Evaluating the Conservation of RNA Packaging Enhancer Elements Across Lentiviruses

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Abstract

It was previously thought that the RNA structural elements within the 5’UTR were the sole determinants of RNA packaging in HIV-1. However, in 2013 Chamanian et al. discovered that the region overlapping the Ribosomal Frameshift Signal (RFS) acted to enhance RNA packaging 50 fold and was thus named the genomic RNA packaging enhancer element (GRPE). To determine if the GRPE was conserved across lentiviruses, using the same approach as Chamanian et al., deletions in similar regions of the SIVmac239 and FIV 34TF10 genomes were done to measure their impact on RNA packaging. No region across the gag-pol ORF of SIVmac239 had any impact on RNA packaging, and the deletion of the RFS in FIV 34TF10 had no impact either, although the RRE was disposable in the SIVmac239 system but necessary in FIV34TF10. This study shows that the GRPE is likely specific to HIV-1.

Keywords
HIV
SIV
FIV
Lentivirus
RNA Packaging
RNA Export
RNA Structure
RNA Stem-loops
Nucleocapsids
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Chapter 1

1 « Introduction »

1.1 « HIV-1, The Causative Agent of AIDS »

In 1981, physicians in New York and San Francisco were puzzled after 41 homosexual men were diagnosed with a rare form of cancer known as Kaposi’s sarcoma. Nine of these patients were tested and discovered to have severe immune defects, specifically with their B and T cell lymphocytes. Initially thought to be a disease specific to the gay population, the immunodeficiency was referred to as Gay Related Immunodeficiency but then renamed to Acquired Immunodeficiency Syndrome (AIDS) since females and heterosexuals in Africa were being diagnosed with a similar immunodeficiencies that led to opportunistic infections. Researchers began taking samples from these patient groups to identify the agent causing these diseases. By 1984, Luc Montagnier and Robert Gallo independently identified the virus causing AIDS, which ultimately became known as Human Immunodeficiency Virus (HIV). HIV was found to preferentially infect CD4+ T helper cells, and over time a depletion of the body’s CD4+ T cell count cripples the immune system, making the patient infected susceptible to secondary co-infections that could prove to ultimately be fatal.

Over thirty years have passed since the identification of HIV and there is still no vaccine or functional cure. In that time, AIDS has been responsible for over 35 million deaths, and at the end of 2016 the WHO reported that 36.7 million people were currently infected with HIV. Thankfully, the development of antiretrovirals has helped suppress viral replication and prevent progression towards AIDS but these drug regimens aren’t
always available to the majority of those infected with the virus and drug resistance still
remains an issue. Therefore, scientists continue to investigate the different parts of the
replication cycle and compare and contrast that with related viruses to try to identify new
targets for treatment and eradication.

1.2 «Retroviruses»

HIV is a Lentivirus, which falls under the *Retroviridae* family. Retroviruses are
enveloped viruses that contain two copies of positive sense single stranded RNA. The
earliest discoveries of retroviruses date back to the beginning of the 20\textsuperscript{th} century when
different groups were able to identify viruses responsible for leukosis and sarcomas in
avian species\textsuperscript{6}. Perhaps the most important discovery in the field of retrovirology was
made in 1970 when Howard Temin and David Baltimore both identified a protein unique
to retroviruses that is able to convert single stranded viral RNA into double stranded
DNA\textsuperscript{7,8}

Temin had proposed a theory that Rous Sarcoma Virus (one of the first identified
retroviruses) replicated through a DNA intermediate step, and although many had
initially doubted his “provirus” theory, the discovery of reverse transcriptase confirmed it. Upon virus entry, reverse transcriptase is able to synthesize proviral DNA from the
viral RNA, which is subsequently integrated into the host chromosome. Integration into
the host cell genome is a hallmark of retroviral infection and contributes to its ability to
establish latently infected cells, leaving a patient asymptomatic for long periods of time
post infection\textsuperscript{9}. This characteristic latent period of infection was seen in patients that were
diagnosed with AIDS which is the reason Gallo and Montagnier decided to look for a
retrovirus.
Retroviruses can be classified as either simple retroviruses or complex retroviruses based on their genome’s protein coding capacity. Simple retroviruses contain three open reading frames (ORFs) necessary for encoding the Gag, Pol, and Env genes and are further subdivided into alpharetroviruses, betaretroviruses and gammaretroviruses. In addition to gag, pol and env genes, complex retroviruses are also able to code for accessory genes that aid in pathogenesis and immune evasion. The complex retroviruses can be further subdivided into deltaretroviruses, epsilonretrovirus, spumaviruses and lentiviruses\^{10}.

1.3 « Lentivirus Evolution»

It comes as no surprise that HIV is the most studied retrovirus due to its devastating effects on the infected human population, but researchers continue to study other lentiviruses to be able to better understand the virus’s evolutionary past as well as develop other animal models to test treatments.

The most abundant strain of HIV and the strain responsible for the epidemic is HIV-1. These HIV-1 strains are further subdivided into groups M, N, O and P, with group M making up the vast majority of human infections\^{11}. A second, less pathogenic, strain of HIV has also been discovered in humans named HIV-2. HIV-2 is less transmissible than HIV-1 and patients infected with HIV-2 typically have a much slower rate of CD4+ T cell decline as well as reduced viral loads in their blood.

The evolutionary past of HIV can be traced back to its closest relative, the Simian Immunodeficiency Virus (SIV)\^{12}. It is believed that HIV evolved through zoonotic transmission of SIV from non-human primates due to bush meat hunting in sub Saharan
Africa. The closest simian relative of HIV-1 was first discovered in two chimpanzees that were in captivity and was named SIVcpz. Further studies confirmed that SIVcpz was the source for the zoonotic transfer of SIV to humans, specifically the subspecies Pan troglodytes troglodytes. Interestingly, SIVcpz does not typically progress to AIDS. The origin of HIV-2 however, has been tracked to another independent zoonotic transfer, from SIV infected Sooty Mangebys in West Africa (SIVsm). These SIVsm infected primates also did not produce AIDS like symptoms however a closely related virus was identified in captive macaques in which the molecular clone, named SIVmac239, results in AIDS and is now used in the majority of macaque studies. The discovery of this virus has been crucial for animal studies since an AIDS like phenotype in non human primates is necessary to be able to gauge the efficacy of a certain treatments and vaccines.

Although SIV is the closest lentivirus relative to HIV, other lentiviruses that infect mammals, including cows, sheep, horses and cats, are also the subject of extensive research. Feline Immunodeficiency Virus (FIV) is one such lentivirus and also causes an AIDS like phenotype in domestic cats across the world. The transmission of FIV is typically through transfer of blood during cats fighting. Recent studies have been tackling the idea of using FIV as another model for vaccine and treatment testing as well as a vector for gene delivery in human cells, therefore understanding the molecular pathways of FIV can also be of great benefit to HIV research and treatment.
1.4 «Lentivirus Genome»

Lentiviruses like all other retroviruses contain gag, pol and env, but it is their additional accessory genes that allow them to evade the immune system and ultimately progress to AIDS (Figure 1). The first open reading frame in the lentivirus genome belongs to the group specific antigen or gag. Gag is translated from the full length unspliced mRNA as a polyprotein in lentiviruses and provides the structural proteins necessary for the assembly of viral particles. In HIV-1, Gag is synthesized as a 55kd polypeptide that contains 4 major subdomains and two spacer regions. At the 5’ end of the Gag polyprotein is the matrix protein (MA). The matrix protein contains a 5’ myristoylation site that is able to interact with phosphatidylinositol-4,5-bisphosphate (P45B) on the inner side of the cell’s plasma membrane. This interaction is responsible for targeting gag to the plasma membrane for virus assembly and budding. FIV matrix is very similar to that of HIV and SIV, in that it is also myristoylated and binds to P45B at the plasma membrane to initiate viral assembly. The next lentiviral subdomain of gag is the capsid domain (CA), which is responsible for the formation of the bullet shaped viral core. In HIV-1 and FIV, CA is 24kDa and thus referred to as p24, while in SIV it is slightly larger and referred to as p27, but all behave in a similar manner. CA contains two helical domains, one at each N terminal and C terminal end. The C terminal domain of CA contains a dimer interface that allows for the multimerization of gag to initiate viral core assembly. The C terminus of MA contains an α-helix that is able to interact with the N terminal domain of CA to target the viral core to the plasma membrane for the assembly and maturation of viral particles. Downstream of CA, is the nucleocapsid (NC) domain of Gag which contains two zinc finger domains which are able to bind
genomic RNA and target it for genomic RNA encapsidation\textsuperscript{25}. The final major subdomain of the gag protein in HIV and SIV is p6, a 6kDa protein necessary for the recruitment of ESCRT machinery that is co-opted by the virus for budding of newly synthesized particles\textsuperscript{26}. FIV does not encode for p6 but contains a similar protein at the 3’ end of Gag known as p2 that is required for efficient budding at the viral membrane\textsuperscript{27,28}.

Overlapping the 3’ end of the gag ORF is the beginning of the \textit{pol} gene. \textit{Pol} is found in all retroviruses and encodes all the enzymatic proteins necessary for viral replication \textsuperscript{29}. In HIV, SIV and FIV, Pol is synthesized as the Gag-Pol polyprotein when the ribosome shifts into the -1 reading frame while translating the full length unspliced mRNA\textsuperscript{30,31}. The \textit{pol} ORF begins with protease (PR) at the 5’ end. PR is autocatalytically cleaved from the precursor Gag-Pol polyprotein and then forms a homodimer with itself\textsuperscript{32,33}. Once in its homodimeric form, PR is able to proteolytically cleave the Gag-Pol polyprotein into its substituents during viral maturation after the virus has bud from the cell membrane\textsuperscript{34}. Both from a structural and functional standpoint, PR is fairly similar between HIV, FIV and SIV\textsuperscript{35}. The HIV and SIV Gag-Pol polyprotein contain similar cleavage sites and can be cleaved by . FIV PR only has 23% amino acid identity with HIV-1 and is not able to recognize and cleave HIV-1 Gag-Pol\textsuperscript{35}. 
Figure 1 Schematic Representation of Lentiviral Genomes (A) HIV-1 (B) SIVmac239 (C) FIV 34TF10.
MA=Matrix, CA=Capsid, NC=Nucleocapsid, PR=Protease, RT=Reverse Transcriptase and IN=Integrase.
The aforementioned reverse transcriptase (RT) is also within the pol gene. RT is first produced as a 66kDa protein but then protease cleaves a 15kDa segment at the C-terminus to produce a 55kDa protein which forms the functional RT heterodimer with the 66kDa precursor. RT is able to convert viral RNA to DNA but with no proofreading ability and a high error rate, resulting in a high mutation rate allowing the virus to evolve at a faster rate and become resistant to treatments. After RT has created the double stranded proviral DNA, the final protein in the pol gene, integrase (IN), is able to translocate the DNA into the cell nucleus and facilitate the integration into the host chromosome. One gene that FIV contains but is lacking in primate lentiviruses is dUTP diphosphotase (dUTPase) an enzyme that is capable of dephosphorylating dUTP, but studies on the protein have been lacking and its function within the FIV lifecycle, amongst other retroviruses, remains largely unknown.

