag exist in the virion core suggests the possibility of an interaction

between mA3 and gGag in the virion that was not observed in the cell. Virions were collected from mA3 cells infected with $gGag^+$ or $gGag^-$ MuLVs 48 hours post infection. The virions were lysed and immunoprecipitated with anti-HA or anti-gGag conjugated to protein G magnetic beads. The immunoprecipitated samples were analyzed by immunoblotting procedures. An antibody to mA3 was able to precipitate mA3 but did not result in the coprecipitation of gGag (Figure 4.5). There are a number of possible explanations for the inability to demonstrate an interaction between mA3 and gGag using the mA3 antibody. These include stoichiometric differences, stearic hindrance, or differences in antibody affinities. In contrast, the reciprocal experiments clearly demonstrated that the mA3 protein was precipitated by antibodies to gGag in virions containing gGag but not from $gGag^-$ virions indicating an interaction between mA3 and gGag in the virion (Figure 4.5).

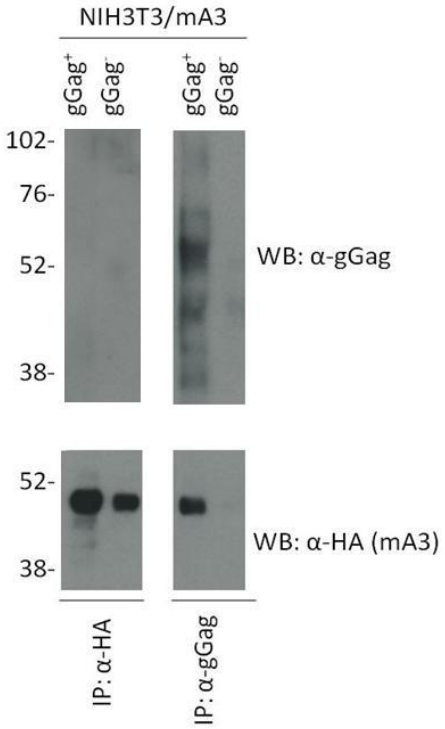

Figure 4.5. Immunoprecipitation of mA3 containing virions. $gGag^+$ or $gGag^-$ MuLVs from mA3/3T3 cells were purified and immunoprecipitated with Protein G beads conjugated with monoclonal anti-HA antibody or the polyclonal rabbit anti-glycosylated gag antibody Resulting complexes were analyzed by immunoblot technique with monoclonal anti-HA HRP-conjugated antibody or the polyclonal rabbit anti-gGag HRP-conjugated antibody.

4.4.6 The cellular mA3 protein does not induce G-to-A hypermutation in viral transcripts

mA3 has been reported to induce hypermutation in the reverse transcripts of several retroviruses including the XMRV and AKV MuLVs as well as HIV resulting in a reduction of infectivity[14,74,110]. Our previous report revealed that both cellular and virion-associated mA3 inhibited the $gGag^-$ MuLV but not the $gGag^+$ MuLV[101]. It is possible that this inhibition was the result of deamination mediated by mA3. To investigate this possibility, viral transcripts from 3T3/mA3 cells infected with $gGag^+$ or $gGag^-$ viruses were amplified by PCR, cloned and sequenced to determine point mutations incurred during infection. Parallel experiments were performed in 3T3 cells devoid of mA3. DNA was isolated early (24 hours) after infection to limit the analyses to transcripts that had undergone a single replication cycle. No evidence of G-to-A hypermutation was observed, nor was there a significant difference in the G-to-A mutation rate between transcripts generated in $gGag^+$ or $gGag^-$ MuLV-infected cells. Surprisingly, a significantly higher overall point mutation rate was observed in transcripts from 3T3/mA3 cells infected with the $gGag^-$ virus compared to 3T3/mA3 cells infected with the $gGag^+$ MuLV while a significant difference in overall mutation rate was not observed in 3T3 cells (Figure 4.6). Examination of the mutation rates for all possible base changes did not suggest the prevalence of a particular type of mutation contributing to the overall mutation rate difference (data not shown). These results suggest that mA3 may exert an influence on reverse transcription independent of cytidine deaminase activity.

