Cloning and characterization of visna vif mutants

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Cloning and characterization of visna vif mutants
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Abstract

Maedi-Visna virus is a lentivirus which affects the lungs and the central nervous system of sheep. The vif gene of maedi-visna virus is necessary for infection of the virus. The Vif protein carries the APOBEC3 proteins to the proteasome, in other words, it uses an ubiquitin ligase to destroy the APOBEC3 proteins. The APOBEC3 proteins are cytosine deaminases which deaminate cytosine (C) to uracil (U) in the minus DNA strand of the virus during reverse transcription. When the cDNA is transcribed to plus strand, adenine (A) pairs with U, leading to G-A hypermutation.

Several mutations have been introduced into the vif gene, and these have led to knowledge of the function of the gene. With this method a cure for AIDS, MVV and other lentiviruses is being investigated. In this text, three mutations have been created, one on W_{98} amino acid, other one in P_{205} and the last one on $IP_{216, 217}$ amino acid.

The infectivity of the $IP_{216,217}$ was examined by using a HIV-1 vector system. Preliminary results indicate that the mutation caused reduced infectivity of the virus.

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INTRODUCTION:

HISTORY:

In the years 1935 to 1951 sporadic cases of previously unknown paralytic disease affecting the central nervous system (CNS) were observed on many farms in the southwestern and southern districts of Iceland. The disease was named visna, meaning shrinkage or wasting in Icelandic, which describes the clinical appearance of the paralyzed sheep. It became evident that the disease had been introduced by an import of Karakul sheep from Germany in 1933. The importation had the goal to improve the Icelandic native breed **(54).**

In the districts where visna first appeared another new sheep disease, pulmonary adenomatosis, had recently been discovered. The chronic progressive pneumonia acquired clinical sign of the disease **(21).**

Maedi and visna were considered two different variants of the same virus, it was called: Maedi-visna virus. The virus is a member of the lentivirus group of retroviruses, and it was the first virus recognized in the slow infection family. It was discovered and described by the Icelandic scientist Sigurdsson in 1954 **(21)**

In 1957-1958 a previously unknown virus was isolated in sheep tissue culture from 5 brains of experimentally infected visna sheep. This virus reproduced visna after intracerebral inoculation into healthy sheep. Later, visna was produced by intrapulmonary inoculation. In addition to the visna lesions in the CNS both intracerebral and intrapulmonary inoculated sheep developed the lung lesions of progressive pneumonia (maedi) **(21).**

By the time that visna was isolated, natural cases of visna had ceased to exist in Iceland due to an extensive slaughtering program to eliminate the 3 diseases. After 3 years, flocks of sheep were removed from the western part to the affected areas. Visna has not reappeared, and neither has adenomatosis, but, maedi reappeared in 1958-1965 in a few outbreaks **(54).**

The conclusion from the Icelandic experience is that visna and maedi are two histopathological and clinical forms of the same viral infection **(54).**

Dr. Björn Sigurðsson was the first scientist who recognized that besides acute and chronic infections another category of infections existed, the ‘slow infections’. He described in sheep as characteristic a ‘long latent period’, not synonymous with incubation period, because the virus is propagated and spread in the body without any clinical signs for months and years followed by a continuous progress of clinical signs after their first appearance leading eventually to severe sickness and death of the sheep **(54).**

Visna, maedi and adenomatosis all had an onset after an incubation period of months or years. In this case, 2-6 years passed after the introduction of a Karakul sheep into a district and the onset of clinical disease in the local sheep **(54).**

Housing sheep from an infected flock with normal sheep often caused infection, but only rarely could an infection be traced to fencing in sheep together outdoors. A very close contact with infected sheep seems necessary for spread of these diseases in nature or in the laboratory **(21).**

The imported Karakul sheep never developed obvious clinical signs of Maedi–visna which raised the question of a breed predisposition. The Icelandic scientists suggested that the native sheep were extremely susceptible. Inquiries revealed that this isolated flock of

Karakul sheep still existed in 1970 in the German Democratic Republic (East Germany). The original flock had been imported in 1901 from Astrachan (Russia). From the exterior they seemed to be the right source to improve the Icelandic native breed. During these years from 1901 to 1970 maedi was never detected in the isolated German flock. The same result was reported from six foreign countries which had received sheep from this flock after 1945 **(54).**

CLINICAL AND PATHOGENESIS:

The major host cells of maedi-visna virus are cells of the monocyte/macrophage lineage, and other lentiviruses, like HIV for example, replicate in $CD4^+$ lymphocytes as well. Infection of these immune cells is the basis for the multiorgan disease that is characteristic of all lentiviral infections. A burst of replication happens in the first few weeks after infection, and during this acute phase the lentivirus spreads throughout the host. Primary sites of viral replication include the lymph nodes, spleen and bone marrow. The virus replication in these organs serves as a reservoir for the posterior spread of infected monocytes and lymphocytes to multiple sites in the body, such as brain, lung, joints, and other organs where the monocytes mature into macrophages and the lymphocytes are activated. The differentiation and activation of the cells serve as activator of the viral gene expression; therefore, virus is then produced in these tissues. This ability to remain latent is one mechanism that the virus uses to evade detection by the host. Another strategy that lentiviruses use is genetic variation **(21).**

The clinical signs of the two diseases are listed in Table 1.

Major clinical signs of maedi	Of visna
Dry cough	Weakness in the hind legs, progressing,
Expiratory dyspnoea	Finally complete paralysis
Body temperature only in terminal stage due to secondary infection elevated	Digestive tract not involved
Emaciation in spite of good appetite and feed intake	No rise in body temperature
Strain dependent mastitis and/or arthritis	Sometimes central nervous disorders

Visna is very insidious in its onset, and only by careful observation can the first signs be recognized in many cases. The sheep may show a slight aberration in gait, especially of the hindquarters; the power to extend the fetlocks is impaired. A fine trembling of the lips is sometimes an early sign, and the head may be turned a little over to one side. The symptoms gradually progress and may end in paraplegia or even total paralysis, so that the animal is unable to rise. All cases eventually die of the disease, but the clinical course is variable. Visna affected sheep gradually lose weight; failure to thrive is sometimes the first sign noted. Both sexes are affected **(21).**

In maedi, failure to thrive is often the first sign noted, then dyspnoea, first upon exercise and later at rest, and a dry cough. There are no general signs of an acute illness. The clinical course is inevitably fatal, as in visna **(21).**

Normally, in both late and early cases of visna and maedi, as it has been explained before, virus is always found in spleen, lymph-node, lung and choroid plexus, often in the

salivary gland and sometimes in kidney, if explants of these organs are cultivated in plasma clots or in trypsinised kidney cells. It is particularly easy to re-isolate a virus from spleen and lymph-nodes, due to its function as primary reservoir, and the virus appears to have great affinity for reticulo-endothelial tissues **(21).**

MOLECULAR BIOLOGY:

INTRODUCTION:

Lentiviruses have similar genetic compositions, with the structural genes gag, pol, and env encoding the core proteins, enzymes (reverse transcriptase, protease and integrase), and envelope glycoproteins, respectively. Besides, lentiviruses have a more complex genetic organization containing additional small open reading frames (ORFs) located between the pol and env genes, as well as exons contained within and at the 3´end of the env gene **(11).**

In addition, regulatory genes modulate the viral cycle in vitro and probably contribute to clinical latency and pathogenic mechanisms in vivo. The regulatory genes tat and rev control viral transcription and RNA transport and translation, vif and vpu regulate production of infectious viral particles. These regulatory genes share little nucleotide or amino acid homology from one lentivirus to another, yet their functions are conserved. Accessory proteins are also important determinants of cross-species transmission and subsequent adaption to new animal hosts **(38).**