The final gene encoded in all retroviruses is the env gene that makes up the viral glycoproteins on the virus envelope. In HIV, SIV and FIV, env contains two subdomains: the surface (SU) glycoprotein (gp120 in primate lentiviruses and gp100 in FIV) domain and the transmembrane (TM) glycoprotein (gp41 in primate lentivirus and gp50 in FIV) domain. The SU domain forms a trimer that is exposed on the surface of viral particles and facilitates binding of the virus to host cell receptors. The TM domain forms a heterotrimer with the SU domain and spans the cell membrane, with a cytoplasmic tail that extends into the cell cytosol. Env is synthesized as a precursor protein in the rough endoplasmic reticulum where it forms trimers and becomes heavily glycosylated as it moves through the Golgi complex towards the cell membrane. As gp160 (HIV, SIV) or gp150 (FIV) moves through the Golgi complex it is proteolytically cleaved by furin, a
host cell protease, into its subdomains which remain associated only by non covalent interactions. The SU subdomain that is exposed on the surface of viral particles determines the tropism of the virus, and interestingly the env gene is the gene with the most sequence diversity among HIV-1 subtypes.

Lentiviruses are complex retroviruses and as such are able to code for accessory genes in addition to gag, pol and env through alternative RNA splicing patterns (Figure 2). These proteins serve a host of different essential functions and in many cases deletions or mutations within these small non-enzymatic proteins can abolish viral infectivity.

Following integration of the viral genome, one of the first genes to be expressed in the HIV and SIV life cycle is the transcriptional activator or Tat protein. Tat is a 16kDa monomeric protein that is able to upregulate transcription of viral mRNA by binding the transactivation response region (TAR) in the 5’LTR of the integrated proviral DNA. Tat is able to recruit the PTEF-b complex to the 5’LTR which in turn activates RNA polymerase II and initiates transcription. Studies have shown that the HIV-1 Tat protein is able to trans activate transcription of the SIVmac239 genome but the SIVmac239 Tat protein is unable to bind the HIV-1 TAR hairpin and activate mRNA transcription. FIV in contrast does not encode for Tat but instead has another gene that is able to transactivate viral mRNA expression named orfA. Basal transcription levels before the binding of Tat to the HIV and SIV genomes are quite low, while FIV has a much higher level of basal transcript being expressed even without orfA binding, yet orfA is necessary for virus replication. The FIV genome does not contain a TAR region in it’s 5’LTR like other lentiviruses and instead the binding of orfA has been mapped to
the 3’ end of the U3 region, which results in a 14-30 fold increase over basal transcription rates\textsuperscript{48}.

Another crucial accessory gene that is expressed early in the lentivirus life cycle is the regulator of expression of virus or Rev. Rev is a protein that is able to bind a specific RNA sequence in the lentiviral genome\textsuperscript{49}. After Rev is expressed, it is translocated back into the nucleus to bind unspliced and partially spliced mRNA at the Rev Response Element (RRE)\textsuperscript{50}. The binding of Rev to the RRE facilitates export of unspliced and partially spliced mRNA through the Crm1 nuclear export pathway. Studies have also shown that Rev binding is necessary for maintaining the stability of the unspliced and partially spliced mRNA and regulating translation\textsuperscript{51–53}.

Some of the accessory genes that lentiviruses able to produce antagonize some of the innate immune mechanisms that host cells use to restrict viral replication. One such antagonist that exists in HIV, SIV and FIV is Vif. Both primate and non-primates produce a apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3 or A3), a family of cytidine deaminases that are able to convert cytidines to uracils \textsuperscript{54–56}. In primates, A3G, A3H and A3F have been shown to restrict lentiviral replication by being packaged within viral particles and creating crippling mutations in the viral genome during reverse transcription and strongly restricting viral replication. In felines, A3Z has been shown to restrict FIV replication in a similar manner\textsuperscript{57}. Vif is able to block A3 activity by binding to it and triggering polyubiquitination which targets the A3 protein for proteosomal degradation.
Figure 2: Schematic of different RNA splicing patterns in Lentiviruses. (A) HIV-1 spliced transcripts responsible for producing vif, vpr, vpu, tat, rev, env and nef. (B) SIVmac239 spliced transcripts responsible for producing vif, vpx, vpr, tat, rev, env and nef. (C) FIV 34TF10 spliced transcripts responsible for producing vif, orfA, rev and env.
Another host factor that restricts viral replication is tetherin, which inhibits the budding of enveloped viruses at the plasma membrane. HIV-1 has evolved to encode Vpu, an 81 amino acid protein that functions as a dimer and is able to antagonize tetherin activity thus increasing budding of viral particles\(^{58}\). Although non-human primates and felines also express tetherin, many (but not all) SIVs and FIV do not contain vpu. FIV env has instead been shown to be involved in evading host tetherins and can effectively bud\(^{59}\). Some SIV strains that are vpu deficient have also developed env genes that are able to antagonize tetherin but some SIV strains contain another accessory gene, Nef, that has recently been discovered to antagonize tetherin\(^{60-62}\).

The Nef protein is one of the first proteins expressed during the infectious cycle of HIV and SIV. Although it has no enzymatic activity, Nef has been shown to serve many crucial roles in the primate lentivirus life cycle, and deletions in the Nef gene in infected patients results in asymptomatic infection\(^{63,64}\). The CD8+ cytotoxic T cells of the adaptive immune system help fight off intracellular pathogens by recognizing infected cells through presentation of foreign antigens on Major Histocompatibility Complex 1 (MHC-1)\(^{65}\). HIV-1 Nef is able to downregulate the surface expression of MHC-1 through two proposed models\(^{66}\). The first model proposes that Nef is able to induce the endocytosis of cell surface MHC-1 while the second model postulates that Nef blocks the transport of newly synthesized MHC-1 towards the host cell surface. SIV Nef has also been shown to downregulate the surface expression of MHC-1 but it is not as well characterized and some strains may downregulate expression through mechanisms distinct from HIV-1 Nef. Another role that both HIV and SIV Nef are able to serve is to
decrease cell surface CD4 expression through endocytosis through an AP-2 and clathrin dependant pathway\textsuperscript{67}. Interestingly, FIV does not contain a \textit{Nef} gene but studies have shown that the orfA protein is also able to downregulate the expression of CD134, the primary entry receptor used by FIV\textsuperscript{17}.

Vpr is another multifunctional, non-enzymatic accessory protein that is highly conserved in HIV and SIV. Vpr is a 14kDa protein that contains a nuclear localization signal that is involved in helping it function as a chaperone for the translocation of the pre-integration complex into the nucleus\textsuperscript{68}. Furthermore, Vpr is able to induce cell cycle arrest in the G2 phase, act as a transactivator for transcription from the LTR and modulate T cell apoptosis. Some strains of SIV (SIVmac239) and HIV-2 also code for a protein structurally similar to Vpr, known as Vpx. HIV-2, unlike HIV-1 is able to transduce myeloid cells, which express a restriction factor sterile alpha motif and HD domain-containing protein-1 (SAMHD-1). HIV-1 is restricted by SAMHD-1 in myeloid cells, but Vpx is able to antagonize SAMHD-1 in HIV-2 and certain SIV strains, allowing them to survive within these cells\textsuperscript{69,70}. FIV does not code for Vpr, but the previously mentioned orfA gene contains a nuclear localization signal and structure similar to that of Vpr, and is implicated in induce cell cycle arrest in the G2 phase as well\textsuperscript{71}. 
Figure 3 Visual Representation of Lentiviral Particle. The conserved viral proteins across *gag*, *pol* and *env* are present to show the enzymes and structures packaged within a mature viral particle. Protruding outward from the viral envelope is the *env* SU domain and embedded within the envelope is the TM trimer. On the inner side of the envelope is the MA protein and further inside is the bullet shaped core made of CA proteins, alongside the protease enzyme occupying space outside the core. Inside the core are the two copies of viral RNA bound by IN and RT. Accessory proteins are not illustrated in this diagram but do get packaged during the budding process.
1.5 « Pre-integration Life Cycle»

The first step in all viral infections is entry into the host cell. The tropism of lentiviruses are determined by the env gene, specifically the surface subdomain. In HIV and SIV, the primary receptor used for virus entry is CD4 and thus HIV preferentially infects CD4+ T-cell. The gp120 trimer is able to bind CD4 on the cell surface which induces a conformational change putting the gp120 trimer in an “open” conformation. Although FIV also preferentially infects CD4+ T cells, the primary receptor it uses is CD134\textsuperscript{72}. HIV and SIV both utilize co-receptors for cell entry, and following the gp120 conformational change, the V3 loop of gp120 is able to bind a chemokine coreceptor which can be either CCR5 or CXCR4\textsuperscript{73}. FIV also utilizes a coreceptor for virus entry, but only uses CXCR4 and no FIV that has tropism for CD134 and CCR5 has been identified\textsuperscript{74,75}. Transmitted HIV is typically CD4.CCR5 tropic but at later stages of infection the viral envelope switches to use CXCR4 as the co-receptor\textsuperscript{76}. CD4.CXCR4 tropic virus is rarely found in SIV infection, but has been shown in vitro. Interestingly, certain strains of FIV are able to mediate cell entry using only the CXCR4 coreceptor, indicating the gp100 trimer is in a more open conformation that allows interaction with CXCR4, bypassing the requirement for initial CD134 binding\textsuperscript{74}. Coreceptor binding then exposes the hydrophobic fusion peptide of the TM domain which is able to insert itself into the host cell membrane and mediate membrane fusion. Once the viral envelope and cell membrane have fused, the viral capsid is released into the cell cytosol.
Figure 4 The Process of Retroviral Reverse Transcription. (A) tRNA Lys3 binds the PBS. (B) Reverse transcriptase begins synthesizing minus strong stop DNA and degrades RU5 region. (C) Newly synthesized DNA strand switches to R region at 3’ end of viral RNA. (D) RT synthesizes DNA towards the PBS and viral RNA is degraded except for PPT. (E)(F) PPT acts as a primer for DNA polymerase extension to generate + strand DNA. (G) + Strand DNA strand switches to the PBS at the 3’ end of the – strand. (H) DNA polymerase completes synthesis of proviral DNA in both directions.
Packaged within the core of lentiviruses, is the reverse transcriptase enzyme that fulfills the crucial role of converting the single stranded viral genomic RNA into double stranded DNA (Figure 4)\textsuperscript{77}. Initiation of DNA synthesis requires an RNA primer and for the reverse transcription of lentiviral genomes cellular tRNA Lys3 is used. tRNA Lys3 is packaged within the viral core and binds the primer binding site (PBS) within the 5’LTR of the viral RNA, and from there RT begins synthesizing the minus strand DNA. RT transcribes the DNA to the 5’ end of the viral genome to create what is referred to as the minus strong stop DNA. RNase H activity of RT is concomitantly degrading the R U5 region of the viral RNA as it is being reverse transcribed which causes the minus strong stop DNA to bind to the 3’ R region of either the same viral RNA or the second packaged copy and continue extending the minus strand DNA back towards the PBS. Again, RNase H degrades the rest of the remaining viral RNA except for the polypurine tract (PPT) region that is resistant to RNase H activity. The PPT lies slightly upstream of the U3 region and serves as the primer to initiate synthesis of the positive stranded DNA. The newly synthesized positive strand DNA extends towards the 3’ end of the viral genome and then switches back to the complementary U5 region of the minus strand DNA at the 5’ end and the positive strand and minus strand DNA are synthesized toward both ends.