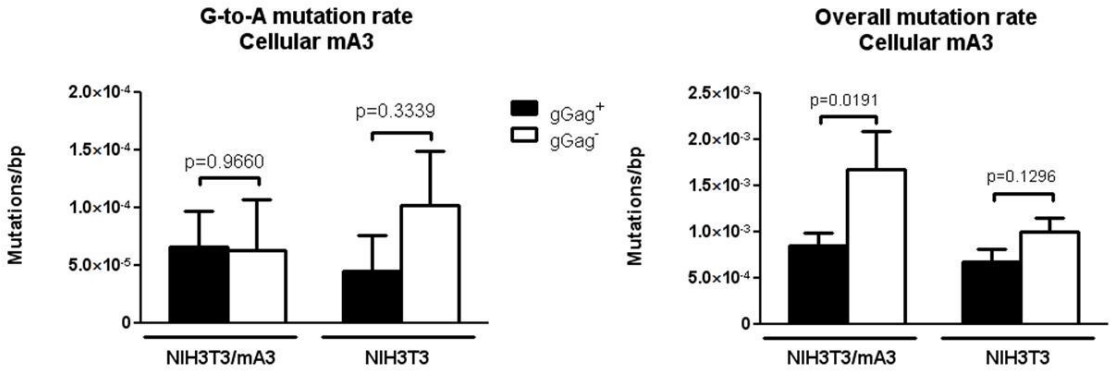

Figure 4.6. The mutation rates of samples exposed to cellular mA3. For analysis of cellular mA3s activity, 3T3 or 3T3/mA3 cells were infected either $gGag^+$ or $gGag^-$. Twenty-four hours after infection cells were lysed and viral sequences amplified by PCR, cloned and sequenced. The mutation rates for each clone were averaged to give the mean mutation rate as well as the standard error. $gGag^+$ NIH3T3/mA3=81 clones, $gGag^-$ NIH3T3/mA3=42 clones, $gGag^+$ NIH3T3=63 clones, $gGag^-$ NIH3T3=78 clones.

4.4.7 Mutation rates in transcripts generated by cell-free virions

Uracil-containing DNA is rapidly degraded in cells by apurinic/apyrimidinic endonuclease (APE)[13,105,185]. The cytidine deaminase activity of mA3 results in a conversion of cytidine to uracil in the single-stranded transcripts generated by RT and it is possible that such transcripts are rapidly eliminated. If this were to occur it would bias the analyses of the mutation rate in infected cells and hypermutation would be apparent in a reaction without cellular factors. To determine if cellular factors mask the true deamination rate, cell-free virions produced from mA3 cells infected with $gGag^+$ or $gGag^-$ MuLVs were isolated and employed in endogenous RT reactions to generate transcripts in the absence of cellular factors. The determination of the G-to-A and overall mutation rates in the endogenous reverse transcription reactions did not reveal evidence of hypermutation nor were significant differences in mutation rates observed between the samples (Figure 4.7). Taken together, these mutational analyses suggest that cytidine deamination is not the main mechanism used by mA3 to inhibit MuLVs in a gGag-dependent manner.

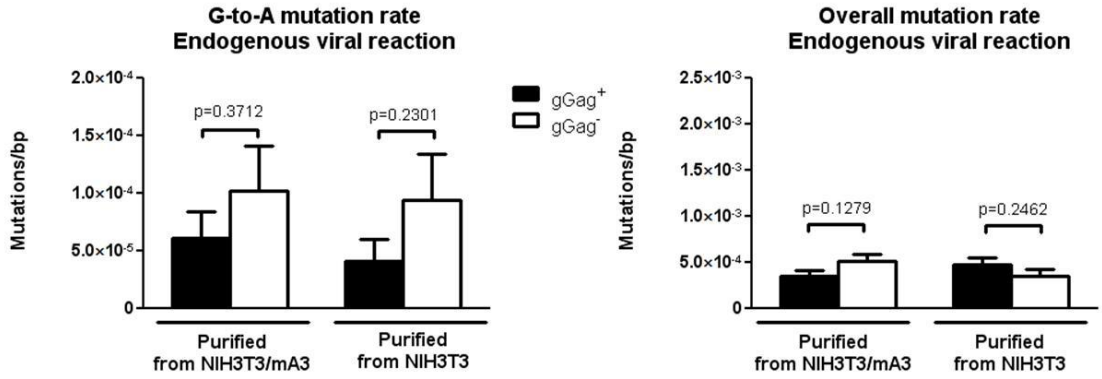

Figure 4.7. The mutation rates of samples in the endogenous viral reactions. For analysis of mA3s activity in a cell free environment, 3T3 or 3T3/mA3 cells were infected either $gGag^+$ or $gGag^-$. Virions were collected and used in cell-free endogenous RT reactions. Viral sequences amplified by PCR, cloned and sequenced. The mutation rates for each clone were averaged to give the mean mutation rate as well as the standard error. $gGag^+$ purified from NIH3T3/mA3=152 clones, $gGag^-$ purified from NIH3T3/mA3=169 clones, $gGag^+$ purified from NIH3T3=164 clones, $gGag^-$ purified from NIH3T3=156 clones.