MOLECULAR BIOLOGY OF MAEDI-VISNA VIRUS:

The ORFs of CAEV and visna virus are biologically related. In addition to the structural genes, these viruses have ORFs that correspond to vif, tat and rev **(11).**

The additional gene products of the lentiviruses contribute to a more complex pattern of gene expression and may also contribute to the pathogenesis of disease. Lentivirus replication can be separated into early and late gene expression. Early gene expression is characterized by the presence of Tat and Rev proteins, all products of multiply spliced viral mRNAs. Despite of this, the structural, enzymatic, and accessory protein, Vif is synthesized from unspliced or singly spliced viral mRNAs during late gene expression **(11).**

The Vif (viral infectivity factor) protein, although not universally found in the lentivirus virion, appeared in early studies to facilitate the infectivity and spread of virus, particularly in primary lymphocyte and macrophage cultures. Other studies, suggested that the effects of the Vif protein were on early events (proviral DNA synthesis) after viral infection Sequence similarities among Vif proteins are low apart from a single highly conserved motif (S/T)LQ(F/Y/R)LA which is a BC-box **(11).**

VIRUS REPLICATION:

Lentiviruses are enveloped RNA viruses that bud from the cellular membrane, producing mature particles. The lentivirus virion core structure is cone-shaped. The virion core is composed of the gag proteins that are proteolytically processed from a gag precursor protein by the virion-encoded protease.

The virion core contains two copies of viral RNA associated with one gag protein. This protein (called NC) contains zinc finger motifs which interact with the viral RNA via these protein motifs. The viral reverse transcriptase and integrase are also associated with the viral RNA. There is also one gene segment in the pol gene between RNAse H and the integrase. Visna virus contains this genetic element. This region encodes a functional dUTPase activity in visna virus which has been found in gradient-purified virions, indicating that these viruses bring this protein into the cell upon infection. The virus-encoded dUTPase is required for efficient replication in macrophages, which are nondividing, terminally differentiated cells. It is known that cellular dUTPase are cell cycle regulated; one of their roles in DNA replication in cells is thought to prevent uracil incorporation into DNA **(11).**

The virion core is surrounded by the viral envelope that is derived from the cellular membrane in which the viral surface and transmembrane proteins have been inserted. In few words, the envelope proteins of lentiviruses are synthesized as a large precursor protein that is cleaved by a cellular protease into the surface and transmembrane glycoproteins. The transmembrane glycoprotein has two hydrophobic domains; one at the amino terminus is responsible for virus-induced cell fusion and is only functional after cleavage of the glycoprotein precursor. The second hydrophobic domain spans the cellular membrane and anchors the glycoproteins in the membrane **(11).**

The first step in the viral life cycle is the interaction of the virion with the target cell; the surface glycoprotein of the virus interacts with specific receptors on the cell surface. Interaction of lentiviruses with their receptors apparently causes a conformational change in the transmembrane envelope glycoproteins that exposes the hydrophobic domain at the amino terminus of the protein. Viral entry appears to be mediated through the fusion of the viral envelope with the cellular membrane **(11).**

After virus entry into the cell, the virus is partially uncoated and the viral RNA is copied by the virion-associated reverse transcriptase, generating a double-stranded DNA copy of the viral RNA genome. In the early steps of DNA replication, activation of the cell is apparently required for complete synthesis of the double-stranded DNA. In addition, cellular factors play a crucial role at these early replication steps since there is a host restriction observed for many lentiviruses at this stage **(11).**

The termini of the long terminal repeats (LTRs) that flank the viral DNA genome are involved in the integration of the viral DNA into the cellular genome; the cell may either remain latently infected, with little or no expression of virus, or be productively infected **(11).**

The LTRs also contain signals for transcriptional activation as well as RNA synthesis, capping, and polyadenylation. Transcription of lentiviral genomes is initially dependent on the presence in the cell of the appropriate cellular transcription factors. The specific cellular transcription factors utilized by a particular lentivirus are in part determinate for its cellular tropism. The macrophage-tropic viruses contain AP-1 and AP-4 binding sites. The cellular factors of AP-1 binding sites in the visna virus LTR are important for transcriptional activation of these viruses in macrophage cell lines, Viruses have sites in the U3 regions that are recognized by other cellular transcription factors. Visna virus utilizes the transcription factors c-Jun and c-Fos (that binds to AP-1 sequences) found in activated or differentiated macrophages **(11).**

The ability of lentiviruses to replicate in nondividing cells distinguishes them from other groups of retroviruses **(11).**

REGULATION OF LENTIVIRUS GENE EXPRESSION BY VIRAL PROTEINS:

Lentivirus gene expression is characterized by early and late phases; however, transcription of the viral RNA always results in the synthesis of a full-length mRNA species that serves as the viral genome as well as the mRNA for the gag and pol genes. Processing of the full-length viral mRNA occurs in the nucleus of the infected cell, and the levels of spliced and unspliced viral mRNAs that are in the cytoplasm of the cell are controlled by the viral regulatory proteins Tat and Rev **(11).**

The tat gene of lentivirus functions to increase viral gene expression at both the transcriptional and posttranscriptional levels. Lentivirus that lack a TAR element (Tat activating region) like visna virus, have Tat proteins that act in a more indirect fashion. The Visna virus Tat protein is a transcriptional activator that contains a typical acidic-hydrophobic domain that does not bind directly to DNA or RNA. The mechanism of the transactivator proteins interacts with cellular proteins to activate transcription. The visna virus Tat protein activates via the AP-1 site most proximal to the TATA box. It also activates transcription of heterologous promoters that contain AP-1 sites **(11).**

The function of the Tat is to increase the expression of viral mRNA during the early phase of gene expression. This early phase of viral gene expression is characterized by the presence of spliced and unspliced viral RNAs in the nucleus but only fully spliced viral mRNAs in the cytoplasm. A critical level of Rev protein is required to shift the early phase of replication to the late phase of viral gene expression. This late phase is characterized by the transport and translation of the structural and enzymatic genes from unspliced and partially spliced mRNAs **(11).**

The Rev protein facilities the export of unspliced viral mRNAs from the nucleus and the association of these viral RNAs with polyribosomes. Rev proteins of visna virus localizes in the nucleolus in infected primary cells in culture **(11).**

The Rev protein functions by binding via its basic domain to highly structured RNA elements present in the env genes of lentivirus **(11).**

The expression of the unspliced viral RNA that encodes the structural proteins of the viral core (gag proteins) and the enzymatic proteins of the pol gene, as well as that of the singly spliced mRNA of the env gene, is dependent on the Rev protein **(3)**

VIF GENE:

The "vif" gene codes for "virion infectivity factor", a protein that increases the infectivity of the MVV particle **(59).**

The protein is found inside all lentiviruses except equine infectious anemia virus (EIAV), and it works by interfering with one of the host system's defenses - a cellular protein called APOBEC3. Basically what happens is that vif sticks to APOBEC3 and encourages the cell to degrade it, preventing it doing its job of sneaking into newly-formed virus particles and making them non-productive (see the APOBEC3 page for more information). Vif itself is a relatively unstable protein with a half-life of ≈ 30 minutes and – like APOBEC3G – is degraded by cellular proteasomes **(1,14)**.

