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## SEQUENCE ANALYSIS OF VPR ALLELES IN HIV LONG-TERM NON-PROGRESSORS

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## ABSTRACT

Five to ten percent of HIV-1 infected individuals remain asymptomatic up to a year after infection. This type of infection is known as a long-term non-progressive (LTNP) infection, and can be characterized as a viral phenotype with a lack of CD4+ T-cell death. This MQP deals specifically with a group of LTNPs known as the French ALT group who have a form of the virus that is considerably less cytopathic. Because mutations in the Viral Protein R (Vpr) region of the HIV-1 genome have been observed to cause similar viral phenotypes, the ALT patient Vpr alleles were investigated. Several Vpr polymorphisms were identified in hypervariable loci, most of which result in significant amino acid changes. In the future, these mutations will be mimiced in wild-type HIV's to determine their effect on viral replication to determine if they are responsible for the LTNP phenotype.

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## BACKGROUND

#### **HIV: Introduction**

The study of human immunodeficiency virus (HIV) is one of the most relevant and exciting research topics today. One of the reasons it is so interesting is because of its worldwide impact (see Figure 1). HIV is a retrovirus first identified in 1983 and is the cause of AIDS. The basic pathology exhibited by individuals infected by this virus is a loss of CD4+ lymphocytes, and a variety of immune disorders (Levy 1998). HIV is present today in two classes and many different subtypes, some more prevalent than others in specific areas of the world. HIV-2, the second class of HIV, is closely related to SIV and is thought to be an earlier form of the virus (Knipe and Howley, 2001).



**Figure 1: HIV Subtype Distribution**. This figure shows the distribution of HIV throughout the world as of 1996 (Levy, 1998).

The disease caused by the HIV virus is characterized by immunological abnormalities, opportunistic infections, and a weakened immune system. HIV infection itself can also cause neurological disorders, and unusual forms of cancer (Knipe and Howley, 2001). The virus is transmitted through fluid transfer, either intravenous or sexual. The virus is present at infectious levels in the blood and genital fluids of infected individuals (Jaffe et al, 1983). HIV-1 is a typical lentivirus, and may have evolved from a similar simian immunodeficiency virus (SIV) found in monkeys and non-human primates (Knipe and Howley, 2001). Genetically SIV (especially from Sooty Mangabeys) and HIV-2 are very similar. This suggests that the HIV-2 may be an older form of the virus. It is hypothesized that HIV developed first in non-human primates, and then switched over to the human population at some time. The HIV virion contains two identical copies of a single stranded RNA genome; each is about 9.2 thousand base pairs long.

#### HIV-1 Genome

Like any lentivirus, HIV has the structural genes Gag, Pol, and Env. These genes encode the proteins that make up the majority of the mature virion. HIV also has a set of regulatory genes, Tat and Rev, (see Figure 2) which control the expression of viral proteins once integration of viral DNA occurs (Levy, 1998). In addition to these regulatory genes HIV also has a set of accessory genes. These genes encode proteins that have evolved to facilitate the virus in its infection, and eradicate or control cellular cofactors poised to inhibit viral activity. The accessory genes are Nef, Vif, Vpu, Vpx, and Vpr (Knipe and Howley, 2001).

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**Figure 2: Diagram of the HIV Genome and Virion Structure**. Part A shows the position of various genes in the HIV genome. Part B shows a diagram of the mature HIV virion. Most HIV genes are arranged in this basic pattern with only slight differences in position due to differences in subtype, or mutation. Taken from (Knipe and Howley, 2001).

## **Viral Entry and Replication**

Viral entry occurs predominantly through the conformational interaction of

surface glycoprotein 120 (gp120) and the CD4 cellular receptor (Capon and Ward, 1991).

After gp120 has come into contact with a CD4 receptor, the gp120 is displaced which

allows the hydrophobic viral gp41 to come into contact with the cell and aid fusion (Moore et al, 1991) (see Figure 3).



**Figure 3: HIV-1 Fusion Model**. This figure shows the role of the HIV-1 surface glycoproteins during fusion. During this stage of infection the viral glycoprotein 41 undergoes a conformational change to facilitate the fusion of the virion, and eventual entrance of the HIV nucleocapsid. Figure taken from (Knipe and Howley, 2001).