4.4.8 Mutation rates in transcripts induced by virion-associated mA3 in infected cells

It has been reported that cellular hA3g reduces the level of accumulated reverse transcripts in infected cells but does not induce G-to-A hypermutation[29]. In contrast, virion-associated hA3g induces G-to-A hypermutation upon infection[26,27]. To determine if virion-associated mA3 exerted a similar affect, naïve *Mus dunni* cells were infected with viruses released from 3T3 or 3T3/mA3 cells. Twenty-four hours post infection viral transcripts were analyzed for point mutations as described above. No evidence of hypermutation was apparent nor were significant differences observed in the G-to-A mutation rate or in the overall mutation rates between the samples (Figure 4.8). It is noted that the experiments determining the mutation rates of virion-associated mA3 are complicated by the procedures employed to generate the viruses. Progeny viruses were obtained after infection of 3T3 cells or 3T3/mA3 and incurred point mutations during this process. Although precise determinations of mutation rate are compromised by this process, hypermutation as a result of virion-associated mA3 would be apparent.

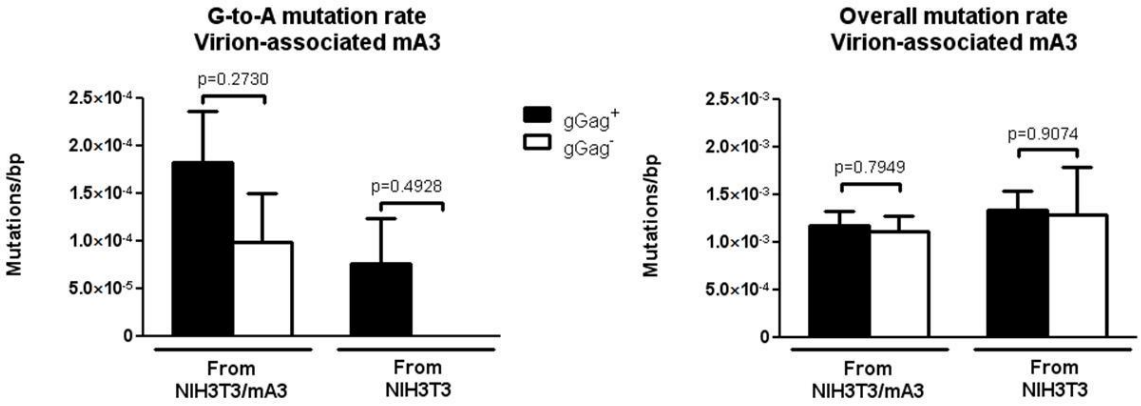

Figure 4.8. The mutation rates of samples exposed to virion-associated mA3. For analysis of virion-associated mA3s activity, 3T3 or 3T3/mA3 cells were infected either $gGag^+$ or $gGag^-$. Virions were collected and used infect *Mus Dunni* cells. Cells were lysed and viral sequences amplified by PCR, cloned and sequenced. The mutation rates for each clone were averaged to give the mean mutation rate as well as the standard error. $gGag^+$ from NIH3T3/mA3=87 clones, . $gGag^-$ from NIH3T3/mA3=67 clones, $gGag^+$ from NIH3T3=84 clones, . $gGag^-$ from NIH3T3=21 clones

4.4.9 Cellular mA3 reduces viral transcript accumulation in a gGag-dependent manner

APOBEC3 proteins have been reported to utilize hypermutation-independent mechanisms to inhibit retroviral replication [4,29,79,100]. In this regard, a partial inhibition of M-MuLV by mA3 was reportedly associated with a reduction in the total amount of viral DNA accumulated in the cell[18]. In our experiments cellular mA3 was shown to inhibit viral replication in a gGag-dependent fashion[101]. It is possible that mA3 exerts an inhibitory effect on reverse transcription resulting in reduced levels of viral transcripts in infected cells. To determine if gGag influences the accumulation of viral DNA in infected cells, 3T3 or 3T3/mA3 cells were infected by $gGag^+$ or $gGag^-$ MuLVs and the level of accumulated transcripts determined. At 24 hours after infection, PCR amplification of viral DNA revealed a striking reduction in the transcript levels in 3T3/mA3 cells infected

with the gGag$^-$ virus compared to levels found in 3T3 cells (Figure 4.9B). In contrast, no significant reduction in transcript levels between the two cell lines was observed after infection by the gGag$^+$ virus. These results suggest a function of cellular mA3 that is inhibited by the presence of gGag that correlates with the reduction of infectivity.