This has been verified in experiments. If you can create a virus with the Vif protein missing (we would call this a "delta-Vif" strain), then it can still infect a cell - but the new virus particles produced from that cell contain APOBEC3 and therefore aren't very effective at infecting other cells **(59).**

The maedi-visna vif gene is necessary for efficient viral replication in cell culture. To proof this, cell cultures of sheep choroid plexus cells (SCP), Fetal ovine synovial (FOS) and macrophages were infected with wild-type virus KV1772 and dVif-1. All three cell types allowed efficient replication of the wt virus KV1772. Growth of dVif-1 was not detected in macrophages, as well as a poor growth in SCP cells (10-fold low of wild type). However, dVif-1 virus replicated to some extent in FOS cells. Macrophages are considered natural target cells of MVV infection. The growth of dVif-1 was highly attenuated in SCP cells but less in FOS cells **(32).**

From here, appear the terms: Permissive and nonpermissive cells: Permissive cells are those cells which replication is not affected by the Vif deficiency; whereas nonpermissive cells are those cells which noninfectious virions are produced by the cells. For a long time it was recognized that nonpermissive cells produce a cellular factor or factors with antiviral activity. Now it is known that this factor is APOBEC3, APOBEC3 triggers substantial G-to-A mutations in the nascent DNA in the absence of Vif **(23).**

It is possible, that, in evolution, mammalian cells have developed a range of enzymes, including cytidine and adenosine deaminases, to destroy invading nucleic acids, and that some of the lentiviruses have acquired and developed, the Vif protein, to counteract these defenses.

The MVV vif gene is required for efficient replication in vivo. Sheep with dVif-1 virus and KV1772 virus were infected. The virus was not isolated from any of the dVif-1 infected sheep, whereas KV1772-inoculated sheep showed high infectivity **(32).**

Increased mutation frequency in Vif-deficient virions. Analysis of sequences from dVif-1 virions grown in FOS and SCP cells showed a greatly increased mutation frequency compared to KV1772. G-to-A transitions corresponding to cytidine deamination of the first strand DNA were most common **(32).**

APOBEC PROTEINS:

INTRODUCTION:

APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide) proteins are host cellular proteins which function by "hitchhiking" with newly produced viral particles until a new target cell is found. Then, during synthesis of the first retroviral DNA strand (minus strand), which is an obligate step in the retroviral life cycle, APOBEC-dependent deamination of cytosine (C) residues results in the accumulation of excessive levels of uracil (U). This pre-mutagenic lesion leads to the demise of the invading retrovirus on its replication, because uracil is recognized as thymine (T) by the viral reverse transcriptase and adenine (A) is incorporated into the newly synthesized second (plus) DNA strand rather than guanine (G). This process of lesion fixation can therefore produce a detrimental level of mutation in the viral genome. A3 proteins have either one or two conserved, zinc

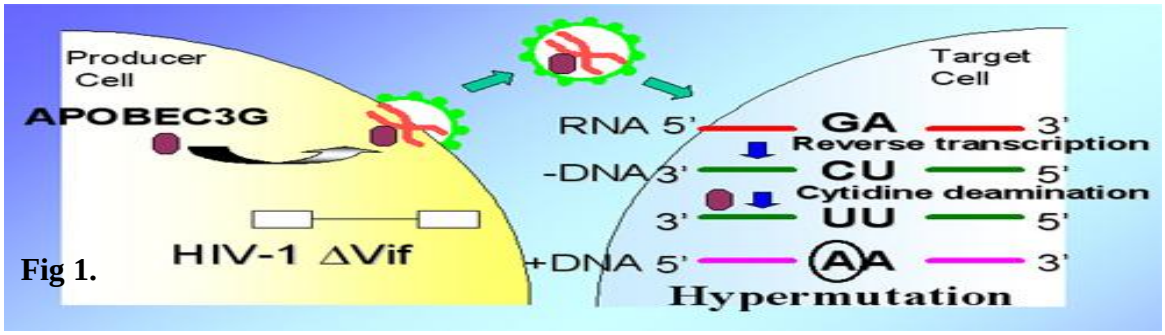

Fig 1.

binding deaminase domains, His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys**(23).** The histidine and the two cysteines coordinate zinc and the glutamate participates in the C→U deamination reaction **(27)**.

The zinc-binding deaminase domains cluster into three distinct phylogenetic groups: Z1a, Z1b or Z2 **(12)**. The active sites of the human DNA cytosine deaminase-competent A3 proteins can be classed as Z1. Most of artiodactyl A3F proteins have a Z1a/Z2 organization. N-terminal Z1a or the C-terminal Z2 domain of the artiodactyl A3F proteins catalyze DNA cytosine deamination **(27)**. The artiodactyls A3F proteins are predominantly cytoplasmatic and they have deaminase-dependent and independent retrovirus activities. **(31)**. All these data suggest that the artiodactyls A3F proteins can be considered evolutionary intermediates between the rodent and primate enzymes. This is in accord with the fact that whereas the primates have seven A3 genes, sheep and cattle have three and the mouse has only one **(23,27,34)**. One important feature among human double domain proteins and artiodactyl double domain proteins are that DNA cytosine deaminase activity resides in the N-terminal Z domain in artiodactyls instead of in the C-terminal Z domain in humans **(27)**. Although accessory proteins acquire the species specificity and avoid the cross-species transmission, it has been shown that some lentiviral Vif proteins lack species specificity. The best example is MVV Vif which triggers the near-complete degradation of the sheep, cow, and cat A3Z3 proteins and partial degradation of the human and rhesus macaque A3H proteins. MVV Vif also restores or improves HIV infectivity in the presence of each mammalian A3Z3 protein. In contrast, the closest relative of this protein, BIV Vif, appears to neutralize only the cow A3Z3 protein **(49)**. All this data suggest that Vif-A3Z3 interaction is not completely species specific but more likely to be host optimized and considerably promiscuous **(49)**.

APOBEC3G belongs to a larger family of bona fide and putative cytosine-deaminase proteins. A3G is one of seven human A3 proteins (A3A to –H) encoded in tandem on chromosome 22 **(12, 26, 30).**

These family members contain a characteristic domain structure. A short α-helical domain is followed by a catalytic domain (CD), a short linker peptide, and a pseudocatalytic domain (PSD). In APOBEC3B, APOBEC3F and APOBEC3G, the entire unit is duplicated to form the domain structure helix1-CD1-linker1-PCD1-helix2-CD2-linker2-PCD2 **(26)**. In APOBECs carrying two deaminase domains (CD1 & CD2), generally only one domain is catalytically active while the second domain is involved in nucleic acid binding and virus encapsidation **(16, 22, 25, 27, 43, 45).**

For example, APOBEC1 is an RNA editing enzyme and is the founding member of the APOBEC family of cytidine deaminases **(58)**. This APOBEC1, in conjunction with APOBEC complementing factor, acts in a highly specific manner and normally deaminates only single cytosine (C^{6666}) on the more than 14,000 nucleotide long apolipoprotein B mRNA to create a premature translational stop codon **(10, 58)**.

There are amino-acid similarities between APOBEC3G and the human mRNA-editing protein APOBEC1 **(57)**. However, APOBEC3G is also similar to AID, a protein that is thought to trigger immunoglobulin gene-diversification. Similar to AID, APOBEC3G can cause C/G to T/A transition mutations in DNA. This phenomenon had therefore been appropriately termed retroviral hypermutation **(23)**. Unlike APOBEC1, which targets single-stranded RNA, APOBEC3G selectively targets single-stranded DNA.

Inter-species comparisons have revealed that APOBEC3G and most of the other APOBEC-family members show more amino-acid-altering (non-synonymous) base-substitution mutations than they show synonymous base-substitution mutations **(50, 65)**.