In addition to CD4 as a receptor, HIV-1 may also use a co-receptor to facilitate viral entry in some cell types (Broder et al, 1993). Chemokine receptors have been found to be important HIV co-receptors. For example, M-tropic HIV infects macrophages using CCR-5 as its secondary receptor (Alkhatib et al, 1996) and T-tropic HIV uses CXCR-4 as a co receptor when infecting T-cells (Berson et al, 1996). Once fusion has occurred, the HIV-1 nucleocapsid enters the cell through a mechanism that is not clearly understood. Once inside the cell, the single-stranded viral RNA is removed from the nucleocapsid and converted to double-stranded, linear DNA by reverse transcriptase

(Figure 4). Reverse transcriptase is prone to errors, which gives the HIV genome its high mutation rate and high degree of variability. It is estimated that the RT enzyme incorporates the wrong nucleotide during replication once in every 10,000 bases.



**Figure 4: The HIV Infection Cycle**. This figure shows the replication cycle of HIV in an infected cell. Once the virus fuses to the cell (1), it enters and casts aside the envelope (2) and then the nucleucapsid. (3). Then it begins to reverse transcribe its single stranded RNA genome (4). Then a circular provirus is made, transported, and incorporated into the chromatin (5). Once viral DNA is incorporated into host cell DNA, replication of HIV viral proteins and genomic RNA is mediated by tat, rev, and nef (6). Complete virions are assembled (8) and can be budded off at the cell surface (9). Figure taken from Levy, 1998.

Once this circular non-covalently bonded viral cDNA copy has been made, it is transported to the nucleus where it is integrated into the chromosomal DNA. Once the provirus is integrated into the host cell's chromosomal DNA, the production of viral mRNA and genomic RNA is mediated by the relative expression of HIV regulatory genes tat, rev, and nef (Knipe and Howley, 2001).

A high expression of Tat, and subsequently high levels of the tat-induced gene products, yields a large amount of viral production. High expression of nef may force the virus into a latent state (Luciw et al, 1987). Viral proteins are made from mRNA, and copies of genomic viral RNA are formed, and incorporated into capsids. After they are synthesized, the Gag and Pol proteins of budding virus undergo proteolytic cleavage before packaged into the virion. Once complete, virions can either bud off of the infected cells surface, or wait inside the cell until the outer membrane is collapsed by apoptosis. The virus can also be transmitted directly from cell to cell inside the body. This method of infection is 100 times more efficient than infection by free virus particles (Sato et al 1992).

#### **HIV Tropisim**

Once inside the body, the virus replicates easily in CD4+ lymphocytes and peripheral blood macrophages. At this point, the virus is considered M-tropic and its main entry is via CD4 and CCR-5, this form of the virus is non-syncytia inducing (NSI). Syncytia are multinucleated cells formed by cell-to-cell fusion during HIV-1 infection (Collman, 1990). At some point in the infection the virus switches its co-receptor usage to CD4 and CXCR-4, and displays a T-tropic behavior where it readily infects and destroys T-cells, which-is responsible for the accelerated T-cell apoptosis and immunodeficiency found in HIV-1 infected individuals. This T-tropic type of HIV is usually found later in infection and is syncytium inducing (SI).

At this point, opportunistic infection can be observed due to the lack of an active T-cell response. Once the virus destroys the immunological ability of the infected individual, they are subjected to severe complications from infections that would have been eradicated by a healthy immune system. Fatalities from advanced HIV are often caused by common viruses and infections like pneumonia and influenza. The virus itself can also cause cancer, dementia and neurological disorders in its later stages.

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### Vpr Background

#### Structure

This MQP focused on HIV-1 viral protein R (Vpr), which is a 96 amino-acid 14kDa auxiliary regulatory protein present in HIV-1, HIV-2 and SIV. Vpr is formed from a singly spliced mRNA, and can be split into three separate parts: the amino terminal, the carboxyl terminal, and the central alpha-helical structures (Figure 5).



Amino terminal

**Figure 5: Structure of the Regulatory Protein Vpr**. This figure shows the proposed crystal structure for the Vpr gene product. Picture Taken from the Protein Data Bank Structure Explorer #1ESX.

Mutations in the Vpr amino terminal affect the virus's ability to package the virion effectively, whereas mutations in the carboxyl terminal affect the virus's ability to augment and control the cell cycle. Finally, mutations in the alpha-helical structures influence the virus's ability to control transcriptional activation, apoptosis, and cellular shuttling (Kino and Pavlakis, 2004).