The newly synthesized proviral DNA must be integrated into the host chromosomes before it can be transcribed to produce viral proteins. The integrase protein is packaged along in the core of the viral particle and as the proviral DNA is being synthesized, the viral capsid begins to come apart\textsuperscript{38}. The first step in viral integration is 3’ end processing, wherein integrase binds the proviral DNA and recognizes a conserved CA dinucleotide in each LTR at the 3’ end of each strand and cleaves the site. This
cleavage exposes a hydroxyl group at the 3’ end of each of the proviral DNA strands. Integrase remains bound to the dsDNA alongside matrix and Vpr to form the pre-integration complex (PIC) in HIV and SIV, which is then translocated into the nucleus of the host cell\textsuperscript{78}. FIV integrase also shuttles the dsDNA after 3’end processing into the nucleus but the structure of the PIC isn’t as well characterized. Once inside the nucleus, the next step in the integration process is strand transfer, a step that has been targeted for many effective antiretroviral drugs. The exposed 3’OH groups at each end of the proviral DNA attack a pair of phosphodiester bonds in the host DNA. The 2 nucleotide overhang at each 5’end of the proviral DNA is removed and the gap in the host DNA is filled in by host machinery and ligated to complete the integration process\textsuperscript{78}.

### 1.6 « Post Integration Life Cycle»

Following integration into the cellular DNA, the viral DNA must be transcribed into numerous transcripts necessary for the production of new viral particles. Lentiviruses have evolved the ability to synthesize numerous proteins through their relatively small genomes by using alternatively spliced transcripts. After integration, transcription is initiated by the binding of host transcription factors to the 5’LTR. NF-kB, Sp1, and TATA box binding protein (TBP) have binding sites within the LTRs of these lentiviruses and initiate basal levels of transcription necessary for the production of fully spliced transcripts that produce Rev and Tat during HIV/SIV infection, and Rev alongside orfA in FIV infections\textsuperscript{51,79}. As previously mentioned, Tat is able to transactivate the transcription of HIV/SIV transcripts by binding to the TAR region of the 5’LTR. Tat binding to the TAR region of the 5’LTR results in the recruitment of pTEFb, which is a kinase complex that contains Cyclin Dependent Kinase 9. CDK9 is able to
recruit RNA polIII and phosphorylate its C terminal domain, activating transcription. pTEFb is also involved in enhancing the recruitment of TPB to the LTR promoter, also increasing transcriptional activity\(^{46,47}\). Another function of Tat that has been shown is its ability to promote chromatin re-modelling, making the region containing integrated provirus more available for transcription. The transactivation of orfA on transcription isn’t as well defined as Tat, but there is a similar increase on both mRNA transcription and subsequently the expression of viral proteins\(^{48}\).

Transactivation of transcription from the 5’LTRs now leads to the production of the partially spliced RNAs (approx. 4kb) that are responsible for encoding for Vif, Vpu and Env in both HIV and SIV strains, as well as Vpx in HIV-2 and SIVmac239\(^{80}\). The partially spliced RNA species in FIV code for Env as well as orfA\(^{71}\). The fully unspliced mRNA is used for the translation of Gag and Gag-Pol, as well as serving as the genomic RNA that is packaged within viral particles.

In eukaryotic cells, after post-transcriptional modification, only the mRNA species that are fully spliced are exported through the nuclear membrane. In a similar vein, this allows the early proteins in lentiviral infection such as Tat, Rev, Nef and orfA be translated first since they are fully spliced transcripts that are able to be transported through the nuclear membrane, whereas the partially spliced and unspliced transcripts are trapped in the nucleus.

Lentiviruses have overcome this restriction by using the Rev protein to facilitate nuclear export of partially spliced and unspliced mRNA species necessary for translation of late proteins. Rev contains a nuclear localization signal that allows it to be transported
back into the nucleus following translation in the cytosol\textsuperscript{51,81}. Once in the nucleus, Rev is able to bind the RRE that is present in the unspliced and partially spliced mRNAs. The RRE is an RNA stem loop structure that is highly conserved in lentiviruses. In HIV and SIV, the RRE sequence lies at the junction of the gp120 and gp41 domains within \textit{env}, while in FIV the RRE structure lies between the 3’end of \textit{env} and the 3’LTR. Rev is able to bind the RRE as a monomer and recruit cellular factors that allow the unspliced and partially spliced mRNA to be transported through nuclear pores with the help of Crm1. Following Rev/RRE binding, Crm1 binds the NES of Rev and additionally bridges binding to RanGTP. This Rev/RRE/Crm1/RanGTP complex is able to interact with the nuclear pore complex and be shuttled through the nuclear membrane with the help of Nucleoporin 98 and Nucleoporin 214\textsuperscript{82}.

Once the Rev/RRE/Crm1/RanGTP complex is in the cytosol, RanGAP1 and RanBP1 bind the complex and induce the hydrolysis of RanGTP to Ran GDP. Following this hydrolysis, Crm1 dissociates from Rev and goes back to the nucleus, allowing importin-B to bind the NES of Rev\textsuperscript{82,83}. The interaction between importin-B and Rev disturbs the interaction with RRE and transports Rev back into the nucleus. The unbound partially spliced or unspliced mRNA is now free to associate with host translational machinery to synthesize viral proteins or become packaged by viral particles.

Once the unspliced mRNA is translocated into the cell cytoplasm, it is the transcript responsible for synthesizing the gag and gag-pol polyproteins. The majority of the time, translation that begins at the \textit{gag} start codon continues until it reaches the stop codon at the end of the \textit{gag} ORF and produces the pr55 gag precursor that is subsequently cleaved into MA, CA and NC\textsuperscript{84}. As the ribosome approaches the
overlapping region of the *gag* and *pol* ORFs it encounters a highly conserved RNA structure known as the ribosomal frameshift signal (RFS)\textsuperscript{30,31}. The RFS is the element responsible for allowing for a frameshift event at about a 5% frequency permitting read through of the *gag* stop codon and allowing for synthesis of the pr160 gag-pol polyprotein. During a frameshift event, the ribosome approaches the RFS and stalls due to the bulky hairpin structure that is formed. This stalling then causes the ribosome to slip on the slippery sequence, which is UUUUUUU in HIV and SIV but GGGAAA in FIV, and fall back into the -1 reading frame, allowing the ribosome to bypass the *gag* stop codon and continue translating the rest of *pol*.

Following cleavage of the gag precursor, MA becomes myristoylated and targets gag to the inside of the cell membrane, where the viral core is able to form and subsequently bud along with pol, the accessory proteins and genomic RNA\textsuperscript{85,86}. Some had initially thought that viral RNA was necessary for the efficient formation of the viral core, but studies showed that transfections with only gag resulted in particle formation similar to wild type\textsuperscript{87}. More recent studies however have been able to show that the presence of viral RNA actually increases the production of viral particles, hinting at the possibility that the NC/RNA complex serves as a point of nucleation for the production of viral particles at the cell membrane\textsuperscript{88}. Once the viral particle pinches off the cell membrane carrying with it an envelope containing the SU and TM domains of env, the particle undergoes the process of maturation in which protease begins cleaving the immature gag-pol polyprotein into its substituents\textsuperscript{84}. The ratio of gag to gag-pol has been shown to be important for maturation since having too much protease present significantly reduces the virus infectivity due to early and excessive cleavage\textsuperscript{89}. 
1.7 « Untranslated RNA Structural Elements»

The lentiviral genome contains regions that have no protein coding capacity but are essential for replication and are highly conserved. The development of structural modelling programs and newer RNA imaging techniques have been crucial in elucidating the structures of these regions and how they impact both RNA-RNA interactions and RNA-protein interactions (Figure 5)\textsuperscript{90,91}. The region upstream of \textit{gag} extending into the 5’LTR is the region that has been most extensively studied in HIV and has been ascribed to have many functions. mFold was one of the first secondary structure prediction tools that helped identify RNA stem loops within the 5’ leader of HIV and newer techniques have been able to show more in depth tertiary structures of the same region in recent years\textsuperscript{92,93}.

As previously mentioned, the first highly structured RNA element that is in the HIV and SIV 5’LTR is the TAR hairpin that acts as a docking site for Tat to transactivate transcription. Directly downstream is the polyA stem loop that serves as the polyadenylation signal that is crucial for mRNAs and this exists at the 3’end since this region is a repeat\textsuperscript{94}. Next is the PBS, where the tRNA Lys3 and RT can bind to initiate the synthesis of minus strong stop DNA\textsuperscript{95,96}.