This phenomenon indicates that the APOBEC-family members have been under a positive-selection pressure, which has facilitated the rapid fixation of amino-acid changes. At present, the double-deaminases-domain proteins have been identified only in mammals. It is curious that the most ancient APOBEC-family members are AID and APOBEC2 **(23)**.

The mechanism of cytosine deamination by APOBEC3G is undoubtedly similar to that of APOBEC1 and AID. Cytosine and cytidine deaminases are characterized by a zinc-binding deaminase motif with the consensus amino-acid sequence His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys (where X denotes any amino acid). Main difference between the aforementioned different deaminases is that APOBEC3G is a double-deaminase-domain protein **(23)**.

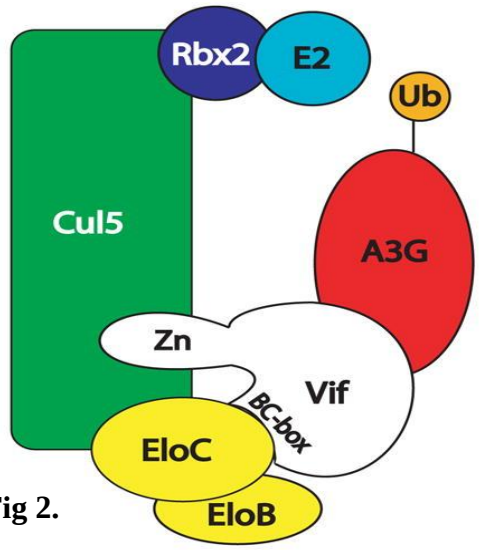

Fig 2.

INCORPORATION OF APOBEC3G INTO VIRIONS:

Based on its mainly cytoplasmatic localization, it is plausible that some APOBEC3G is simply engulfed by the budding retrovirus **(39,40, 53, 55, 56)**. Second, the incorporation of APOBEC3G into viral particles might be facilitated by a cellular protein. Third, APOBEC3G might bind a component of the virus itself.

In recent studies, it shows that Gag (group specific antigen) protein of HIV-1 can interact with APOBEC3G. Two groups mapped this interaction to the nucleocapsid region of Gag **(2, 55)**, whereas a third group showed that this region was dispensable for the incorporation of APOBEC3G into virus-like particles **(55)**It is plausible that the Gag-APOBEC3G interaction is not direct but occurs, instead, through an RNA-containing complex **(55)**. Whether this is the sole route used by APOBEC proteins to gain access to the virion has yet to be determined. Indeed, the current consensus view is that A3G packaging is determined by specific interactions between the N-terminal CDA domain of A3G and the nucleocapsid (NC) region of Gag that are also dependent upon the binding of A3G to RNA **(3, 7, 31, 35, 42, 51, 55, 64).**

APOBEC3G DESTRUCTION IS MEDIATED BY VIF:

The specificity of protein degradation is mediated by members of the E3 ubiquitin ligase family, including the cullin-based E3 ligases. Cullins form a scaffold on which other components of the E3 ligase organize to bring the substrate into close proximity with the E2 ubiquitin-conjugation enzyme **(6, 8, 13, 20, 28, 33, 47, 48)**

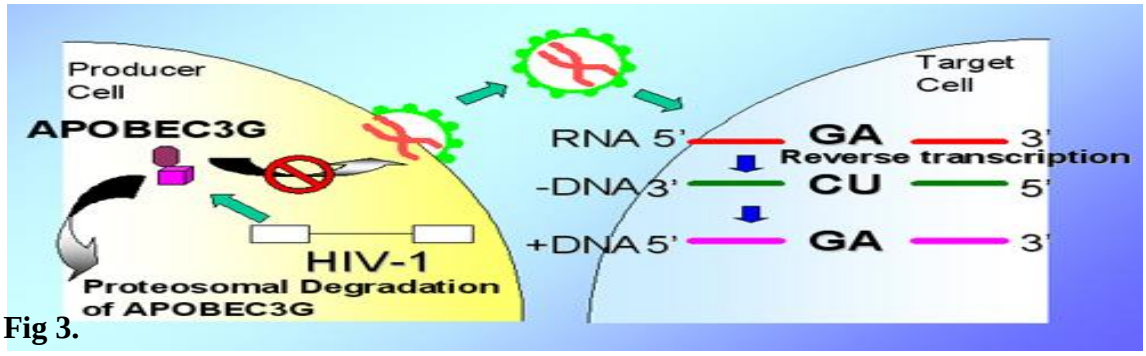

Fig 3.

Ubiquitin-ligase is a complex that contains Vif and several cellular proteins such as ElonginB and ElonginC, Cullin-2 (Cul2) and Ring-box-1 (RBX1) or Cullin-5 (Cul5) and Ring-box-2 **(63)**. Vif might also function to impede APOBEC3G protein synthesis, but this possible role is less well-defined **(29, 53)**.

Cul2 and Cul5 ubiquitin ligases each include the heterodimeric Elongin BC complex, composed of the 112-amino acid ElonginC protein, and the 118-amino acid ElonginB protein. This complex functions as an adaptor that links a BC-box substrate recognition subunit **(37).** The BC-boxes have a consensus sequence, required for binding to the Elongin-BC complex. This motif is [STP]LXXX[CSA]XXXφ, which has residues among 140 to 155 of Vif. Binding of Elongin BC to the BC-boxes is governed by interaction of an invariant leucine at the N terminus of the BC-box with a hydrophobic pocket created by residues in the C-terminal half of ElonginC **(37).** Besides, phosphorylation of Ser-144 in Vif´s BC-box inhibits Elongin C binding, suggesting that A3G degradation may be sensitive to regulation by as yet unidentified signaling pathways **(41).** Structurally, the Vif BC box adopts a loop-helix structure, with the helix starting at the second residue (L145) in the consensus SLQYLA sequence. Both the loop and the helix form a largely hydrophobic interface that binds EloC **(5).** A notable difference between the cellular and viral BC boxes is that the highly conserved cysteines and arginine residues in the middle of the cellular BC box are replaced by alanine (A149) and leucine (L150) in Vif **(36, 62).**

Assembly of Elongin BC-box proteins into Cul5 and Cul2 ubiquitin ligases can be determined by the presence of specific motifs, termed "Cullin-boxes" located downstream of the BC-box. The Cul5-box corresponds to the C-terminal portion and has the consensus sequence φXXLPφPXXφXX(Y/F)(L/I) **(24, 28).** The central LPφP might play a particularly important function as a determinant of Cul5 binding **(28)**. The conserved hydrophobic residue, Val, at the N terminus of the Cul5-box is important for Cul5 binding **(37)**. Talking about Vif, the conserved hydrophobic residues, mostly leucines, which Vif posses in the Cullin box point toward a different face of the EloC H4 helix, this interacts with the BC box. Besides, it is found that the Vif Cullin box-EloC interaction is required for the binding of Cul5 but not EloBC **(5)**.

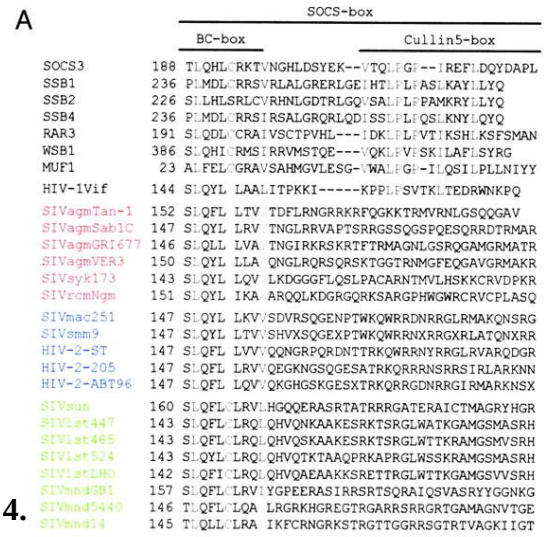

Fig 4.