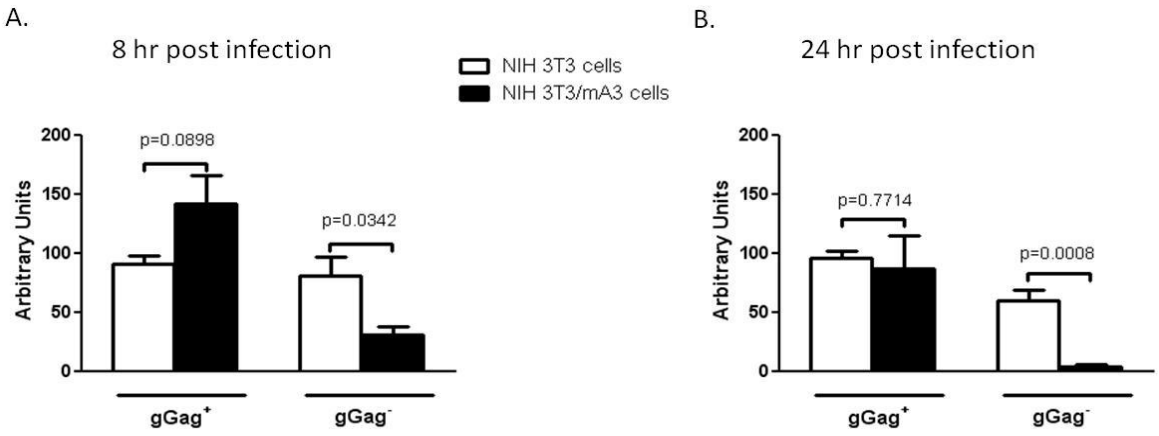

Figure 4.9. Quantification of viral transcripts in infected cells expressing mA3. gGag$^+$ and gGag$^-$ viruses were used to infect 3T3 and mA3 cells. Eight **(B)** and twenty-four **(A)** hours after infection cells were lysed and viral sequences amplified by PCR. GAPDH PCR products were used as loading controls and samples were quantitated using a standard curve.

At 24 hours after infection it is expected that most viral DNA in the cell exists as integrated proviruses in the host genome. Thus, the reduction in viral DNA at this time could reflect processes that occurred after reverse transcription rather than an effect of mA3 on reverse transcription itself. To examine this possibility, viral transcript levels were determined 8 hours after infection. At this time, very little, if any, viral DNA would be in the form of integrated proviruses and would more closely reflect immediate products of reverse transcription. These analyses also revealed a significant decrease in the level of transcripts in 3T3/mA3 cells compared to 3T3 cells when infected with the gGag$^-$ MuLV (Figure 4.9A). A similar difference in the level of transcripts in these cells was not observed upon infection with the gGag$^+$ virus. These results suggest that the

reduction in viral transcripts in cells infected by the gGag$^-$ virus in the presence of cellular mA3 is the result of an effect of mA3 on reverse transcription rather than a subsequent process during infection.

4.4.10 Viral transcript levels in cells infected by viruses containing mA3

Our previous studies indicated that virion-incorporated mA3 is also inhibitory to infection in a gGag-dependent manner[101]. In these studies a substantial reduction in the infectivity of individual virions (specific infectivity) was observed. The experiments above suggest that the reduction in transcripts of gGag$^-$ viruses in the presence of cellular mA3 occurs at the level of reverse transcription rather than at a later stage of infection. If the inhibition of specific infectivity is due to inhibition of reverse transcription occurring at a post-entry stage of infection, then infection of naïve cells by an equal number of gGag$^+$ or gGag$^-$ MuLVs containing mA3 would be expected to result in a reduction of transcripts of the gGag$^-$ compared to the gGag$^+$ virus. Alternatively, it is possible that the mechanism of inhibition by cellular mA3 differs from that of virion-associated mA3 and might involve post-transcriptional events. These analyses were undertaken to determine if the decrease in specific infectivity of mA3-containing gGag$^-$ virions observed in earlier experiments (Chapter 3) was a result of events occurring early in infection, before or during reverse transcription or, alternatively, at a later stage of infection subsequent to reverse transcription. To examine this possibility naïve *Mus dunni* cells were infected with gGag$^+$ and gGag$^-$ MuLVs harvested from 3T3 or 3T3/mA3 cells and normalized to equal levels of virions. Amplification of viral DNA 24 hours after infection revealed that viral transcripts were virtually undetectable in cells infected with gGag$^-$ MuLVs