Following the primer binding site is what has been described as the major packaging signal: a series of four stem loop structures that have been implicated in the efficient packaging of RNA. Extensive studies have been done on this region of the genome to try and fully understand the interactions and sequences that are involved in the packaging of specifically two copies of unspliced mRNA into budding viral particles. These stem loops have been designated as SL1-SL4\textsuperscript{96}.
The RNA genome that is packaged within viral particles is a dimer, and SL1 has been identified as the structural element in the 5’LTR responsible for initiating dimer formation between two identical RNA copies and thus is also referred to as the Dimer Initiation Site (DIS)\textsuperscript{97,98}. DIS is a 49 nucleotide RNA hairpin structure that has a 9 nucleotide loop at the tip. Six of these 9 nucleotides are a palindrome which allows them to Watson Crick base pair with the DIS of another unspliced mRNA strand. The formation of the interaction is known as a kissing loop\textsuperscript{98}. Following the formation of the kissing loop, adjacent palindromic sequences are then able to also base pair with the adjacent RNA strand to further stabilize the dimer formation. The 6 nucleotide palindrome is highly conserved and only two hexanucleotide sequences have been identified in infected patients dependent on subtype. Subtype B and D contain a GCGCGC palindrome, while all other subtypes contain GUGCAC\textsuperscript{99}. SIVmac239 also contains a hexanucleotide palindrome sequence that is able to form a kissing loop with another RNA strand, with the sequence GGUACC\textsuperscript{100}.

SL2 in both HIV and SIV is a hairpin that contains the major 5’ splice donor sight in the apical loop and is commonly referred to as the splice donor (SD). Studies have shown that although the GGUG tetraloop of SD has a high affinity for binding the NC domain of gag, it has been shown through mutational analysis that it is the GGUG tetraloop at the apex of the SL3 (psi) hairpin that is responsible for binding NC to package genomic RNA\textsuperscript{101}. The presence of SD before SL3 provides an explanation for the selective packaging of unspliced mRNA over the spliced mRNAs since the SL3 loop would be removed. SL4 is the final stem loop in the major packaging signal and contains the gag start codon at the base of the hairpin. SL4 has been shown to be involved in
forming long range interactions to facilitate RNA conformations conducive to packaging that will be discussed later\textsuperscript{92}.

The structures of the 5’ leader of FIV has not been studied to the same degree as primate lentiviruses, and therefore the functions of some of the stem loops have yet to be fully elucidated. SHAPE analysis has shown that the secondary structure of the 5’ leader in FIV is very different from that of primate lentiviruses\textsuperscript{102}.

In FIV, there are 9 stem loops upstream of the gag start codon whose functions are not as well defined, and there is no definitive sequence that has been identified for binding to the NC domain of gag to facilitate RNA packaging. As necessary for all retroviruses, there is a PBS that exists between SL1 and SL2 where tRNA can bind to initiate reverse transcription. The major splice donor falls between SL4 and SL5, just slightly upstream of the gag start codon\textsuperscript{103}.

Like all lentiviruses, FIV also packages dimerized RNA but interestingly the DIS in FIV has been mapped to SL5 which falls within the \textit{gag} ORF and contains a palindromic sequence that has been hypothesized to initiate loose dimer formation in a similar manner to SL1 in primate lentiviruses\textsuperscript{103}.

The specific sequences as well as secondary and tertiary structures that form within a strand of lentiviral unspliced mRNAs are not only important for RNA-RNA interactions but RNA-protein interactions as well. The binding of Rev to RRE and Tat to TAR are well documented and accepted RNA-protein interactions that are crucial for replication, but recent studies have shown that even host restriction factors are able to bind highly structured RNA sequences, such as APOBEC3Hs affinity for duplex RNA
within the 5’UTR\textsuperscript{104,105}. Other host proteins have been shown to recognize viral RNA in a sequence dependent manner. APOBEC3G has been shown to bind preferentially to G rich and A rich regions of RNA while Zinc Finger Antiviral Protein (ZAP) binds preferentially to regions that are high in CG dinucleotides\textsuperscript{106,107}. One of the best examples of the combination of the sequence and structure specific recognition of viral RNA is the binding of NC to unspliced mRNA to facilitate packaging.

As previously mentioned, the zinc fingers, that are comprised of two conserved CCHC motifs, within the NC domain of Gag is able to bind with high affinity to the G rich loop of psi in the 5’UTR\textsuperscript{101}. There is evidence that the binding of NC to psi helps maintain and stabilize the dimerized form of unspliced mRNA that is known to be packaged, but whether or not NC binds RNA before it dimerizes or after is still debated\textsuperscript{108,109}.

SIVmac239 NC is structurally very similar to that of HIV-1, and also contains 2 of the CCHC motifs that are capable of interacting with RNA. When experiments were done to compare the NC and psi interactions across the viral species, HIV-1 NC and SIVmac239 NC showed greater affinity for HIV-1 psi compared to TAR RNA, while neither NC proteins showed a greater affinity for SIVmac239\textsuperscript{110}. This would imply that there is some other mechanism at play that is influencing SIVMac239 NC to preferentially bind the unspliced mRNA to facilitate packaging. Although there are some differences in the structure of FIV NC, the two zinc finger domains are still present, and it is known that functionally they are very similar, but the binding specificity for FIV RNA has not yet been effectively characterized\textsuperscript{28,111}. 
1.8 "Models of RNA Packaging"

Although the RNA structural motifs and RNA-protein interactions involved in the process of RNA packaging in HIV has been well documented, there still remains the question of determining the destiny of unspliced mRNA for either translation or packaging.

As is this case with many RNA strands, they are constantly in a structurally dynamic state, meaning that the conformations they adopt can switch depending on their surroundings or binding partners. Since the unspliced mRNA is used for translation of Gag and Gag-Pol, it was initially hypothesized that two conformations existed: one that is translationally competent while another that is conducive to being packaged within viral particles and these two conformations have been termed the Long-Distance Interaction (LDI) conformation and the Branch Multiple Hairpin (BMH) conformation, respectively (Figure 5A).

The different elements within the 5’UTR that have been previously described can only function if they form a conformation in which the sites of interactions are exposed. In the BMH conformation, the AUG start codon of gag forms a duplex with the U5 region of the 5’ leader, allowing the DIS, SD and psi to form the correct hairpins. In this state, the apex of the DIS stem loop is exposed, allowing the palindromic sequence to form the kissing loop with another unspliced mRNA in the BMH conformation. By allowing the unspliced mRNA to form a dimer, it was believed that this conformation was the catalyst for sending this RNA species down the path of RNA packaging. Additionally, by adopting the BMH conformation, ribosomes can not access the Gag start codon since it is occluded.
Figure 5 Dynamic Structural Models of HIV-1 5'UTR and Lentiviral RFS Structures. (A) The Long Distance Interaction conformation in equilibrium with the Branched Multiple Hairpin conformation that promotes RNA Packaging. (B) Comparison of the conformations proposed from the Summers lab with the U5-DIS promoting translation and the U5-AUG and Three-way junct models promoting packaging. (C) The different stem loops formed downstream of the slippery sequence of HIV-1, SIVmac239 and FIV 34TF10.
In the LDI conformation, the DIS palindromic sequence instead forms a duplex with the polyA hairpin. When the 5’UTR adopts this conformation, the DIS palindrome is not able to interact with another unspliced mRNA to form a dimer. Additionally, in this conformation, AUG does not form a duplex with U5, granting access to the ribosome to begin translating the gag ORF. Interestingly, soon after these studies were done, the LDI structure was disproven to be responsible for promoting translation of Gag and Gag-Pol, since mutations that disrupted the equilibrium of the LDI/BMH structures did impact RNA dimerization and packaging but had no impact on the production of Gag or Gag-Pol.

As technology has advanced, studies have been able to better elucidate the conformations of unspliced mRNA in addition to getting a better understanding of the localization of RNA-Gag interactions that facilitate genomic RNA packaging. In 2011 Summers’ group began using Nuclear Magnetic Resonance (NMR) imaging to detect the structural elements that were at play during the process of RNA packaging. In their study, they proposed an alternate conformational switch in which the U5 region interacts with the gag AUG to form packaging enhanced but translationally repressed conformation. In 2015 the group expanded on this model and were able to generate 3D images to reveal the tertiary structure of the packaging signal which adopts a three way tandem junction structure and is quasi-tetrahedral in shape. In this model, similar to the BMH conformation, the U5-AUG duplex sequesters the Gag codon, as well as the splice donor site within the three-way junction, to inhibit translation and mRNA splicing, while DIS and psi are protruding outwards to promote dimerization and NC interaction (Figure 5B).
1.9 « Discovery of GRPE»

Most of the major studies on RNA packaging have focused on the 5’UTR region of the HIV genome due to the fact that mutations within the region will have no impact on the production of viral particles. Studying RNA structural elements within the protein coding region is more difficult due to the presence of multiple ORFs and therefore any viral defects could be attributable to defective protein production. It is however crucial to study the RNA structural elements within the protein coding sequence since they may provide important insight into certain aspects of the viral lifecycle.

In 2013, Chamanian et al were able to show that indeed there exists an element overlapping both the gag and pol ORF that is necessary for efficient packaging of genomic RNA into viral particles\textsuperscript{114}.

This finding was the result of attempting to optimize the infectivity of a heterodiploid virus production system. One of the tools that the Arts lab uses for cloning within full length HIV is yeast based homologous recombination\textsuperscript{115}. Using a selectable marker, one can easily insert specific sequences within the full-length HIV genome by creating overhangs around that are homologous to the sequences flanking the target sequence you are attempting to replace. By creating a double stranded break in the region that is being replaced by the desired insert, yeast are able to recognize the regions of homology around the insert and use double stranded break repair to stitch together the insert and the vector. The issue that arose while using this system was that since the full-length HIV genome contains LTRs, these would be recognized by the yeast as regions of homology and instead the whole protein coding sequence of HIV would be kicked out. To alleviate this problem, the 5’LTR was deleted upstream of the PBS and the resulting
vector was termed near full length NL4-3 (nfl). This nfl construct was able to produce viral particles since the full protein coding sequence was in the vector as well as package its RNA since SL1-SL4 were also present. However, this viral particle was non-infectious since the R U5 region was not part of the packaged RNA and therefore RT would be unable to create minus strong stop DNA in the first step of reverse transcription. To be able to study the infectivity of these different clones, a short complementing (cplt) vector containing the R region of the 5’LTR extending just past the start codon of gag was co-transfected so that this short RNA could be packaged alongside the nfl RNA and initiate reverse transcription to create minus strong stop DNA, which would then strand switch to the nfl vector and complete reverse transcription. Based on Hardy Weinberg Equilibrium, it was hypothesized that this complementation system would have 50% of the infectivity compared to a vector that contained the full-length genome since 50% would be homodiploid for one RNA or the other, while 50% would be heterodiploid and be able to produce proviral DNA and infect target cells. The progeny virus actually turned out to only be about 0.1% the infectivity of a transfected full length clone. This lead to attempting to optimize the system by extending the RU5 cplt vector towards the 3’end of the genome to identify at what point the hypothesized infectivity level would be reached. Constructs that extended to the 3’ end of gag and further showed the expected level of infectivity, and it was believed that this discrepancy in infectivity was due to inefficient packaging of the complement vector which turned out to be true. Through mutational analysis and fine mapping, the region responsible for this enhanced packaging was found to overlap the ribosomal frameshift signal necessary for the production of the gag-pol polyprotein. Deletion or perturbation of the structure of this stem loop resulted in a near
50 fold decrease in the amount of packaged genomic RNA and a significant drop in virus infectivity, and thus the region was termed the Genomic RNA Packaging Enhancer Element.