The complex Vif-EloBC-Cul5 reveals two potentials Vif-Cul5 interfaces. The first interface is observed between the PPLP downstream region in the Vif Cullin box and Cul5 loop 2. The second potential interface is found between the Vif HCCH zinc finger motif and Cul5 loop5 (residues 118 to 134) **(5).**

Although sequences downstream of the BC-box have been shown to be needed for interaction with Cul2, it has been difficult to define a specific sequence motif that correlates with Cul2 binding **(28)**. The Cul2-box is located ≈8-23 amino acids C-terminal to the BC-box and in each protein is evolutionarily conserved in sequence and position. This motif shares some sequence similarity with the Cul5-box and has the consensus φPXXφXXXφ, where the first position is most frequently a Leucine **(37)**.

Therefore, there are little similarities among residues conserved in Cul2- and Cul5-boxes **(28)**. Residues conserved in the Cul2-boxes of many BC-box proteins are a subset of those conserved in the Cul5-box. Many Cul2-boxes include a single LP, IP, or VP in place of the conserved Cul5-box LPφP motif, which has been proposed to play an important role in Cul5 recruitment **(37).**

SIV_{AGM} (African Green Monkey simian immunodeficiency virus) Vif and other non-human primate lentiviral Vif proteins lack a clear Cul5-box. Although the SLQ_xLA motif in certain SIV Vif proteins matches well with a cellular BC-box consensus sequence **(6)**, this motif in other SIV Vif proteins, including SIV_{AGM} Vif, is highly divergent from the consensus BC-box sequence **(6)**. SIV Vif proteins interact efficiently with human ElonginC and Cul5, because human and AGM have identical ElonginC and Cul5 amino acid sequences. ElonginC and Cul5 are both highly conserved among mammals and vertebrates. Therefore, despite the absence of a Cul5-box and highly divergent BC-boxes, primate lentiviral Vif proteins from multiple lineages can assemble with Cul5-ElonginB-ElonginC complexes **(33)**. Both Cul2 and Cul5 use ElonginB and ElonginC as adaptors to bridge substrate receptor molecules in the E3 ligases **(13, 47, 48)**. Visna virus and BIV also contain a BC-box like motif (SLQxLA). Visna virus and BIV Vif interact with ElonginB and ElonginC **(33)**. Cul2 and Cul5 assemble with their respective substrate receptor proteins through a common adaptor complex, ElonginB-ElonginC. Cul5-box or Cul2-box is responsible for cullin selection.

Vif proteins selectively recruit Cul5 but lack a Cul5-box, suggesting the existence of other motifs in these Vif proteins. Sequence analysis of HIV/SIV Vif proteins identified a highly conserved $Hx_5Cx_{17-18}Cx_{3-5}H$ motif just upstream of the BC-box (SLQxxA motif), and has the potential to coordinate zinc, which may participate in the assembly of Vif with Cul5-E3 ligase. BC-box is responsible for the formation of the Vif-ElonginC-ElonginB complex but is not sufficient to recruit Cul5 into the complex: Both the BC-box and HCCH motif are required to form the complete Vif-Cul5-ElonginB-ElonginC complex **(33)**.

Non-primate lentiviral Vif proteins, which lack this HCCH motif, displayed reduced interaction with Cul5. Sequence analysis of non-primate lentiviral Vif proteins from BIV, Visna virus, and feline immunodeficiency virus indicated the absence of the HCCH motif that is present in HIV/SIV Vif proteins. When compared with HIV-1 Vif, Visna virus and BIV Vif interacted inefficiently with Cul5. Interaction of Visna virus with various cullins was also examined. Visna Vif was coimmunoprecipitated with Cul2 and to a lesser extent with Cul5 **(33)**. This finding is in sharp contrast to that for SIV Tan Vif and HIV-1 Vif **(47)**, which preferentially interacts with Cul5. Visna Vif was not coimmunoprecipitated with Cul1 or Cul3, which do not interact with ElonginC **(33)**. Non-primate Lentiviral Vif proteins that lack the HCCH motif, such as Visna Vif, do not selectively recruit Cul5. It is possible that the BC-box downstream sequences from SIV Tan Vif contain a degenerate Cul5-box and mediate interaction with Cul5 **(33)**.

DEGRADATION-INDEPENDENT INHIBITION OF APOBEC3G:

Vif has additional functional properties that prevent the encapsidation of APOBEC3G into virions n a degradation-independent manner. The most striking observation in that respect is the recent identification of a degradation resistant form of APOBEC3G. Degradation resistant APOBEC3G was still packaged into vif-deficient HIV-1 virions and had antiviral properties. Surprisingly however, Vif prevented the packaging of this APOBEC3G variant and restored viral infectivity **(46)**.

There are different mechanisms that can adjust with the degradation-independent process that occur:

1. Vif can affect the enzymatic activity of APOBEC3G in the absence of proteosomal degradation.
2. Vif prevents packaging of APOBEC3G through competitive binding to a common packaging signal.
3. Vif promotes or accelerates the transition of APOBEC3G from LMM to HMM conformation.

Undoubtedly, all of this mechanism coexist together **(17).**

MUTATION IN VIF:

Several mutations have been introduced in the vif gene. The effects are from slight infectivity to complete disruption of the normal function. Some important examples are explained below.

In the BC- box, mutation of SLQ to AAA significantly reduced the ability of mutant SIV_{AGM} Tan Vif-AAA to recruit Cul5-ElonginB-ElonginC **(33)**. Recruitment of Cul5-ElonginB-ElonginC by SIV_{AGM} Tan Vif is critical for its function. As a result, SIV_{AGM} Tan VifAAA could not exclude AGM-A3G from released SIV_{AGM} Tan-virions. Unlike WT SIV_{AGM} Tan Vif, the SIV_{AGM} mutant vif could no longer suppress the antiviral activity of AGM-A3G. Therefore, the SLQxLA motif in SIV_{AGM} Tan Vif, despite divergence from the BC-box consensus sequence, plays an important role in binding to Cul5-ElonginB-ElonginC and is required for SIV_{AGM} Tan Vif activity in degrading AGM-A3G and excluding it from released virions. Indeed, the L145 contributes substantially to the hydrophobic interface between Vif and EloC by fitting into a hydrophobic pocket formed by EloC helices 3 and 4 **(5).**

The capsid of retrovirus has been shown to be a target for cellular restriction factors in various virus and cell systems. The addition of two mutations in two different strains of MVV, one in gag (CA) and another one in Vif, showed different results. The CA mutation affected replication in macrophages somewhat, but with the addition of the vif mutation, replication in macrophages was markedly reduced. The mutation in Vif on its own did not affect replication in macrophages **(20)**.

The CA mutation is an L-R mutation in amino acid 120 of MVV CA. This is a position corresponding to a K131 in helix 7 in the N-terminal domain of HIV-1 CA protein **(15, 61)** whereas the P-S mutation in Vif is in a proline-rich region at the C-terminal end of the protein that has been shown to be important for association with cellular membranes and interaction with the Gag precursor $Pr55^{gag}$ **(4, 18, 19)**

Therefore, Vif has been shown to play a role in the stability of the core of HIV-1 and the interaction between Vif and CA may either be indirect or too transient to be detected by standard methods that are used for detecting protein-protein interactions. It is possible that the two mutations are unrelated and that the effect is additive. However, since the mutation in vif did not have a detectible effect on replication on its own, it is tempting to speculate that there is an interaction of CA and Vif in the replication of the virus. The mutations may define a novel host restriction factor that targets the capsid and is counteracted by Vif. Another possibility would be that the CA mutation slows the growth of the virus by destabilizing the core, thus making it more susceptible to host proteins in general **(20)**.