#### Vpr Function

Vpr is not needed for viral replication, but is required for regulation of apoptosis and the infection in some non-dividing cell types (Jian and Zhao, 2003). The Vpr gene product is packaged in high copy into the virion and has been shown to play a roll in the active nuclear translocation of the HIV-1 pre-integration complex (Lucia et al, 2000). This is important because the Vpr gene product can also transport the pre-integration complex inside the nucleus of non-dividing cells. Infection of non-dividing cells allows HIV to enter more cell types inside the body such as highly specialized cells, as well as brain cells. This suggests that an intact Vpr gene is needed to give the virus the ability to infect and destroy T-cells, as well as macrophages (Velpandi et al, 2002). It can also be said that an intact Vpr gene is needed for the virus to regulate apoptosis (Samosundaran et al, 2002).

In general, apoptosis is the regulated mechanism of controlled cell death, which is essential for the normal development and homeostasis of multi-cellular organisms. The cell can undergo apoptosis when stressed or damaged. This destruction of defective cells provides a defense against viral infection and oncogenesis (Lucia et al, 2000). When a cell undergoes apoptosis, a calcium dependent endogenous nuclease fragments cellular DNA into small nucleotide units. After this occurs, the cell undergoes a convolution of the cellular surface, and forms protuberances that pinch off into apoptotic bodies (see Figure 6).

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**Figure 6: Apoptosis Model.** This figure shows a normal cell undergoing apoptosis. During apoptosis the cell compacts and segregates its chromatin, followed by convolution of the nuclear and cellular envelops. Once the cellular envelope becomes deformed, it pinches off into smaller round bodies that can be ingested by macrophages. Figure taken from (Levy, 1998, pp 129).

These apoptotic bodies can either float freely, or be eventually consumed by macrophage cells. Early in infection apoptosis can be delayed so as to give the virus more time to replicate and assemble virions, before the cell is burst and the virions are released. However later in infection it is important to induce apoptosis to burst the cell and release more active virus to try and infect other cells (Razvi and Welsh, 1993). Exactly how the virus controls apoptosis is not clearly known, however it is known that during HIV-1 infection there is a large increase in apoptosis of immune cells (B and T lymphocytes) (Gougeon et al, 1996). It is this phenomenon that is responsible for the CD4+ T-cell loss that causes disease and opportunistic infection in HIV patients. Apoptosis can even be induced by soluble viral proteins. These proteins can either be from viral gene expression inside an infected cell, or viral proteins outside the cell can be taken inside, and eventually force the cell into apoptosis. Thus, it can be said that apoptosis is an important cellular function for HIV to mediate as the infection progresses (Lum et al, 2003).

#### Vpr Mutations

Specific mutations in Vpr have been categorized from patient isolates. Mutations in Vpr have been known to alter phenotype in a distinct way. Specifically mutations in the alpha helical regions of the gene product impact the virus negatively, impairing its ability to induce apoptosis and mediate infection. This mutated form of the virus is unable to deplete T-cells unlike wild type (see Figure 7) (Somasundaran et al, 2002).



**Figure 7:** Cytopathicity of Wildtype LAI Strains Versus Mutated Patient Vpr Alleles. This figure shows the number of viable cell vs. time after infection for various HIV isolates. Vpr's I-IV are mutated non functional Vpr alleles inserted into wildtype LAI virus. The virus with these inactive non-coding Vpr alleles can not decrease the amount of viable cells; these viruses cannot efficiently cause infected cells to undergo apoptosis as does wildtype virus (clear diamonds). Figure from (Somasundaran et al, 2002).

This lack of CD4+ T-cell destruction in Vpr mutants confers a specific viral phenotype to patients infected with this form of the virus. Since the virus does not deplete their T-cells as quickly, they are known as long term non-progressors (LTNP).

Individuals infected with a form of the virus that cannot efficiently kill T-cells do not get as much damage to their immune system. Typically these patients live longer. Another example of a severe Vpr mutation is the Q3R mutation, which occurs at the Nterminus of Vpr. This mutation affects the cytopathic and apoptotic ability of the virus. The Q3R substitution occurs at the third amino acid in the gene product, where a Glutamine is switched to an Arginine. The Q3R form of the virus is not very cytopathic when compared to a non-mutated virus (see Figure 8).