containing mA3 (Figure 4.10). These results are consistent with a process of mA3 inhibition of reverse transcription which is circumvented by gGag.

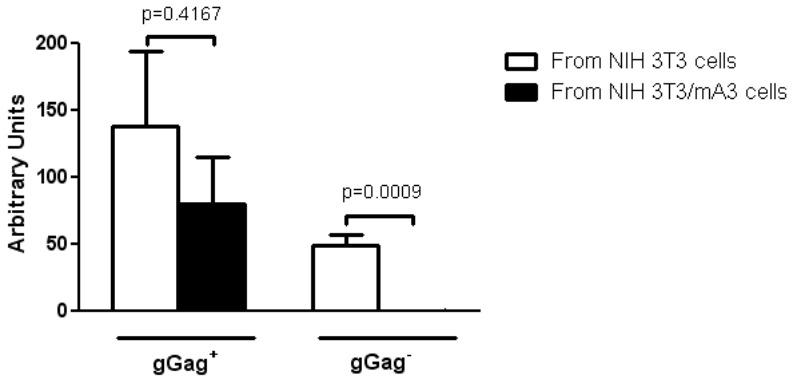

Figure 4.10. Quantification of viral transcripts in cells infected by virions containing mA3. $gGag^+$ and $gGag^-$ viruses (amount normalized with p30 quantification) from 3T3 or mA3 cells were used to infect *Mus Dunni* cells. Cells were lysed after 24 hours and viral sequences amplified by PCR. GAPDH PCR products were used as loading controls and samples were quantitated using a standard curve.

4.5 Discussion

In this chapter we investigated the mechanisms of the MuLV gGag-dependent escape from mA3 antiviral activity. We found in our experiments that mA3 was not depleted from infected cells. This differs from the way HIV uses its Vif protein to facilitate the proteolytic degradation of hA3g in infected cells[34]. Further suggesting the absence of a direct interaction in an infected cell, neither coimmunoprecipitation nor colocalization between mA3 and gGag was observed. Thus, an indirect interaction between mA3 and gGag in an infected cell is hypothesized to be responsible for the gGag-dependent inhibition of replication seen in mA3 cells.

Our analysis of the localization of gGag in the virion revealed that gGag is localized within the core along with mA3. This was a surprising result as gGag is

described as a transmembrane protein in an infected cell and would be expected to be localized with the envelope[65,147]. Nonetheless, this colocalization in the virion between gGag and mA3 could facilitate the abrogation of mA3 inhibitory activity. Coprecipitation experiments revealed that gGag and mA3 do interact in mature virions. The importance of this localization and interaction of mA3 and gGag in the virion is unknown, but may further signify a protective role for gGag from mA3 activity. Further experimentation will be necessary to delineate the importance of interactions of mA3 and gGag in the virion.

Previous studies of the action of APOBEC3 proteins on retroviral replication have predominantly examined the activity of virion-associated hA3G or mA3[17,20,22,23,34,77,96,100,124,126,128,132,179]. Our approach enabled the analysis of the action of mA3 expressed in the cell, which perhaps more closely reflects a natural retroviral infection. In our initial studies, cellular mA3 exerted a marked inhibition of virus infection in a gGag-dependent manner[101]. This result was somewhat unexpected considering that the incorporation of gGag in virions is thought to be quite low[63]. Here, the analyses of G-to-A mutation rates of viral transcripts in mA3-expressing cells were not significantly different between cells infected by the $gGag^+$ or $gGag^-$ viruses. However, a significant increase in the overall mutation rate was observed after infection by the $gGag^-$ MuLV. Further, the level of viral transcripts in mA3-expressing cells was strikingly reduced when infected with the $gGag^-$ virus. These results are consistent with our results of the effect of cellular mA3 on infectivity.