Other mutation, this time $W_{89}R$ has been shown to attenuate the replication of CAEV **(52)** and could be expected to be defective in APOBEC3-vif interaction analogous to HIV Vif, which requires various Trp residues in the N- and central part of Vif for APOBEC3 interaction **(60)**. It is tested that FOS cells are somewhat more permissive both for dVif and the Vif $W_{98}R$ mutant.

MATERIAL AND METHODS:

VIF MUTATED GENE FORMATION:

VR1012 Vifopti plasmid (codon-optimized MVV Vif in pVR1012 vector (Kan resistant)) is called optimized due to the sequence was changed to the most frequently used codons in mammalians. This plasmid has been spliced with the restriction endonucleases: AccI, BamHI and MfeI (New England Biolabs Inc.) achieving a plasmid with two cutting places with which Vif mutated sequences was sticking together, creating new plasmid with the Vif mutated gene sequence inside.

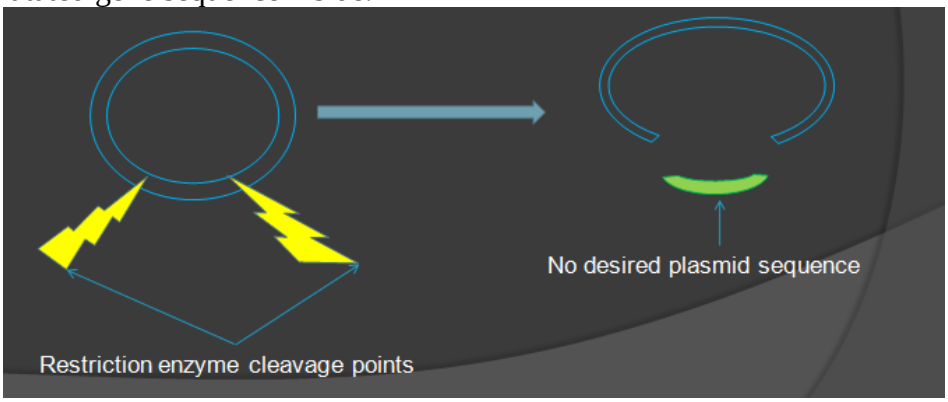

Fig 5.

The restriction enzymes used for the cleavage of the plasmid are:

- BamHI: ……..

  5′...G▼GATCC...3′
  3′...CCTAG▲G...5′

- AccI: ……..

  5′...GT▼MKAC...3′
  3′...CAKM▲TG...5′

- MfeI: ……....

  5′...C▼AATTG...3′
  3′...GTTAA▲C...5′

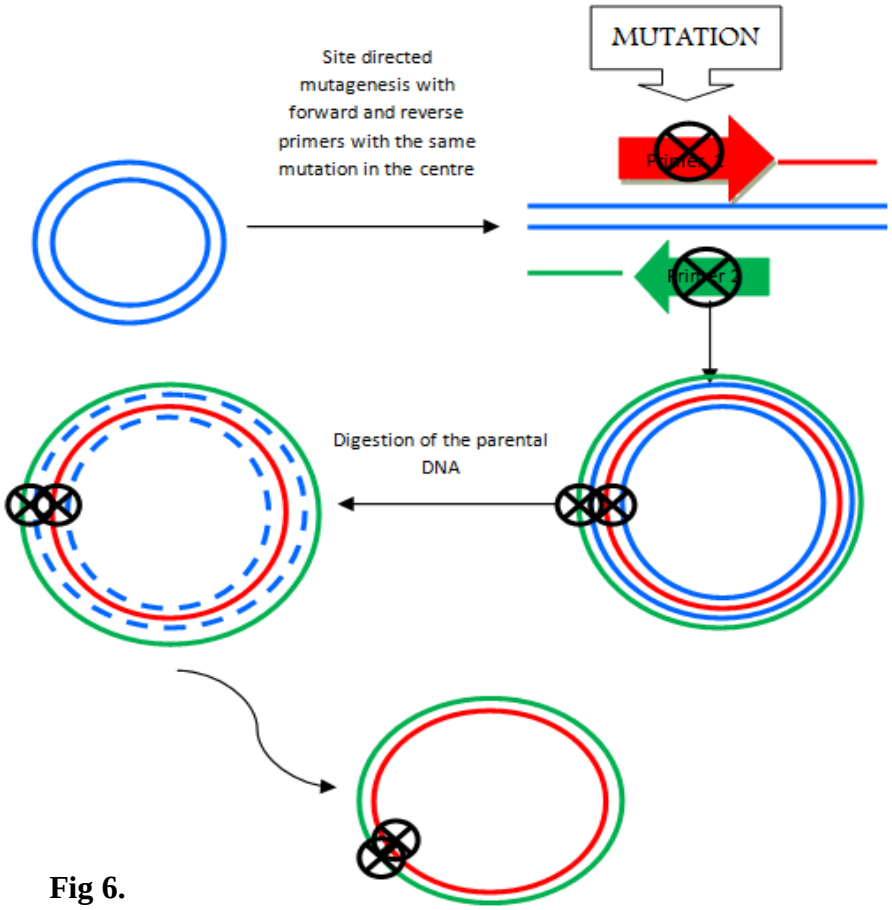

Fig 6.

The mutations created were:

- $W_{98} \rightarrow R$
- $P_{205} \rightarrow S$
- $IP_{216,217} \rightarrow AA$
-

These mutations were created with several PCR in which two different bands were joined two times, acquiring only one band which contains the mutation sequence. The mutation was achieved because the same mutation was within of the two primers.

The primers used for the PCR were:

- ✓ Vifopti1forward: 5´-GCG GTG CTG TTA ACG GTG G-3´
- ✓ Vifopti2reverse: 5´-CAG GGT GTG TCA CAG AGA AGG GG-3´

When the Vif mutated sequence and the plasmid had been cut with the same two endonucleases enzymes; two different methods to join the sequence could be used.

In the first one, each sequence and plasmid are isolated from the agarose gel, this time, with the kit "QIA quick kit to extract DNA in agarose gel" from QIAgen©. Since extracted, all the mix is adding together with the T_4 DNA ligase enzyme. A new plasmid with the new sequence inside is obtained with the reaction.

In the other one, the plasmid, which it has been already extracted from the gel, is added with a mix of the Vif mutated sequence band which has been cleavage with the restriction enzymes and the aforementioned enzymes. The endonuclease enzymes have to be inactivated passing for 80ºC for 20 min. All this mixture is added with the T_4 DNA ligase enzyme with the same result.

The first method has the advantage that the amount of DNA which is included in the mixture, and thence, joined with the ligase, is maximum, the amount of DNA is optimized during all the process, and likely the ligation may be done.

On the other hand, with the second method, apart from the problem of the lost of DNA during all the process, there is a great problem; the mismatch. This mismatch is a problem which is due to the union of the plasmid at the band in the wrong way, i.e. two plasmids joined, two sequences joined, and so on.

ESCHERICHIA COLI THERMAL SHOCK-BASED TRANSFORMATION:

Since the mutations had been prepared, Escherichia Coli, which is called DH5α, is transformed with the thermal-shock method.

Only those cells which have the kanamycin resistant gene inside of this genome, and therefore, the new plasmid, can survive in the kanamycin Luria-Bertoni medium.