**Figure 8: Replication of wt LAI versus Q3R**. The wt LAI virus (dark circles) has a larger decrease in viable cell count when compared to LAI with the Q3R polymorphisim (open boxes). LAI Q3R also produces higher levels of p24 than wild type LAI. This suggests that there is more replication and less apoptosis occurring with the Q3R virus. This figure is from (Somasundaran et al, 2002).

Cytopathicity describes the virus's ability to induce cell death and apoptosis. This lack of cell death found in the Q3R form of the virus suggests that a functional Vpr is needed for the virus to be cytopathic (Somasundaran et al, 2002). This lack of cytopathicity and apoptosis shows that the Q3R mutation reduces the virus's ability to induce apoptosis, because this polymorphism harms the Vpr gene product, which plays a role in controlling apoptosis and cell death. From studying the differences in phenotype between Vpr-negative viruses and wild type viruses, it has been found that Vpr plays a role in the nuclear localization of the pre-integration complex as well as apoptosis, which is lowered considerably when the virus has a non-functional Vpr. This tells us that the Vpr gene product may regulate apoptosis (see Figure 9).



**Figure 9: Effect of Vpr Mutations on Apoptotic Index**. This figure shows apoptotic ability of truncated, non functional Vpr versus wild type LAI Vpr. Vpr-negative virus shows less apoptosis; this suggests that Vpr plays a large role in mediating this cellular process (Somasundaran et al, 2002).

#### French ALT Background

This MQP research is based around the study of an unusual group of HIV patients who are infected with the virus but do not exhibit normal viral phenotype. These patients have high viral loads yet also retain high levels of CD4+ T-cells (Candotti et al, 1999). Normally patients that have a steady viral load and normal viral replication have low levels of CD4+ T lymphocytes, but these patients show high T-cell counts even with relatively high viral loads, as shown by Figure 10 below.



**Figure 10:** Long Term Asymptomatic HIV-1 Infection. This figure shows plasma RNA load of each of the patients in the French ALT group. The patients are grouped initially by starting viral load (entry listing). The total amount of HIV plasma RNA remains relatively the same even after a year. This steady level of plasma viremia and lack of CD4+ T-cell death allows us to classify these patients as long-term asymptomatic individuals. Filled circles denote patients who had a significant increase in viral load, while clear circles denote those who have not increased significantly. Figure is from Candotti et al, 1999.

This form of HIV from the ALT patients exhibits a lack of viral cytopathicity and Vpr dependent apoptosis, but it can replicate efficiently and is also able to enter cells, but cannot destroy cells through apoptosis. Viral levels in plasma do not increase significantly overtime, probably due to the lack of cell rupture and cytopathicity normally associated with wild type HIV. It is the main focus of this MQP to investigate the Vpr alleles of the virus from the French ALT group, looking for polymorphisms. These polymorphisms in Vpr may be responsible for the long term asymptomatic infection, and lack of Vpr dependent apoptosis. This would explain the peculiar phenotype of the virus found in the French ALT Study Group.

The correlation between non functional Vpr and lack of apoptosis suggests that the French ALT group of patients, who exhibit low levels of apoptotic activity, may have mutations in their Vpr alleles. This similarity is the cause for the investigation of the ALT Vpr alleles. Specifically polymorphisms that cause truncations or premature stops in the gene product, or convert the hydrophobicity of a key aa, can cause the Vprphenotype.

Not much is known about the viral accessory proteins and their exact role in successful infection. At this point it is known that a non functional Vpr allele can cause the virus to loose the ability to cause apoptosis, but will not stop the virus from replicating (Somasundaran et al, 2002). This resembles the phenotype of the LTNP's (long term non-progressors) discussed above. However it is not known completely which specific sequence polymorphisms in Vpr alleles result in augmented viral phenotypes of the ALT patients. There are certainly new mutations in Vpr that can be shown to confer the LTNP status. This MQP study and sequence analysis of the Vpr alleles will allow us to look into certain Vpr mutations and their effect on viral cytopathicity, infectivity, and apoptosis. Hopefully some new mutations can be uncovered in this group of asymptomatic HIV-1 patients. Learning where on the gene mutations occur and how these mutations change phenotype will allow us to better understand the role of the Vpr gene product during infection.

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## **PROJECT PURPOSE**

The purpose of this MQP is to investigate HIV-1 infection in the French ALT Group of long term non-progressors, especially characterizing various Vpr alleles that may be responsible for the unusual phenotype. The phenotype of the virus from these patients is very strange since the virus replicates efficiently but does not decrease viable T-cell count by inducing cell death the way wild type virus does. The HIV-1 Viral protein R (Vpr) is known to play an important role in nuclear import and apoptosis, so is the target protein in this project. If mutations in the French ALT Vpr are found, then future experiments can be performed to test the effects of mimicking these mutations in wt isolates on viral replication and apoptosis.