As with the discovery of any functional element of a viral genome, it is important to compare and contrast it to closely related ancestors as well as ones that are further away on a phylogenetic tree. By doing so, you can get a better idea of the conservation of such elements, the point of their evolution, as well as being able to narrow down the specific function and pathway of the element. In the case of the GRPE, due to the high similarity and elements involved in the RNA packaging process across lentiviruses, we set out to test whether a GRPE element exists in SIVmac239 as well as FIV 34TF10, a common strain of FIV.
Chapter 2

2 « Methods»

2.1 « Yeast Based Homologous Recombination »

To introduce different mutations, and create large deletions, we harnessed the ability of *Sacharomyces Cerivisae* to perform double stranded break repair on a transformed plasmid by introducing a dsDNA insert with regions of homology flanking the location of the break. The backbone of the constructs used for yeast cloning are based on pcDNA 3.1 (Invitrogen) with the addition of three specific genes. For replication of the transformed plasmid within the yeast it must be recognized as an artificial chromosome, therefore an autonomously replicating sequence (ARSH4) and a yeast centromere sequence (CEN6) were inserted into the pcDNA 3.1 backbone to facilitate plasmid replication during mitosis. Additionally, the β-isopropylmalate dehydrogenase (LEU2) gene was added to the backbone as well to allow for selection and growth on leucine deficient growth media.

The cut plasmid DNA and insert DNA were introduced into the yeast through the lithium acetate transformation method. Yeast are initially grown in 50mL of yeast extract peptone dextrose (YPD) media overnight in a shaking incubator at 30°C for 16 hours. The yeast were then pelleted by centrifuging the culture at 4000g and resuspended in 1mL of sterile water. The yeast suspension was pelleted again by centrifugation by spinning at 4000g for 5 minutes and resuspended in 400µL of TE/LiAc solution made of 1x TE (10mM Tris-Cl pH 7.5 and 1mM EDTA pH 8.0) and 1x Lithium Acetate (pH 7.5). 50µL of the yeast suspension is added to 3µg of linearized plasmid, 1µg of insert DNA,
50μg of denatured Salmon Sperm DNA (Thermo Cat. No. 15632011) and 300μL of polyethylene glycol (8 volumes 50% PEG400, 1 volume 10x TE and 10x LiAc). The solution was incubated for 30 minutes at 30°C in a shaking incubator for one hour and then heat shocked for 15 minutes at 42°C. Following heat shock, yeast were pelleted by centrifuging at max speed for 10 seconds and resuspended in 100μL of water and plated on appropriate media. Plates are allowed to grow 2-5 days until visible colonies formed.

Colonies are picked and inoculated in a 5mL culture overnight, then pelleted at 4000g. The pellet is resuspended in 200μL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) and added to 0.3g of glass beads in a 1.5mL microcentrifuge tube and 200μL of phenol/chloroform/isoamylalcohol. The mixture is vortexed for 2 minutes and centrifuged for 5 minutes at 14000rpm at room temperature. 100μL of the aqueous phase is transferred to a new 1.5mL microcentrifuge and mixed with 170μL of water, 30μL of 3M sodium acetate, 700μL of 100% ethanol and vortexed for 5 seconds. The mixture is centrifuged for 10 minutes at 14000 rpm at room temperature, then pelleted and washed with 700 μL of 70% ethanol. The mixture is pelleted again, supernatant is removed and the pellet is air dried for 5 minutes. The crude DNA pellet is resuspended in 20μL of water and then transformed into Thermo Electrocompetent Stbl4 bacterial cells according to manufacture protocol. Bacteria is plated on ampicillin plates and grown overnight. The resulting colonies are then screened by either restriction digest, PCR or Sanger sequencing.
In the yeast cloning system, Orotidine-5′-phosphate decarboxylase is what serves as the selectable marker for inserting different genes. URA3 is a catalytic enzyme that allows for the growth of yeast on uracil deficient media. When yeast are being selected for the insertion of the URA3 gene, they are grown on CMM media lacking leucine and uracil (CMM-Leu-URA). URA3 also produces a toxic metabolite in the presence of 5-Fluoroorotic acid, therefore when a gene is being inserted in place of the URA3 gene, yeast are plated on CMM media with 5-FOA but lacking leucine (CMM-Leu+5FOA), to negatively select for the desired product.

2.2 « Generation of SIVmac239 Heterodiploid System for testing RFS Impact»

The two vectors required for the production of heterodiploid infectious SIVmac239 virus were provided by Dr. Yong Gao (Figure 6A). The vector responsible for produces all viral proteins was termed SIVmac239 nfl, which contained the SIVmac239 genome from the PBS to the end of the 3′LTR was cloned into the pREC backbone for use in the yeast recombination system. The second vector, which allows for the initiation of reverse transcription, contains the SIVmac239 genome from the R region in the 5′LTR to the end of the pol ORF in the pREC backbone and was called SIVmac239 U5gag-pol. To evaluate the impact of the ribosomal frameshift signal on RNA packaging within the SIVmac239 genome, the frameshift stem was deleted in the U5gagpol vector. Using overlap extension PCR, primers were designed to create a 58 base pair deletion beginning at the slippery sequence (2351-2409). Two fragments were generated in the first round of PCR amplification using an annealing temperature of 55°C and an extension time of 1 minute with the following primer pairs: SIV delRFS fwd/SIV
delRFS pol and SIV delRFS rev/SIV delRFS gag. The two fragments from the first round of PCR are used as the template at equal copy number for the second round of PCR using an annealing temperature of 58°C and extension time of 1 minute 30 seconds with the SIV delRFS gag/SIV delRFS pol pair (Figure 6B). The U5gagpol vector and mutated PCR product are digested at the BamHI SbfI cut sites and then ligated together using NEB T4 Ligase according to the manufacturer’s protocol (Cat. No. M0202). The ligation mixture was transformed into Thermofisher Electromax Stbl4 competent cells according to manufacturers protocol, plated on LB Agar plates with ampicillin and colonies were screened by Sanger sequencing.

2.3 « Making Large Scale Deletions in SIVmac239 U5gagpol »

To elucidate potential RNA structural elements in SIVmac239 that may impact RNA packaging outside of the region of the ribosomal frameshift signal, URA3 was inserted at different locations along the gag-pol ORF to disrupt any RNA structures encompassing the region (Figure 6C). First, primers were used to amplify the URA3 gene from the pREC URA3 vector as a template, containing 40bp overhangs with homologous flanking the desired region of insert into the pREC SIVmac239 U5gag-pol vector. PCR products were generated using Platinum Taq protocol and cycled with an annealing temperature of 58°C and an extension time of 2 minutes. BamHI was used to cut within the gag gene and two different PCR products were used to insert URA3 to replace the majority of gag or the 3’ half. SbfI and Bsu361 were used to make single cuts within pol to insert URA3 at the 5’ third and in place of RT respectively. The cut plasmid was
transformed into yeast along with the correct insert and plated on CMM-Leu-URA plates. Colonies were screened to confirm correct insert by sequencing.

Yeast recombination was used to introduce the mutant ribosomal frameshift signal in a near full length viral clone. Overlap extension PCR was used to introduce point mutations into the ribosomal frameshift signal. Two 40bp primers were created that contained the desired mutations that were reverse complements of each other. Two more primers were created to amplify PCR products with the mutation primers. One PCR product contains the mutated RFS at the 3’ end and one with the mutated RFS at the 5’ end. These two products were added at equivalent copy number into another PCR reaction using the 5’ and 3’ primers, allowing the two products from the first round of PCR to anneal to each other and generate the full length insert containing the mutated RFS. The target vector for cloning was obtained from the Viral ARTS library which was a near full length construct containing URA3 in place of the p6 gene. The vector was cut at the SacII site within the URA3 gene and transformed into yeast alongside the mutant RFS insert and plated on CMM-Leu+5FOA plates. Colonies were picked after 72 hours and screened by sanger sequencing.
Figure 6 Cloning Schematic for Generation of Mutant SIVmac239 U5gag-pol Vectors. (A) Visual representation of the vectors used in the heterodiploid SIVmac239 system consisting of a U5gag-pol vector and an nfl vector. (B) Approach to deleting RFS in U5gag-pol by digestion and ligation with PCR product containing deleted RFS. (C) Approach to creating U5gag-pol deletion vectors by amplifying URA3 with primers containing overhangs flanking target sequence to introduce products through homologous yeast recombination.
Figure 7 Schematic of Cloning Strategy to generate FIV 34TF10 vectors for evaluating impact of RFS. (A) Binding of PCR primers (arrows) to generate fragments containing pREC homologous overhangs required for cloning into pREC backbone using yeast based homologous recombination for FIV 34TF10 U5gagpol and FIV 34TF10 nfl. (B) Deletion of RFS in FIV 34TF10 U5gagpol and FIV 34TF10 nfl using QuickChange XL II Site Directed Mutagenesis.
2.4 « Generation of FIV 34TF10 Heterodiploid System to evaluate impact of RFS »

To generate the insert for the FIV U5gag-pol vector, primers were designed that would amplify the FIV34TF10 molecular clone from the beginning of the R region in the 5’LTR to 273bp past the 3’ end of the gag ORF (Figure 7A). Each of the primers contained a 40bp overhang homologous to the pREC backbone to allow for effective yeast recombination. The pREC/URA3 vector contains only the URA3 gene, and therefore was used as the target to insert the FIV U5gag-pol sequence into the pREC vector. pREC/URA3 was cut with SacII and transformed into yeast alongside the FIV U5gag-pol insert and plated onto CMM-Leu+5FOA to select for recombinants with URA3 replaced by the insert sequence. Colonies were picked and screened by PCR.