After that, the DNA of the bacteria which have grown on the Petri plates are extracted and purified with the kit "Plasmid DNA purification using the QIAprep Spin miniprep kit and a microcentrifuge" from QIAgen©.

SEQUENCING:

First of all, the purified DNA is measured with a spectrophotometer which is neccesary to know the concentration of DNA in the sample. With the known amount of DNA, sequencing preparation may be done. Sequencing is running with the desired sample which has been prepared before. The sequencing system used was ABI PRISM sequencing (Applied Biosystems). The resulting sequence is displayed with the program "Sequencher 4.9".

VIF P_{205}→S MUTATION SEQUENCE:

CTGTNATNCNGAAAGNTNNNNNNAAAAANAAAANAANTCCCNCCATAAANAAAAAAN
AANCCCCAGCATGCCTGCTATTGTCTTCTTTATCCCCCCCCTTGCTGTCCTGCCCCACCCCACCC
CCCAGAATAGAATGACACCTACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGA
CAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGC
TGGCAACTAGAAGGCACAGCAGATCTGGATCCCTACAGATCCTCTTCTGAGATGAGTTTTTGTT
CCTCAAAAATGCTCTCCATCTCCCAACACTCCTGCAGACTCCAGGGGATGGTGTATCCCATTGG
AGACCTGCACCATGAGAACTTTTGCACAGTCACCCTCCGTGCCCTCCAAAGAGGCATGACCTGG
AACACATGGATCCTTAGCGAGGTGAACAGGGCAAGTCTCTGCAGGCTTCTGGGGTTAGTGTTTT
TGACAATCTCGCCCTGGATGCAGCTTTTGCACAGGTCCCACCTGTGCTTCCCAATCATAAACTCT
CTGATGTCCCACCTGGTCTTGTTACAGCTGCAAAGGGCAATCCGCCAGTGGAACCAAAATTGGT
TCTCTTTTCCCTTGTAATCTCCGGGGGCTCTCGTACCACACCCCAATTGTCTTCCATTCCCCCTGG
GANTTGTATGGCCAACGTTGCNAAANTGATCTGAACGATCANGTCCAAATCCCTTCTGATTTCA
TCTTCCATGCTTCCCGAAGNTTCCCAATAATCCTAANCCTGANTTTCTTCNAAAATTAACTT

VIF W_{98}→R SEQUENCE:

CTAAATCCAAAAANTANACCCTNAGAAAGNAAATCTNTCCCATANANNGAAAAATCCCCAGCA
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ATGANNGCTACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTG
GCACCTTCCAGGGTCAAGGNAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAG
AAGGCACAGCNTATCTGGATCCCTACAGATCCTCTTCTGAGATGAGTTTTTGTTCCTCAAAAATG
CTCTCCATCTCCCAACACTCCTGCAGACTCCAGGGGATGGTGTATCCCATTGGAGACCTGCACC
ATGAGAACTTTTGCACAGTCACCCTCCGTGCCCTCCAAAGAGGCATGACCTGGAACACATGGAT
CCTTAGCGAGGTGAAGCAGGGCAAGTCTCTGCAGGCTTCTGGGGTTAGTGTTTTTGACAATCTC
GCCCTGGATGCAGCTTTTGCACANGTCCCACCTGTGCTTCCCAATCATAAACTCTCTGAAGTCCC
ACCTGGGTCTTGTTACAGCTGCAGAAGGCAATCCGCCAGTGGAACCAGAAATGGTTCTCTTTTC
CCTTGTANTCTCCGGGGCTCTCGTACCACANCCCAATTGCNTNCATTCCCCTGGGGANTTGTACT
GCCGAACGTTGCNAAAATTGATCTGACGATCAAGTCCAAATCCNTCTGAATTCCAACTTCCACG
GCTTCCTGAAGTTCCCAAATTCCAAACCCTGANTTCCTTCANAAAATCACTTANGG

VIF IP$_{216,217}$→AA SEQUENCE:

TCCCAGGGGAATGGAAGACAATTGGGGTGGGTACGAGAGCCCCGGAGATACAAGGAAAAGAG
AACCAATTNTGGTTCCACTGGCGGATTGCCNTNTGCAGCTGTAACAAGACCCAGGTGGGACATC
AGAGAGTTTATGATTGGGAAGCACAGGTGGGACCTGTGCAAAAGNTGCATCCAGGGCGAGATT
GTCAAAAACACTAACCCCAGAAGCCTGCAGAGACTTGCCCTGCTTCACCTCGCTAAGGATCATG
TGTTCCAGGTCATGCCTCTTTGGAGGGCACGGAGGGTGACTGTGCAAAAGTTCCCATGGTGCAG
GTCTCCAATGGGATACACCGCCGCCTGGAGTCTGCAGGAGTGTTGGGAGATGGAGAGCATTTTT
GAGGAACAAAAACTCATCTCAGAAGAGGATCTGTAGGGATCCAGATCTGCTGTGCCTTCTAGTT
GCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACT
GTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGG
GGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGG
ATGCGGTGGGCTCTA

FLUORESCENCE-ACTIVATED CELL SORTER (FACS):

Virus genome is modified removing env gene and adding GFP gene, with which the cells infected for the aforementioned virus acquiring fluorescence and it can be measured with a flow cytometry device. A pseudoenvelope is constructed for the viruses' genome, therefore, the virus can infect the cell once, but, because of the lack of the env gene, the virus cannot encode new envelope, and, obviously, it may not infect cells for second time. With this technique, it is possible to measure the infectivity of the virus with high-yield results. Those cells which shown fluorescence have been affected for the virus, and those cells which does not present it, have not acquired the virus in its matrix.

RESULTS:

VIF MUTATED GENE $IP_{216, 217}$→AA:

IP is considered a conserved motif sequence of Cul2-box which binds with BC-box proteins; in instead of the LPφP conserved motif of Cul5 with binds with HIV-1 Vif. A mutation in this sequence should show an interrupt in the union between Vif and BC-box proteins. In our data, it is displayed that there is less infectivity, but less difference that we expected. This data suggests that there are more sequences which interact in the union of Vif-Cullin2-EloBC. It is possible that there is a sequence with similar function of HCCH in Cullin5-box of HIV-1 virus type 1 Vif gene.

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 Strand=Plus/Plus

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Query  57   AAAGAGAACCAATTNTGGTTCCACTGGCGGATTGCCNTNTGCAGCTGTAACAAGACCCAG  116
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Sbjct  506  AAAGAGAACCAATTCTGGTTCCACTGGCGGATTGCCCTCTGCAGCTGTAACAAGA-CCAG  564

Query  117  GTGGGACATCAGAGAGTTTATGATTGGGAAGCACAGGTGGGACCTGTGCAAAAGNTGCAT  176
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Sbjct  565  GTGGGACATCAGAGAGTTTATGATTGGGAAGCACAGGTGGGACCTGTGCAAAAGCTGCAT  624

Query  177  CCAGGGCGAGATTGTCAAAAACACTAACCCCAGAAGCCTGCAGAGACTTGCCCTGCTTCA  236
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Query  237  CCTCGCTAAGGATCATGTGTTCCAGGTCATGCCTCTTTGGAGGGCACGGAGGGTGACTGT  296
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Sbjct  804  AGTGTTGGGAGATGGAGAGCATTTTTGAG  832
```

VIF MUTATED GENE P_{205}→S:

It is shown that there is an interaction between the capsid of the virion and the dysfunction of this interaction affects the infectivity of the virus. One mutation in Gag (CA) affects the infectivity slightly on its own, and another one in proline-rich region of Vif does exactly the same. But, when both of the mutations are together, the effect is markedly high. This highlights that the two mutation are additive and there are connections among this two regions. In our case, only the proline-rich region mutation of Vif has been added, for this reason, a slight effect has been recollected. This data suggest that this mutation has effect for itself but this one is really slight.