Our studies have revealed that that virion-associated mA3 reduced the specific infectivity of virions in a gGag-dependent manner[63] which was reflected in levels of viral

transcripts in infected cells. This observation is not without precedent, as another report indicated that mA3 may also possess deaminase-independent activity[18]. While a high number of G-to-A mutations was not seen in M-MuLV, the transcript levels were reduced in an amount comparable to the reduction in infectivity[18]. This suggests that the gGag of M-MuLV may not completely block the action of mA3. It is noted that analyses of other MuLV sequences reveal extensive differences in their gGags including deletions and amino acid substitutions (data not shown). In this regard, XMRV exhibits termination codons in the sequence encoding gGag and has been reported to be susceptible to the action of mA3.

Inhibition of MuLVs by cellular or virion-associated mA3 occurs in a gGag-dependent manner. The precise mechanism by which virion gGag abrogates the action of mA3 is unclear. Considering the high infectivity to particle ratios reported for many retroviruses[165], it is possible that a large number of "non-infectious" virions may nonetheless enter the cell and provide a sufficient amount of gGag protein to effect inhibition of cellular mA3. Alternatively gGag present in the virion could protect the virus from inactivation by mA3. Such an effect could be operative to protect against both cellular and virion-associated mA3. Our results strongly suggest that the mechanism of inhibition by mA3 involves an effect on reverse transcription of the viral genome and is largely independent of G-to-A mutation. It is possible that gGag exerts a stabilizing effect during reverse transcription or perhaps in the integrity of other virion components. It is also possible that gGag acts in a stearic manner preventing an interaction of mA3 with the RT or viral RNA. In the absence of gGag, there is an increase in the overall point mutation rate and a reduction of the level of transcripts in infected cells in the presence of

mA3. These two effects may be linked, thereby causing both a decrease in transcription efficiency as well as a decrease in RT fidelity. Further studies will help elucidate the protective effect of gGag against mA3 activity.

CHAPTER 5: Summary

5.1 Discussion

HIV evades the action of hA3G through expression of an accessory protein, Vif, however the means by which exogenous MuLVs evade the action of mA3 was unclear[28,162]. We have shown that gGag acts as an antagonist of cellular and virion-associated mA3 *in vitro* and have found gGag to be essential for *in vivo* replication in mA3 expressing mice. The mechanistic basis for these observations is largely independent of G-to-A mutation but is associated with a reduction of reverse transcripts in infected cells.

Inhibition of infectivity by mA3 was observed in two distinct contexts in our *in vitro* system: during the initial infection in which viruses devoid of mA3 infect cells expressing mA3 (cellular mA3); and during the subsequent infection in which viruses containing mA3 (virion-associated mA3) infect naïve cells not expressing mA3. Inhibition by cellular mA3 is not without precedent. It has been reported that M-MuLV[123] and mouse mammary tumor virus[138] are partially inhibited by cellular mA3. HIV has also been reported to be inhibited by cytoplasmic hA3g[196]. Nevertheless, it was unexpected that cellular mA3 exerted a gGag-dependent effect on MuLV infectivity in the absence of virion-associated mA3 considering that the level of gGag incorporated into the virion is low compared to that which is expressed in an infected cell[54,63]. Furthermore, the small amount of gGag in the virion would be expected to associate with the viral envelope as a type 2 transmembrane protein[65,147]. Our experiments involving the localization of gGag in the virion shows clearly that it is associated with the virion core. There are many possible functional consequences of this localization. One of these is that gGag may serve

to stabilize the structure of the virion. It has been reported that gGag is involved in virion release and that gGag$^-$ M-MuLV exhibits an abnormal morphology during virion budding[121]. Mature virions also differed from the wild-type virions by envelope lipid content but did not exhibit detectable morphological differences. In agreement with a number of other studies with different MuLVs, the gGag-deficient M-MuLV was not replication-defective *in vitro* but replication was severely limited *in vivo*[121].

A number of mechanisms by which cellular mA3 might interact with gGag were examined in these studies. The HIV-1 Vif protein mediates the degradation of hA3G in infected cells and it was possible that the presence of gGag might lead to mA3 degradation[28,162]. However, no significant difference in the level of mA3 expression was detected in the presence or absence of gGag. Thus, viral gGag does not lead to the proteolytic degradation of mA3 *in vitro*. The inhibition of cellular mA3 by gGag may involve a direct interaction of the proteins that delays or prevents mA3 from exerting its effect. In this regard, immunoprecipitation experiments using antibodies directed at either protein did not result in coprecipitation of the proteins. It is possible that interactions between cellular mA3 and gGag may exist but be too weak or fleeting to be detected by immunoprecipitation techniques. If the proteins do interact in infected cells, it is possible that colocalization to the same cellular compartment might be detected by confocal microscopy; however, no specific colocalization of the two proteins was detected when examined at various times after infection. In the absence of evidence supporting a direct interaction, it was surmised that there may be an indirect interaction between cellular mA3 and viral gGag that enables the virus to escape the activity of mA3.