## METHODS

### Sample Information

Purified HIV samples were obtained from peripheral blood cells of a French ALT Group of long term non-progressors (Somasundaran et al, 2002). The samples are all classified as HIV-1 and they are all subtype B. Our lab obtained these 33 samples by mail from Dr. Candotti (Umass).

### Plasmid Vector Selection

Once these HIV samples were obtained, a plasmid pIRES2-EGFP vector from BD Biosciences ® was selected containing a GFP ORF, as well as a Kanamycin resistance gene shown on the vector map (see Figure 11).



Figure 11: Plasmid Vector Map (pIRES vector map BD Biosciences)

The vector also has an internal ribosome binding site to allow protein production. The MCS area on Figure 11 depicts the multiple cloning site of the vector which was cut out and replaced by the ALT Vpr alleles. The MCS section removed was site 615 to 662. When this section was removed, the 96 amino acid Vpr allele amplified from PCR was inserted in its place.

#### Preparation of Vector DNA

After the pIRES vector was chosen, LB media was inoculated and a large scale plasmid DNA preparation was done using the Qiagen Maxi prep protocol, with no major changes in the recommended protocol. This vector will carry the VPR allele from each of the numbered ALT samples.

#### Construction of Vpr Primers

Vpr complementary nucleotide sequences were designed using a map of the HIV-1 genome. These oligonucleotides were ordered from Integrated DNA Technologies, Inc. Oligonucleotides upstream and downstream from the Vpr gene were constructed to serve as the template for amplification. To specifically amplify only Vpr from each patient sample, nested PCR was used. Nested PCR is a simple dual amplification, an initial round of amplification is followed by a second round using primers internal to the first set. The primers used for Vpr amplifications are shown in Table 1 below:

PCR Round	Primer Name	Primer Sequence
1	HVPR 3 Forward	CAA TAC TTG GCA CTA GCA GCA TT Position 5057 in HIV LAI
1	HVPR 2 Reverse	TTA GGC TGA CTT CCT GGA TGC TT Position 5467
2	VXI1 Forward	CTT TGC CTC GAG TTA CGA AAC TGA CAG AGG A, Position 5107
2	VBI Reverse	CTT CCT GGA TCC TTC CAG GGC TCT AGT CTA G, Position 5451

**Table I: Primers for Cloning ALT Vpr Alleles** 

This table shows the sequence and position of each primer used in the Vpr amplifications. These primers were constructed using a complete LAI HIV-1 subtype B sequence taken from the Los Alamos database.

#### PCR

Vpr was amplified by PCR by nested PCR. In this nested PCR there were two rounds of amplifications, the first of which consisted of twenty cycles, and used the first set of outside primers. The second round had thirty cycles, and used the second internal set of inside primers. These primers included restriction sites for BamHI and XhoI. These restriction sites were included so the amplified DNA could be easily cut and inserted into the pIRES vector. Typically 0.25  $\mu$ g of HIV DNA was used as template in each reaction. After the nested set of amplifications, the PCR products were purified using a standard Qiagen PCR purification column, eluted in 30  $\mu$ l.

#### Restriction Digests

Purified PCR products and pIRES vector were cut with both BamHI and XhoI. These Biolabs ® restriction enzymes were selected on the assumption that they would exhibit the least amount of non-specific digestion. After the restriction digestion, plasmid vector was mixed with each digested Vpr allele, and the mixture was purified again using a standard Qiagen PCR purification column, and eluted in 30 µl

#### Ligation

After elution from the column, this combined vector and VPR product was ligated using Biolabs ® T4 DNA Ligase in 10 µl reactions. The ligation reaction was allowed to proceed at room temperature overnight.

#### **Transformation**

To transform the DNA into bacteria cells Invitrogen  $\circledast$  stbl2 competent cells were put on ice and combined with 1.5 µl of ligation reaction, heat shocked at 42°C for 30 sec, and allowed to recover at 30°C for an hour. These cells were then plated on Kanamycin plates and left to grow overnight at 37°C. Once the plates had incubated sufficiently, 4 colonies from each plate were streaked onto new plates and set aside to incubate a 37°C overnight.