```
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 Strand=Plus/Minus

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Query  374  TGGAGACCTGCACCATGAGAACTTTTGCACAGTCACCCTCCGTGCCCTCCAAAGAGGCAT  433
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Query  434  GACCTGGAACACATGGATCCTTAGCGAGGTGAA-CAGGGCAAGTCTCTGCAGGCTTCTGG  492
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Query  493  GGTTAGTGTTTTTGACAATCTCGCCCTGGATGCAGCTTTTGCACAGGTCCCACCTGTGCT  552
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Sbjct  355  CCAATATTCGTAGCCCTGATTTTCCTTC  328
```

VIF MUTATED GENE W_{98}→R:

It is thought that there are some essential trp along the N-terminal and central part of Vif which somehow interact with APOBEC, this data has been tested in HIV-1 and showed in CAEV. Therefore, a mutation in some of this neccesary Trp could highlight a partial or total disunion between Vif and APOBEC proteins. Our data backs this theory, displaying a high loss of infectivity. This data suggest that this Trp, with may be other ones, creates some interactions (weak or strong links) which helps the union between APOBEC and Vif by a protein-protein interaction.

```
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Sbjct  356  CCCAATATTCGTAGCCCTGATTTTCCTTCACAAACATCAGCTTA  313
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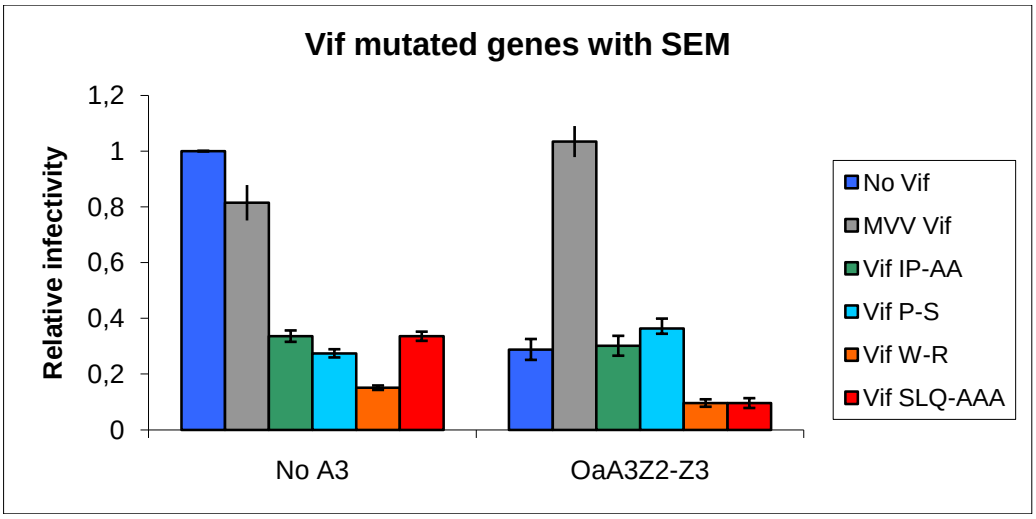
FACS (FLUORESCENCE-ACTIVATED CELL SORTING):

The data is highlighted in relative infectivity terms. The histogram shows the error in basic of standard error.

Fig 7.



Vif mutated genes and their infectivity. Vif wild type presents the highest level of infectivity. In sort of infectivity from higher to lower: Vif P-S > Vif IP-AA > Vif SLQ-AAA (previous data) = Vif W-R.

DISCUSSION:

The main reason for using codon-optimized Vif rather than normal vif frame was that two HIV-1 accessory proteins, Vpu and Vif, are notoriously difficult to express autonomously in the absence of the viral Tat and Rev proteins. These two genes are firmly related with genes Tat and Rev. The formation of the codon-optimized Vif removes the block of Rev and Tat and allows the expression of the genes, mostly due to the stabilization of the mRNA. No differences have been observed in the transcription of the aforementioned codon-optimized genes **(44).**

Due to this previous research, we decided to use Vif codon-optimized for our study, avoiding the problem of the Tat and Rev-dependent expression. Satisfactory results were achieved in all the assays. Besides, previously to this project, the codon-optimized was tested in a the SLQ→AAA mutation, with positive outcome.

Our project suggests that mutation of P_{205} mutation has slight effect for itself, showing an interaction with other mutations that decrease the infectivity of Vif at a higher rate that this mutation alone. IP_{216} has effects as well in the infectivity of the gene, but less than we expected, suggesting that even mutating this Cul2-box conserved-motif, they would have more motives which bind with Vif. W_{98} has the highest effect in the infectivity of all the mutations of our study, highlighting a likely interaction between Trp of Vif protein and the APOBEC protein.

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APPENDIX:

>codon-optimized MVV Vif in pVR1012 vector (Kan resistant)

PRIMER **MfeI** **BamHI** **AccI**

MUTATION IP→AA **MUTATION P→S** **MUTATION W→R**

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAG
ACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGG
CGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAG
AGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGC
GTAAGGAGAAAATACCGCATCAGATTGGCTATTGGCCATTGCATACGTTGTAT
CCATATCATAATATGTACATTTATATTGGCTCATGTCCAACATTACCGCCATGT
TGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTT
CATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCT
GGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCC
CATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTAC
GGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCC
CCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACAT
GACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTAT
TACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTG
ACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTT
GGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG
ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTC
GTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCC
ATAGAAGACACCGGGACCGATCCAGCCTCCGCGCCGGGAACGGTGCATTGGA
ACGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGACTCTATAG
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CACCCCCGCTTCCTTATGCTATAGGTGATGGTATAGCTTAGCCTATAGGTGTGG
GTTATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTTCCATTACTA
ATCCATAACATGGCTCTTTGCCACAACTATCTCTATTGGCTATATGCCAATACT
CTGTCCTTCAGAGACTGACACGGACTCTGTATTTTTACAGGATGGGGTCCCATT
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TATTAAACATAGCGTGGGATCTCCACGCGAATCTCGGGTACGTGTTCCGGACA
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CTCCAGCGGCTCATGGTCGCTCGGCAGCTCCTTGCTCCTAACAGTGGAGGCCA
GACTTAGGCACAGCACAATGCCCACCACCACCAGTGTGCCGCACAAGGCCGTG
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GTTGTATTCTGATAAGAGTCAGAGGTAACTCCCGTTGCGGTGCTGTTAACGGTG
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CCGTCGTCGACGCCACCATGCTCTCCAGTTACAGGCACCAGAAGAAATATAAA
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GTCCACGCCCCTGGTTCTTAGTTCCAGCCCCACTCATAGGACACTCATAGCTCA
GGAGGGCTCCGCCTTCAATCCCACCCGCTAAAGTACTTGGAGCGGTCTCTCCCT
CCCTCATCAGCCCACCAAACCAAACCTAGCCTCCAAGAGTGGGAAGAAATTAA
AGCAAGATAGGCTATTAAGTGCAGAGGGAGAGAAAATGCCTCCAACATGTGA
GGAAGTAATGAGAGAAATCATAGAATTTCTTCCGCTTCCTCGCTCACTGACTCG
CTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT
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CCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA
GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGC
TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC
TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTC
GTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTC
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CATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCGAGCAAGACGTT
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GTC