To generate the FIV nfi vector capable of producing all viral proteins and also transcribing an usnpliced mRNA capable of being packaged within virions, the insert was amplified in two parts and yeast double recombination was used to generate the final product. To generate the first fragment, primers were designed to amplify the FIV 34TF10 genome from directly after the 3’ end of the U5 region to 2000bp into the pol ORF, with the 5’ end of the fragment containing a 40bp overhang homologous to the pREC backbone. The second fragment that was amplified beginning 1900bp into the pol gene, providing 100 bp of homology between the two fragments, to the end of the 3’LTR in addition to 40bp of overhang homologous to the pREC backbone. The two PCR products were transformed alongside the SacII digested pREC/URA3 vector and plated on CMM-Leu+5FOA plates to select for recombinants. The yeast will recombine the two PCR fragmenets together due to the 100bp of homology within the pol gene and
recombine the combined PCR fragment at each end due to the regions homologous to the pREC background.

To test whether or not the RFS has any effect on the ability of an RNA strand to be packaged, the FIV U5gag-pol vector was deleted from the slippery sequence to the end of the RFS stem loop (Figure 7B). Primers were designed containing a 30bp deletion (1881-1910) that would bind the region flanking the RFS and used with the Agilent Quickchange XL II Site Directed Mutagenesis Kit (Cat. No. Cat. No. 200521) according to manufacturer’s protocol. The mutated vector was transformed into ThermoFisher Electromax Stbl4 cells and colonies were screened and confirmed by sequencing.

2.5 « Cell culture and transfection»

Human embryonic kidney 293T cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 100μg/mL penicillin/streptomycin. Cells were split at a ratio of 1:10 every 3 days. U87.CD4.CCR5 cells were grown in DMEM containing 10% FBS, 100μg/mL penicillin/streptomycin, 300μg/mL G418 and 1μg/mL of puromycin. U87 cells were split every 3 days at a 1:3 ratio. Crendell Reese Feline Kidney cells were also grown in DMEM with 10%FBS and 100μg/mL penicillin/streptomycin, and were split every 3 days at a ratio of 1:10.

To produce viral particles through transfection of human embryonic kidney 293T cells, first 3x10⁶ cells were plated on a 100mm cell culture dish and allowed to grow overnight. Once cells were at about 60% confluency, cells were transfected. In each experiment, the near full length plasmid and complementing 5’LTR construct were transfected at an equal copy number adding to a total of 24μg of total DNA per reaction.
The 24μg of plasmid DNA was added to 1mL of DMEM alongside 72μL of FuGENE 6 reagent (Promega) in a 1.5mL microcentrifuge tube, vortexed, incubated at room temperature for 15 minutes and added to cells. At 8 hours post transfection, the culture dish supernatant was removed and 17 mL of fresh media was added so as to remove any unincorporated plasmid/liposome mixtures. Virus supernatant was harvest and purified at 48 hours post transfection.

2.6 «Virus Purification»

Following 48 hours post transfection, the 17mL of viral supernatant were transferred to a 50mL conical tube and centrifuged at 1500 RPM for 10 minutes at 4°C to pellet any cellular debris. The remaining supernatant was aspirated using a 60mL syringe and clarified through a 0.45μM pore size filter. The remaining volume was transferred to an 100KDa Amicon Ultra-15 Centrifugal Filter Unit (Cat. No. UFC910024), and centrifuged at 3700 RPM for 12 minutes at 4°C. The volume remaining in the filter unit was transferred to a new 1.5mL microcentrifuge tube, and topped up with PBS to fill to the brim of the tube. The 1.5mL centrifuge tube was spun at 32000g for 1 hour. The remaining supernatant was discarded, and the viral pellet was resuspended in 600μL of PBS, aliquoted, and stored at -80°C until used.

2.7 «Reverse Transcriptase Assay»

Reverse transcriptase activity of viral harvests was used as a relative measure of viral particles. 10μL of virus supernatant was added to 20μL of RT assay buffer (50mM Tris-HCl, 5mM DTT, 5mM MgCl2, 150mM KCl, 0.05% NP-40 and oligo-dT poly-rA) and incubated at room temperature for 30 minutes. Next, 5μL of RT buffer containing
radioactive dTTP at a concentration of 4μCi/mL was added per well and the mixture was incubated at 37°C for 2 hours. Following incubation, 10μL of the mixture was spotted onto DEAE filter mats, and allowed to dry for 10 minutes. The mat was subsequently washed on a shaking incubator five times for 1x saline-sodium citrate buffer for 5 minutes each, followed by two, five minute washes with 85% ethanol. The filter mat was then placed on a 65° heat block to dry for 15 minutes. Once dry, the filter mat was exposed overnight on a Phosphor screen and imaged using the Bioquant 800 Phosphorimager and analyzed using ImageJ software.

2.8 « Infections for Real TCID50»

Infectivity assays for SIVmac239 and FIV 34TF10 were done using human U87.CD4.CCR5 and CrFK cells respectively. Cells were plated in a 96 well plate at a density of 4,000 cells per well and allowed to grow overnight. To measure the infectivity of progeny virus, 300μL of virus supernatant was diluted 1:3 In a 96 well plate 7 times, and 50μL of virus was added onto the plated U87 cells in triplicate. 24 hours post infection, supernatant was removed and 150μL of fresh media was added. RT assays was done on day 6 post infection, wells were scored + or – based on RT activity and the Reed Meunsch method was used to calculate Real TCID50/mL.

2.9 « RNA Extraction and qRT-PCR»

Viral RNA was extracted from 140μL of concentrated virus supernatant using Qiagen QIAmp Viral RNA Mini Kit (Cat. No. 52906) according to the manufacturer’s protocol and eluted in 70μL of water. The extracted RNA was treated with DNAsen to
remove any residual plasmid DNA using the Ambion Turbo DNA-free Kit (Cat. No. AM1907) following the manufacturer’s instruction.

To synthesize cDNA for quantification by qPCR, the viral RNA was subject to reverse transcription PCR using AccuScript Hi-Fi Reverse Transcriptase (Cat. No. 200820) using the corresponding reverse qPCR primer. Initially, 5μL of RNA was added to 2.0μL of 10mM dNTPs, 2.0μL of 10x AccuScript RT buffer, 1.0μL of the designated RT-PCR primer at a concentration of 10μM as well as 7.25μL of water and subject to the following cycling conditions: 88°C for 1 minute, 65°C for 10 minutes and 25°C for 5 minutes. While samples remained in the thermocycler at 25°C, added in order to each sample was: 2μL of 100mM DTT, 0.25μL of RNAseOut and 0.5μL of AccuScript RT enzyme. The reaction was then cycled at 42°C for 90 minutes, 70°C for 10 minutes and held at 4°C.

The quantification of genomic RNA copy number was done through real time qPCR using the TaqMan system. Primers and probes were acquired from EuroFins and are found in Table 1. Each reaction contains 4μL of each primer at 10μM, 2μL of probes at a concentration of 10μM, 8.25μL of ThermoFisher TaqMan FastAdvanced Mastermix (Cat. No. 4444557), 1.25μL of water and 5μL of synthesized cDNA. Reactions for each sample were run in triplicate alongside a 10 fold serial dilution of plasmid DNA with known copy number to generate a standard curve for calculating RNA copy number from cT values. The RT-qPCR reaction is then run on the QuantStudio Real Time PCR platform using cycling conditions of: denaturation for 5 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 62°C for 1 minute. The raw data was exported and a
standard curve was generated, from which the Ct values of the experimental samples were interpolated to get RNA copy number.

2.10 « Nuclear/Cytoplasmic RNA Fractionation»

300,000 cells were plated per well in a six well plate and allowed to grow overnight. Once at ~70% cells were transfected for 24 hours with the necessary plasmids. At 24 hours post transfection, cells were treated with 10μM Leptomycin B to block the Crm1 pathway. 12 hours later, cells were harvested, pelleted at 1500g, washed with PBS and pelleted again. The cell pellet was then resuspended in 5 pellet volumes of cytoplasmic extraction (CE) buffer (10mM HEPES, 60mM KC, 1mM EDTA, 0.075% NP40, 1mM DTT and 1mM PMSF adjusted to pH 7.6) and placed on ice for 3 minutes. Next, the samples were centrifuged at 2000 RPM for 4 minutes to produce a pellet containing the nuclei and the supernatant containing the cytoplasmic fraction. The cytoplasmic fraction was moved to another tube and placed on ice. Next, the nuclei was washed with 100μL of CE buffer without NP40 and pelleted again. Supernatant was removed and the pellet was resuspended in 2 pellet volume of nuclear extraction buffer (20mM Tris-Cl, 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 1mM PMSF and 25% glycerol adjusted to pH 8.0). The mixture was then incubated on ice for 10 minutes, vortexing periodically and then transferred to a new tube. RNA was extracted from each fraction using the ThermoFisher PureLink RNA Mini Kit (Cat. No. 12183020) according to manufacturer’s protocol.
2.11 «Platinum Taq PCR Protocol»

All PCR reactions were done using the Thermofisher Platinum Taq DNA polymerase kit (10966-018). Each reaction contains: 5μL of 10x Platinum Taq PCR Buffer, 1.5μL of 50mM MgCl2, 1μL of 10mM dNTPs, 0.5μL of each forward and reverse primer at a concentration of 20μM, 0.4μL of Taq Polymerase, 10ng of template and water to bring the final reaction mixture to a total volume of 50μL. The PCR reaction was subject to the following thermocycling conditions: initial denaturation for 2 minutes at 95°C followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 58°C, extension at 72°C for 1min/kb of the final product length, followed by a final 10 minute extension step at 72°C and hold at 4°C.
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Chapter 3

3 « Results »

3.1 « Deletion of RFS in SIVmac239 has no Impact on RNA Packaging or Virus Infectivity »

Based on the presence of the GRPE element overlapping the RFS of HIV-1, we used a similar system as Chamanian et al (2013) to identify whether this function is conserved in the SIVmac239 genome. In the Chamanian paper, results showed that the perturbation of the structure of the frameshift stem resulted in a defect in packaging. Therefore, the frameshift stem of the RFS within the SIVmac239 genome was deleted downstream of the slippery sequence in the U5gag-pol vector. This vector and the wild type U5gag-pol vector were co-transfected in 293T cells with a ngl vector to produce viral particles containing the two subgenomic RNAs (Figure 8A). 48 hours post transfection, culture supernatant was harvested, concentrated and subject to radioactive RT assays to measure the relative amount of viral particles. There was no significant difference in RT activity in cell culture supernatant indicating that both conditions were producing nearly the same amount of viral particles. Each condition had RT activity down to a dilution of 1:256 indicating a robust amount of virus production (Figure 8B).