#### Recombinant DNA Preparation

Once these re-streaked colonies had grown a thick lawn, small scale DNA preps were prepared with Qiagen mini prep columns, these preps were eluted in 30  $\mu$ L.

#### Recombinant Plasmid Screening

Another BamHI/XhoI digestion was performed to confirm the presence of the patient Vpr allele. After the digest was complete another agarose gel was run, using an

amplified Vpr band as a control. When positive bands were observed, colonies from that plate were taken to inoculate 200 mL of LB culture for a large scale DNA prep.

#### Large Scale Recombinant DNA Preparation

After inserts were confirmed, large scale DNA peps, grown 200 mL cultures, were performed to obtain enough DNA for sequencing. For innoculum a full loop of bacteria was placed in 200 mL of LB Broth. In addition to the desired recombinant bacteria, 50mg/mL Kanamycin was added to insure only the growth of the desired plasmid. The cultures were allowed to shake overnight at 37°C. Bacteria were pelleted, and plasmid DNA isolated via Qiagen maxipreps as described above.

#### Sequencing

Sequencing was done with Big Dye version 3 at the Umass CFAR sequencing center. One microgram of sample DNA was added to primer 5X diluent and enzyme. The samples, went through the sequencing program on the thermo-cycler. Sequencing reactions were cleaned up using Qiagen Dye removal columns. Sequencing was done for four separate clones of each sample, these sequences were compared with each other to rule out Taq-induced mutations during the PCR. Sequences were interpreted and corrected as electropherograms using ABI prism. Then sequences were translated and aligned using Mac Vector. Only the most concise and accurate sequencing of patient samples are represented in the final alignments.

## Construction of Controls

Three HIV controls were constructed to compare against the plasmids that were constructed with patient Vpr alleles. These controls were wt LAI virus Vpr, LAI Vpr Q3R polymorphism, and a LAI clone with a Vpr-negative phenotype. Control Vpr's were ligated and inserted into the pIRES vector in the same method described above for patient alleles.

## **RESULTS**

The purpose of this project was to sequence the Vpr gene from several HIV isolates from the French ALT LTNP group, and to analyze the effect of any mutations on the Vpr protein to determine whether any mutation could help explain the LTNP phenotype. Out of approximately 30 patient clones made and sequenced, the best eight isolates showing the most reliable and reproducible sequence data are shown in Figure 12 below.