Although the mutation data did not reveal a substantial cytidine deaminase

activity for cellular mA3, a higher overall mutation rate was observed with gGag$^-$ than with gGag$^+$ MuLVs. This may reflect a functional role of gGag in promoting the fidelity or stability of reverse transcription and counteracting a destabilizing action of mA3. Such a role is consistent with the location of gGag in the virion and suggests it may interact with RT, RNA or other virion components in the reverse transcription complex to stabilize the reverse transcription reaction. It has been reported that APOBEC3 proteins exert deaminase-independent inhibitory activity on retroviral replication, though the mechanism by which it is accomplished is poorly defined. In this regard a recent study reported that M-MuLV was partially inhibited by mA3[18]. An elevated G-to-A mutation rate was not observed, however, the transcript levels were reduced in an amount comparable to the reduction in infectivity. Similarly, we observed a striking inhibition of viral transcription levels after infection of cells with the gGag$^-$ virus. While the gGag$^+$ transcript levels remained consistent between 3T3/mA3 and 3T3 cells, the gGag$^-$ transcript levels were significantly reduced in the presence of mA3. A number of studies have reported varying susceptibilities of MuLVs to mA3[110,123,162,188] . Differences in the susceptibility of MuLVs to inhibition by cellular mA3 could reflect differences in the efficacy of their respective gGags to counteract mA3. In this regard, a comparison of the gGags of different MuLVs reveals subtle to extensive differences which include deletions as well as amino acid substitutions (data not shown).

We observed that progeny virions released from mA3 expressing cells were also inhibited in a gGag-dependent manner when tested on cells not expressing mA3. It is possible that both gGag$^+$ and gGag$^-$ MuLVs incorporate mA3 into virions at similar levels but that the incorporation of mA3 is qualitatively different between the MuLVs. The

location or association of mA3 with virion components may be altered in response to the presence of gGag. The observation that mA3 is located in the virion core for both the $gGag^+$ and $gGag^-$ MuLVs argues against this possibility. The presence of both gGag and mA3 in the virion core may allow for an interaction between the two proteins. In contrast to the immunoprecipitation analyses in infected cells, coprecipitation of mA3 and gGag was observed in the virion. The proximity of mA3 and gGag to each other in the virion or the presence of a binding intermediate, such as the RNA genome or other binding partners may facilitate coprecipitation in the virion but not in the cell. Further experimentation will reveal if viral RNA or other proteins are associated with mA3 and gGag.

The binding interaction observed in these analyses may function to prevent mA3 from exerting its antiretroviral activity on the virus during reverse transcription upon infection. No statistically significant differences of the mutation rates were observed between any of the viruses regardless of virion incorporation of mA3 or gGag. However, the transcript level was severely reduced in *Mus dunni* cells infected with the $gGag^-$ viruses produced from 3T3/mA3 cells. In these experiments the level of virus used to infect the *Mus dunni* cells was adjusted to the number of virions rather than the infectivity. Assuming that the presence of mA3 in the absence of gGag does not alter the initial events of viral receptor binding and fusion to enter the cells, it is likely that the process of reverse transcription was altered in the infected cells. Conversely, in cells infected with the wild-type virus possessing gGag and mA3, inhibition was presumably prevented by the interaction of gGag and mA3.

The results of the *in vitro* studies were corroborated in our *in vivo* investigations.

There was a striking inhibition of replication *in vivo* of the gGag$^-$ MuLV compared to the gGag$^+$ MuLV in mice expressing mA3. In mice lacking mA3, the level of Gag$^-$ replication was restored to the high levels of gGag$^+$ MuLV. These results were obtained in both the 129/Ola and C57BL/6 genetic background. Development of reagents, such as a specific mA3 antibody, will facilitate *in vivo* studies as one could determine beyond mRNA levels the amount and type of mA3 expressed in tissues and the amount that is incorporated into virions. The development of specific tags and/or refinements in the purification of virions from mice may also make it possible to more precisely determine the amount of gGag incorporated into virions.