The relative RT activity was used to normalize the amount of virus for quantifying RNA copy number and infecting cells to measure infectious titers between the different conditions. Viral RNA was extracted, reverse transcribed and subject to qPCR using specific primers to measure the copy number of each subgenomic RNA that was packaged within the viral particles. There was no significant difference between the amount of WT U5gag-pol RNA and dRFS U5gag-pol RNA being packaged. Both of the
U5gag-pol RNAs were packaged with similar quantities as the nfl RNA, falling within the $10^6$ to $10^7$ copies/μL (Figure 9B, 9C).

In the heterodiploid genome system, viral particles that contain both the U5gag-pol RNA and the nfl RNA are able to infect target cells. The U5gag-pol RNA initiates reverse transcription to produce minus strong stop DNA that will then strand switch to the R region of the nfl RNA to continue the reverse transcription process. Real TCID50 assays were done to measure the infectious viral titers in each condition by doing 8, 3 fold serial dilutions and infecting human U87.CD4.CCR5 cells. Infected wells were identified by RT assay, scored + or – and the infectious titer was calculated using the Reed Muench method. There was no significant difference in infectious titers between cotransfection with WT U5gag-pol or dRFS U5gag-pol, which is reflective of the amount of U5 RNA being packaged (Figure 9D). Thus, there is no RNA element in the RFS downstream of the slippery sequence that impacts RNA packaging or infectivity in SIVmac239.

### 3.2 « Deletions Across the gag-pol ORFs of SIVmac239 have no impact on RNA Packaging or Virus Infectivity »

Since the deletion of the frameshift stem in SIVmac239 did not have an impact on its RNA packaging efficiency, there remained the possibility that an enhancement element lied outside of the initially probed region. To address this, larger regions across the U5gag-pol vector were replaced by the URA3 gene to disrupt any RNA structural elements within the region. The URA3 gene was amplified with primers containing overhangs homologous to the regions flanking the desired deletion location within the U5gag-pol vector. Yeast recombination was used to insert these URA3 fragments into the
U5gag-pol vector at the specified locations (Figure 8A) by creating single restriction enzyme cuts within the region where deletion is desired. Four different deletions were successfully made in the U5gag-pol vector with two deletions within the gag ORF and two within the pol ORF, and named U5gag-pol del1-4.

In the same conditions as the comparison of WT U5gag-pol and dRFS U5gag-pol, the new deletion mutants were cotransfected with SIVmac239 nfl and supernatant was harvested and concentrated after 48 hours (Figure 8A). Following concentration, again, one aliquot was used for RT assays to measure relative particle production between the different conditions. Again, there was no significant difference in supernatant RT activity between the cotransfection of WT U5gag-pol and the U5gag-pol deletion mutants (8B). Like the dRFSU5gag-pol mutant, there was also no significant difference in packaged RNA from the U5-gag-pol deletion mutants and the WT U5gag-pol mutant (Figure 9B).

The bipartite genome system was made so that deletions could be made within the protein coding sequence of one RNA while the other would provide all proteins necessary to produce viral particles. It is important to note that U5gag-pol del1 can’t produce any gag, U5gag-pol del2 can’t produce capsid or nucleocapsid, U5gag-pol del3 can’t produce protease and U5gag-pol del4 can’t produce RT. The WT U5gag-pol vector contains the intact protein coding sequence necessary for the production of the proteins that can produce budding viral particles that could be detected by RT assays. Although the U5gag-pol vectors are being packaged and thus travel through the cytoplasm, when the WT U5gag-pol vector is co-transfected with the nfl vectors there is no increased supernatant RT activity compared to the deletion mutants, indicating that the U5gag-pol RNAs are not being used for translation and are only packaged as genomic RNA.
As expected, with no difference in the amount of U5gag-pol RNA being packaged in the deletion mutants, the virus infectivity is not significantly different from the WT U5gag-pol vector (Figure 9D). In mutants such as U5gag-pol del1 and del2, during the reverse transcription process, if there is recombination after the first strand switch event within the pol region of the genome, the integrated provirus would be defective since they would contain large deletions. The fact that there is no significant difference in the infectivity levels between the deletion mutants and the WT U5gag-pol vector indicates that recombination following the first strand switching event is negligible. Although there are very high RNA copy numbers of genomic RNA in these samples, the infectious titers were typically under $10^3$ infectious units/mL which is relatively low (Figure 9D). This low level of infection is most likely due to the use of the human U87.CD4.CCR5 that does not have the macaque receptors that SIVmac239 has evolved to utilize.
**Figure 8 Production of Heterodiploid Genome Viral Particles.** (A) Co-transfection of SIVmac239 nfl and different mutant U5gag-pol vectors to produce viral particles. URA3 replaces del1 from position 633-1204, del2 from 633-2069, del3 from 2056-2760 and del4 from 2696-3556. (B) Representative autoradiograph of RT assay done on a dilution series of produced viral particles, 48h post transfection, to measure relative particle count and bar graph illustrating RT activity relative to WT cotransfection conditions, n=5.
Figure 9 Packaged Genomic RNA and Infectivity of U5gagpol Deletion Mutants. (A) Schematic of SIVmac239 vectors used for transfections and binding location of qPCR primers. (B) Copy number of SIVmac239 U5gagpol RNA packaged within viral particles from cotransfections derived by RT-qPCR and normalized by RT activity, n=5. (C) Copy number of SIVmac239 nflu RNA packaged for each U5gagpol vector co-transfection condition derived by RT-qPCR and normalized by RT activity, n=5. (D) Infectivity of progeny virus for each U5gagpol vector co-transfection condition by Real TCID50 assay, n=2.
3.3 « Generating Heterodiploid Genome System to Evaluate Impact of RFS deletion in FIV34TF10 »

The RNA structures that are implicated in packaging genomic RNA in the FIV genome are not as well defined as those of primate lentiviruses, therefore we set out to see if a GRPE like element also overlaps the FIV 34TF10 RFS. To setup the bipartite genome system, primers were designed to amplify the FIV34TF10 genome and yeast recombination was used to insert them into the pREC expression vector. To generate FIV34TF10 U5gag-pol vector, the FIV34TF10 genome from the 5’ R region into just past the gag orf was inserted into the pREC backbone. To create the FIV 34TF10 nfl vector capable of producing viral particles, the genome downstream of the U5 region was PCR amplified in two segments and yeast double recombination was used to insert both segments into the pREC background. Next, the RFS of the FIV U5gag-pol vector was deleted using site directed mutagenesis to be able to evaluate the impact the absence of the structure had on genomic RNA packaging. The construction of these vectors was done by Dr. Paul Wille.

Following the same experimental approach as for the SIVmac239 experiments, the FIV U5gag-pol WT and dRFS vectors were co-transfected with FIV nfl in 293T cells (Figure 11A). 48 hours post transfection supernatant was harvested, concentrated and assayed for RT activity. There was no significant difference in the supernatant RT levels, indicating that there was no difference in viral particle production levels as expected. RT activity was detectable in the supernatant of both conditions even to a dilution of 1:256, indicating a strong level of viral particle production (Figure 10B).
The relative RT activity of the viral supernatants was used to normalize the amount of input virus for infection of CrFK cells to quantify infectious viral titers through Real TCID50 assays. Interestingly, the progeny virus showed extremely low levels of infectivity in both conditions. There were near undetectable levels of supernatant RT activity in the 1:3 dilution, which is contrary to what was expected based on the very high levels of RT activity in the supernatant harvested from the 293T cell transfection (Figure 10B). This result indicates that although there is a high level of viral particles they are severely hindered in their ability to infect.
Figure 10 Production and Infectivity of FIV 34TF10 in Heterodiploid Genome System. (A) Production of viral particles by cotransfecting a U5gag-pol vector (WT or dRFS) alongside nfl. (B) Autoradiograph of RT assay on produced viral particles, in a 1:4 serial dilution series. (C) Autoradiograph of RT assay for Real TCID50 assay done to measure the infectivity of produced viral particles. (D) Graph comparing the RT activity of viral particle produced post transfection vs post infection, n=3.
Figure 11 RNA Packaging and Nuclear Export of FIV 34TF10 Heterodiploid Vectors. (A) Schematic of vectors used to produce RNA measured in viral particles and nuclear/cytoplasmic fractions with qPCR binding locations. (B) RNA copy number of subgenomic RNAs packaged within viral particles for different cotransfection conditions, n=3. (C) RNA copy number of subgenomic RNAs in nuclear and cytoplasmic fractions of transfected 293T cells treated with Leptomycin B for inhibiting Crm1 nuclear RNA export, n=2.
3.4 « Defective Packaging and Export of FIV U5gag-pol RRE Deficient Subgenomic RNA »

To address whether or not this defect in infectivity of produced viral particles was caused by reduced RNA packaging, viral RNA was extracted, reverse transcribed and subject to qPCR to measure the amount of each subgenomic RNA being packaged. Although there was no significant difference in the amount of between the mutant and WT U5gag-pol RNA, there was almost a 2 log decrease in the amount of packaged U5gag-pol RNA, WT or dRFS, compared to the nfl RNA (Figure 1B). With such a low amount of U5gag-pol RNA being packaged, there would be a large decrease in virus infectivity since reverse transcription cannot be initiated and therefore explains the low infection rates in both conditions.