		10	20		30	40	1	50	60	70	
LAI Vpr Control	AGGAAA	AGCEC	AGAAGA	AAGGGO	AA	AGGGAG	AAAA	TGAAGGA	AGAGO	TTAGAGE	GUNTA
Vpr - Control	AGGAASA	AGESSE	AGAAGA	AAGGGG	AA	AGGGAG	AAAA	CAADGGA	AGAGE	TITCTAGAGG	GERMA
Q3R Vpr Contrc	AGGAAGG	GGGGGG	AGAAGA	AAGGG	A A	AGGGAG	AAAA	TGAADGGA	AGAGA	TTTAGAGE	
4068	ADGGAAUA	AGGGGGS	AGAAGA	AAGGGC	λλ	AGGGAG	AAAA	GAADGGA	AGAGAG	PITTE AGAGG	
8009	ACAACA	AGGGGG	AGAAGA	AAGGG	AA	AGGGAG	AMAAG	GAADGGA	GERACA	AGAGG	
8013	ACCAACA	AGEGEE	AGAAGA	AAGGG	A A	AGGGAG	A A AA	GAADGGA	AGAGA	DE DE A CACE	
5002	ATGGAASA	AGGGGG	AGAAAA	AAGGG	A AZ	AGGGAGO	AMAAA	GAADGGG	AGAGAGA	AGAGO	
02011	ACCAACA	AGCELL	AGAAGA	AAGGGC	A A	AGGGAG	A A AA	GAATEGA	AARAR		
060011	ADGGAAG	AGEEDE	AGAAGA	AAGGG	AA	ACCCAN	AAAAA	GAGOGGA	ANARAR	TTAGAGG	
04034	AGGAAGA	AGCOBS	AGAAGA	AAGGGC	AA	ACCAC	A A AA	AADCCA	AGAGAG		
04064	AGGAAAA	AGCORD	AGAAGA	AAGGG	AA	AGGGAG	AAAA	GAADGGA	ACACA		
consensus	ADCCARCA	AGOOD	AGAAGA	AARGG	AA	ACCAL					
	80	90	100		110	1	20	130	140	150	
LAI Vpr Control	SO AGAAOGAA	90 G 🐨 🖬 G 🖬 📆	100	TICTAG	110 A T T T	G G G D G G A	20 13 G G	130	140 A A C A C A C A C A C A C A C A C A C A		REGER
LAI Vpr Control Vpr - Control	SO AGAATGAA AGAATGAA	90 G G T G T T G G T G T D	AGAAT	TTOCTAC		GG D A	20 <b>T</b> G G <b>T</b> G <b>C</b> A <b>T</b> G G				GGGGG
LAI Vpr Control Vpr - Control Q3R Vpr Contrc	50 A G A A G G A A A G A A G G A A A G A A G G A A		100 AGAA13 AGAA17 AGAA17	TTCCTAGO TTCCTAGO		G G C A G G C A G G T A	20 7 G G 7 G A G G 4 G G 6 G A G G 6 G G 6 G G 7 G G 7 G G G 7 G G G 7 G G 7 G G G 7 G G G G G G G 7 G G G G G G G G G G G G G G G G G G G	130 7 7 A G G G C 7 7 A G G G C			
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LAI Vpr Control Vpr - Control Q3R Vpr Contro 4068 8009	90 A G A A T G A A A G A A C G A A A G A A C G A A A G A A C A A A G A A C A A		A     G     A     A     T     G       A     G     A     A     T     G       A     G     A     A     T     G       A     G     A     A     T     G       A     G     A     A     T     G       A     G     A     A     T     G       A     A     A     A     T     T			G G G G A G G G G A G G G G A G G G G A G G G A G G G A A	20 T G G T G G T G G T G G G G G G	130 7 7 A C C C C 7 7 A C C C C C C C 7 7 7 A C C C C C C C 7 7 7 A C C C C C C C 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7			
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LAI Vpr Control Vpr - Control Q3R Vpr Contrc 4068 8009 8013 5002	SD         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         A         G         A         C         A         A         G         A         C         A		A         G         A         A         F         F           A         G         A         A         F         F           A         G         A         A         F         F           A         G         A         A         F         F           A         G         A         A         F         F           A         G         A         A         A         F         F           A         G         A         A         A         F         F           A         G         A         A         A         F         F           A         G         A         A         A         F         F				20 3 G G 3 G A 5 G G 4 G G 5 G G 5 G G 6 G	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	140       A     A     A     A     A     A     A       A     A     A     C     A     A     A       A     A     A     C     A     C     A       A     A     A     C     A     C     A       A     A     A     C     A     C     A       A     A     A     C     A     C     A       A     A     C     A     C     A     C       A     A     C     A     C     A     C       A     A     C     A     C     A     C       A     A     C     A     C     A     C       A     A     C     A     C     A     C       A     A     C     A     C     A     C	150 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	
LAI Vpr Control Vpr - Control Q3R Vpr Contrc 4068 8009 8013 5002 02011	30           A         A         A         C         A         A         C         A		A     G     A     A     B     B       A     G     A     A     A     B       A     G     A     A     A     B       A     G     A     A     A     B       A     G     A     A     A     B       A     G     A     A     A     B       A     G     A     A     A     B       A     G     A     A     A     B       A     G     A     A     C     B       A     G     A     A     C     A		110 A 1 2 4 A 2 7 7 A 3 7 A 4 7 A 7 A 7 A 7 A 7 A 7 A 7 A 7 A		20 7 G G 7 G A 7 G G 7 G G	130           0         7         A         G         G         G           0         7         A         G         G         G           7         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G           9         7         A         G         G         G	140       A     A     T     A     T     T     T     T       A     A     T     A     T     T     T     T       A     A     A     T     A     T     T     T       A     A     A     T     A     T     T     T       A     A     A     T     A     T     T     T       A     A     A     T     A     T     T     T       A     A     A     T     A     T     T     T       A     A     A     T     A     T     T     T       A     A     A     T     A     T     T     T	150       126       A       B       A       A       A       B       A       A       A       B       A       A       B       A       A       B       A       B       A       A       B       A       B       A       B       B       B       B       B       B       B       B       B       B       B       B       B       B	
LAI Vpr Control Vpr - Control Q3R Vpr Contro 4068 8009 8013 5002 02011 060011	30         A         A         C         A         A         C         A		A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I       A     G     A     A     I		110 A 4 5 A 5 A 5 A 5 A 5 A 5 A 5 A 5 A		20 3 G G 4 G G 5 G G 5 G G 6 G G 6 G G 6 G G 6 G G 6 G G 6 G G 7 A	130           Image: Constraint of the state of			
LAI Vpr Control Vpr - Control Q3R Vpr Controc 4068 8009 8013 5002 02011 060011 04034	30         A         C         A         C         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         A         C         A         C         A         C         A         C         A         C         A         C         A		A     G     A     A     F       A     G     A     A     F       A     G     A     A     F       A     G     A     A     F       A     G     A     A     F       A     G     A     A     C       A     G     A     A     C       A     G     A     A     C       A     G     A     A     C       A     G     A     A     C       A     G     A     A     C       A     G     A     A     C       A     G     A     A     C		110       A     1		20 X G G G A A G G G G G G G	130           1         1         1         1         0         0         0           1         1         1         0         0         0         0         0           1         1         1         0	140           A         A	150 A G A A A 7 1 7 A G A 7	
LAI Vpr Control Vpr - Control Q3R Vpr Contro 4068 8009 8013 5002 02011 060011 04034 04084	30         A         A         C         A		A         C         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G         A         A         T         T           A         G				20 11 G G 17 G	130       1     1       4     6       4     6       5     4       6     6       6     6       7     6       6     6       7     6       7     6       8     6       9     6       10     6       11     6       12     6       13     6       14     6       14     6       15     6       16     6       17     6       18     6       19     6       10     6       10     6       10     7       10     7       10     7       10     7       10     7       10     7       10     7       10     7       10     7       10     7       10     7       10     7       10     8       10     8       10     8       10     8       10     8       10     8        10     8	140           A         A         A           A		
LAI Vpr Control Vpr - Control Q3R Vpr Contro 4068 8009 8013 5002 02011 060011 04034 04064 <i>consensus</i>	30         A								140           A         A         A		