5.2 Future directions

Based on the localization of gGag in the virion core, gGag may serve a function in stabilizing the mature virion or the reverse transcriptase complex. To this end, a recent report indicated that gGag exhibits some functional similarities to the Nef protein of HIV[149]. Nef is an accessory protein of HIV that exhibits many different functions including increasing the efficiency of reverse transcription[168]. Nef is incorporated into virions and facilitates replication both *in vitro* and *in vivo*[19,94,95,144]. Nef-deficient viruses exhibit reduced infectivity which can be restored by complementation with gGag. Indeed, the complementing activity of gGag was discovered in cells which had been inadvertently infected with an MuLV[149]. It is unknown if Nef has an influence on the action of APOBEC3 proteins nor is it clear if Nef can complement the effect of gGag on MuLV replication Further studies of the action of Nef may provide insight into the precise mode of action of gGag on the activity of mA3.

The susceptibility of the gGag$^-$ MuLV to cellular mA3 may occur by an indirect

mechanism. In this regard, it has been reported that gGag is involved in virion release and that gGag$^-$ M-MuLV exhibits an abnormal morphology during virion budding[121]. It is conceivable that mature virions may be altered from an mA3-resistant to an mA3-susceptible phenotype. If an altered mA3-sensitive phenotype exist, then it may be possible to restore it by infecting cells that contain a gGag construct or by superinfecting cells with another MuLV encoding a functional gGag. Indeed, Low et al[121] showed that expressing a gGag construct in a retroviral packaging cell line restored the gGag$^-$ budding virion phenotype to a more common spherical one. It is of interest to determine if the expression of gGag in a cell would restore the infectivity of progeny virions on mA3 expressing cells. Further, if complementation in *trans* could be demonstrated, it might be possible to compare the efficacies of different MuLV gGag proteins.

In the *in vitro* studies, the gGag$^+$ MuLV was not inhibited, however, there has been a number of studies that report partial to marked inhibition of other gGag$^+$ MuLVs[109,122,162,187]. One report compared the inhibitory effects of mA3 on M-MuLV and AKV MuLV. AKV was inhibited to a greater extent than M-MuLV[108]. Inhibition of AKV by mA3 exhibited G-to-A mutations whereas M-MuLV did not[107,162]. The susceptibility of MuLVs to inhibition by mA3 could represent a difference in the efficacy of gGags to counteract mA3. Comparing the gGag sequences of multiple MuLVs revealed differences in their amino-terminal fragments. It is of interest to determine if the inhibition of mA3 by gGag of viruses utilized in this study can be generalized to other gGag$^-$ viruses. This could provide insight into differences in the activities of gGag proteins from different sources. As mentioned above, it may also be possible to directly compare efficacies of different MuLVs through complementation studies.

Some studies have noted that there are allelic differences between different mouse strains in the both the levels and isoforms of expressed mA3. Some mice express the full length protein, while others express a Δ exon 5 or a Δ exon 2 splice variant[111,139,162,163]. There are conflicting reports on the inhibitory activities of these variants. In our studies, there was a gGag-dependent inhibition in 129/Ola mice, which predominately produce the full-length version, and in C57BL/6 mice, which predominately produce the Δ exon 5 splice variant. It is unknown if there are differences in the activities *in vivo* as the expression levels are different between mouse strains. In order to address this issue, it will be necessary to conduct parallel *in vitro* studies using cells expressing full length mA3 as well as the spice variant forms of the protein.

During the course of these investigations it was noted that some uninfected $mA3^{-/-}$ mice developed a proliferative disease at 6 to 8 months of age. Spontaneous proliferative diseases in mice are often accompanied by the expression of endogenous retroviruses. It will be of interest to determine if the expression of endogenous viruses is demonstrable in $mA3^{-/-}$ mice that have developed proliferative disease.

In closing, the studies conducted for this dissertation are of particularly interest in light of recent reports indicating cross-species retroviral infections from mouse to humans[120,166,193]. In some studies, these infections are evidenced by the detection of endogenous gGag-deficient MuLVs. Infection of mice by $gGag^{+}$ viruses may inhibit the activity of mA3 and facilitate the mobilization of endogenous MuLVs through pseudotyping. Certain MuLVs such as amphotropic and xenotropic MuLVs encode intact gGag proteins and can infect humans. Pseudotyping of endogenous MuLVs by these viruses could account for the transmission of endogenous gGag-deficient MuLVs.

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