In the construction of the U5 containing vectors throughout this study, as well as much of Chamanian *et al* (2013) study, the RRE was not included. The RRE has been shown to be required for the export of full length unspliced mRNA into the cytoplasm of the cell through the Crm1 pathway. Full length unspliced mRNA is the RNA species that is incorporated into viral particles, and thus to be packaged this RNA must be translocated into the cytoplasm. In the case of our SIVmac239 U5gag-pol vector, although there was no RRE, the RNA was being packaged at similar levels as the nfl RNA indicating that it was being efficiently transported to the cytoplasm. To test whether our FIV U5gag-pol vector was not being packaged due to nuclear export as a result of the missing RRE, we compared the nuclear and cytoplasmic fractions of cells transfected with both vectors to see if the U5gag-pol vector was trapped in the nucleus. 293T cells were transfected with both the U5gag-pol vector (WT or dRFS) alongside the FIV nfl
vector. At 24 hours post transfection, cells were treated with Leptomycin B, an inhibitor of the Crm1 to confirm determine whether the vectors were being transported through the Crm1 pathway. 36 hours post transfection cells were harvested, nuclear and cytoplasmic fractions separated, and RNA extracted from each fraction. The RNA was reverse transcribed and used for qPCR to quantify RNA copy number for the different RNA species. As expected, there were comparable levels of FIV nfl RNA in the nuclear and cytoplasmic fractions, and treatment with Leptomycin B resulted in almost a log decrease in cytoplasmic FIV nfl RNA levels, confirming that the unspliced mRNA is being transported through the Crm1 pathway (Figure 11C). In contrast, there is about a log decrease in the amount of U5gag-pol RNA in the cytoplasm compared to the nucleus, explaining the low amount of U5gag-pol RNA being packaged (Figure 11C). The treatment of Leptomycin B resulted in slight increase of U5gag-pol RNA in the nuclear fraction accompanied by a slight decrease in the cytoplasmic fraction. Together, this result suggests that although the RRE is disposable for U5gag-pol vector for SIVmac239, it is required for FIV 34TF10.
Chapter 4

4  « Discussion »

Lentiviral RNA packaging has been studied for decades but the finer details of the process still remain unclear. The overwhelming majority of the efforts to understand lentiviral RNA packaging have been on HIV-1, specifically the RNA structures within the 5’UTR. Extending these studies to other lentiviruses helps us better understand their evolution while also providing better insight when designing tools and optimizing different animal models.

The identification of the GRPE was an important discovery in the field of HIV-1 RNA packaging since it highlighted the importance of probing within the coding sequence of lentiviruses for structures that serve multiple functions. The logical next step was to look for similar elements in other lentiviruses to determine whether or not the GRPE is a conserved element.

Since SIVmac239 is one of the most prevalent clones used for primate models of infection and shares a fairly recent common ancestor with HIV-1, it was the first candidate to test whether or not the GRPE element is conserved. The results showed that no sequence within the gag-pol ORFs had any impact on the packaging of genomic RNA. At this point, it is unlikely that any other RNA elements within the coding sequence of SIVmac239 would have any impact on RNA packaging either since U5gag-pol RNA was packaged at a similar level to the nfl RNA.
HIV-1 and SIVmac239 only share about 50% sequence homology so it is understandable that the RNA structures that form would differ not only in shape but to a certain degree their functional capabilities. Chamanian et al (2013) showed that the impact of the GRPE relied on the frameshift stem loop maintaining the correct conformation and if mutations were done that still kept the stem loop structure intact, there was no impact on RNA packaging. The frameshift stem loop of SIVmac239 is distinct in size from that of HIV-1 therefore an RNA-RNA interaction or an RNA-protein interaction with the frameshift stem loop might not be able to occur due to this difference in stem loop size. The 5’UTR of SIVmac239 is also structurally distinct from HIV-1 and although both regions have been considered to be the major determinants of packaging, the exact process is most likely contains some differences. These differences could account for the inability for any element within the coding sequence to enhance the packaging efficiency of unspliced mRNA in SIVmac239.

SIVmac239 is very closely related to HIV-2, the significantly less pathogenic counterpart to HIV-1. It would not be a stretch to think that HIV-2 also does not contain a GRPE element due to it’s high sequence homology with SIVmac239, whereas SIVcpz could very well have a GRPE like element since it is so closely related to HIV-1. Perhaps the increased pathogenicity of HIV-1 compared to HIV-2 is that it has gotten better at packaging it’s genome, therefore leading to greater infectivity. To address this hypothesis, experiments can be done to calculate the absolute level of RNA per viral particle to see if one virus packages their genome with greater efficiency than the other. Our study relied on RT assays to get relative viral particle production between transfection conditions, but by using p27 and p24 ELISAs, one could calculate the
RNA/capsid ratio to get a more accurate measure of RNAs packaged per particle. Since the RFS is a region that is responsible for two ORFs as well as promoting ribosomal frameshifting, perhaps in the case of SIVmac239, the selective pressures of the host immune system required specific mutations at the protein coding sequence that outweighed the need for the region to enhance RNA packaging.

Although SIVmac239 did not exhibit any need for an additional RNA enhancing element within the protein coding sequence, we still wanted to test the FIV RFS in the same system. There was no difference in the amount of FIV U5gag-pol RNA that was packaged when the RFS is deleted, however, there was a severe impairment of virus infectivity, regardless of which U5gag-pol vector was cotransfected with FIV nfl. It turned out that both U5gag-pol RNAs were being packaged at a quantity near two log less than the FIV nfl RNA, thus limiting the template for which reverse transcription could be initiated to produce proviral DNA. We postulated that this might be due to poor export of the U5gag-pol RNA through the nuclear membrane into the cell cytoplasm due to the absence of the RRE. Cells were transfected with both vectors and nuclear/cytoplasmic fractionations were done to compare the RNA levels within each fraction to see if the U5gag-pol vector was impeded in nuclear export. The results showed a large reduction in cytoplasmic RNA of the U5gag-pol vector compared to the nuclear fraction, while this reduction was not apparent in the FIV nfl RNA, indicating that the RRE is most likely necessary for nuclear export and by proxy, RNA packaging.

This result was interesting since the SIVmac239 U5gag-pol vector that was used was also missing the RRE element but was being expressed in the cell cytoplasm at the same level as the SIVmac239 nfl RNA. Although this RNA was being expressed in the
cytoplasm, it was not being translated since the amount of viral particles produced and measured by RT activity did not decrease when gag was replaced with URA3 in the U5gag-pol vector. This could imply that there are certain negative regulatory elements within the gag-pol ORFs that inhibit protein translation in the absence of Rev/RRE binding. One way of identifying these potential elements would be to use the U5gag-pol URA3 deletions to insert GFP in frame in the different locations to simultaneously remove the potential negative regulatory element and also have a phenotypic read out (fluorescence).

The fact that the SIVmac239 U5gag-pol vector is packaged efficiently but not translated could be beneficial in the design of lentiviral gene delivery systems. Many of these systems rely on export elements such as that of the constitutive transport element (CTE) of the Mason-Pfizer monkey virus in their transfer vector, but perhaps the SIVmac239 system could provide an alternative. Before its application, an important experiment that needs to be done is to add the 3’LTR to the U5gag-pol vector to see if it retains efficient RNA export, since there could be potential negative regulatory elements in the 3’LTR that suppress export of unspliced mRNA in the absence off the Rev/RRE interaction.

Clearly, the FIV 34TF10 genome would not be as easily applied in the context of this system. It is unclear why in the absence of RRE the SIVmac239 U5gag-pol is exported but for FIV it is not. There are most likely negative regulatory elements that exist that have not yet been identified that are implicated in trapping the RNAs in the nucleus. The 5’ leader of the FIV genome vastly differs from those of primate lentiviruses, and the function of many of these different structures has not yet been
assigned. One possibility is that one of the structures downstream of the splice donor site within the 5’UTR could be a negative regulatory element, since the elements upstream of the splice donor exist on fully spliced, RRE deficient, transcripts that are efficiently transported. Making point mutations within some of the stem loops in the 5’ leader that would disrupt the structure in the FIV U5gag-pol vector and measuring the ability of these mutants to be transported could shed more light on what other elements could be involved in nuclear RNA transport. One study had shown that FIV gag localizes to the nucleus after translation and acts as a shuttling protein for export of nuclear localized proteins through the Crm1 export pathway. One idea that they had proposed based on these findings is that perhaps NC interacts with genomic RNA within the nucleus and the ribonucleoprotein is shuttled through the nuclear membrane and becomes packaged in viral particles\(^{116}\). Our study disproves this theory since there are similar levels of FIV nfl and FIV U5gag-pol RNA in the nucleus but this does not hold true in viral particles, and therefore encapsidation of FIV genomic RNA does not initiate within nucleus of infected cells. Unfortunately, the compromised nuclear export of the U5gag-pol RNA does not allow us to conclusively claim that the RFS has no impact on RNA packaging.

Therefore, the logical next step is to clone the RRE back in to U5gag-pol RNA to definitively see if the RFS plays any role. Efforts have been made to generate a U5 containing vector that spans from the 5’ R region to the RRE in FIV 34TF10 using the yeast recombination system and including the RFS deletion. Unfortunately during the recombination or transformation process, the U5 region gets deleted and yields negative RT-qPCR data. RT assays done on transfection supernatant, however, show double the amount of RT activity compared to just the RU5 gag-pol vectors (data not shown). This
makes sense, since the transcript is being exported efficiently and being translated into
viral particles at a similar frequency as the U3 containing nfl vector, thus producing
double the amount of viral particles. In the future, other cloning strategies must be used
to try and incorporate the U5 region into these vectors for effective probing of the
genomic RNA and infectivity studies.

These studies not only shed light on the function, or lack thereof depending on the
context, of certain RNA structures in SIVmac239 and FIV 34TF10, but they also provide
some comparison to help narrow down how exactly GRPE enacts its function. Although
it was never our hypothesis, other studies have shown that the GRPE has no increased
binding affinity to HIV-1 NC than random RNA species. There could potentially be other
protein binding partners for the GRPE other than NC, since it has recently been shown
that full length unspliced mRNAs form granules with other host proteins such as DDX6
and ABCE1\textsuperscript{117}. It is imperative that more studies are done to look into potential binding
partners by doing RNA pull downs followed by mass spectrometry.

Furthermore, there is also a strong possibility that the GRPE is able to improve
packaging efficiency through an RNA/RNA interaction. Perhaps the GRPE is involved in
a long range interaction with the 5’UTR that helps maintain a conformation that is more
conducive to RNA packaging instead of protein translation. One way to test this would be
to replace the SIVmac239 5’UTR with that of HIV-1, as well as swap the ribosomal
frameshift signals, and see if there is an increase in packaging efficiency, since
SIVmac239 NC is able to interact with HIV-1 psi and cross package it’s RNA. Another
possibility that requires exploration is that the GRPE could be involved in maintaining
the stability of RNA dimers. It has been well characterized that the DIS is responsible for
forming the initial loose kissing dimer, but it is known that the RNAs form tighter interactions when they are being packaged. There is a six nucleotide palindrome that exists at the base of the P3 stem loop of the RFS in HIV-1, and there is evidence that a pseudoknot can form at the RFS, which could potentially expose this region in a way that could interact with the RFS of an adjacent RNA, possibly strengthening the dimer interaction. More mutational analysis followed by salt titrations to measure dissociation rates of the dimers would help elucidate or rule out this hypothesis.

In conclusion, the evolution of different lentiviruses has caused the divergence of function in many of the RNA structural elements within their protein coding sequence. This study highlights the importance of comparing and contrasting these elements across lentiviruses to gain a better understanding of the role they play in their respect viral life cycles.
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