**Figure 12: DNA Sequence Alignment of 300 bp of the Vpr Allele from 8 ALT Patients and 3 Controls.** These 8 patients had the most clear and concise sequencing data, confirmed by sequencing multiple patient clones. In this base alignment there are four colors, each of which represents a different base. The first control is LAI HIV, the second is a Vpr mutated virus that does not code for a functional Vpr protein, and finally the third control is the Q3R virus discussed above. Polymorphisms can be clearly seen when comparing numbered ALT patient lanes to the wild-type LAI patient control (upper reference sequence).

Patient and control Vpr sequences were examined first as electropherogram print outs obtained from the Umass CFAR sequencing facility. These electropherograms were edited if necessary (using multiple cloned allele sequences to ensure that any polymorphisms shown represent true changes in the ALT samples, not Taq-induced mutations) then imported from the original sequence files to text files. This was done with ABI prism. After they were imported from ABI prism, sequences were then entered into Mac vector, aligned, and translated. The DNA alignment shows some variability between isolates. For example, position 93 for isolate 5002 contains a C instead of a T as in the wild-type LAI reference sequence. Another example of some variability is the change at position 110 for isolates 5002 and 8009. In this case a C is substituted instead of an A. Mutations at the single base level may induce real amino acid changes, or they can be silent mutations not predicted to alter protein function much. Even amino acid substitutions can be relatively silent, which can occur when relatively un-important amino acid substitutions occur.

To determine whether the base substitutions alter amino acid composition, the patient Vpr Alleles were analyzed for open reading frames using Mac Vector's translate function, then a second alignment was constructed (Figure 13). This translated Vpr amino acid alignment shows each patient sample translated along with the three controls (see Figure 13 below).

		- Allerander	10	20			30		40			50			
Q3R Control	M	APET	GPOB	PHNEWTL	LLL	L	BAVE	FP	IWLGL	GOR	IYBT	YG	DT	A	G -
Vpr - Control	M	APED	GPOR	PHNEWTL	LLP	E L K	AV	FP	IWLHAW	LAA	TYL	IN L	WG	S	G
LAI Vpr	ME	APED	GPOR	EPHNEWTLI	LL	LK	NDAVRU	F P	IWLBGL	GOR	IYET	YG	DT	A	G -
4068	M	APED	GPOR	PRNEWTL	LLE	L	NAVEL	FP	IWLGL	GQU	IYET	YG	T	A	G -
8009	ME	APED	GGPOR	EPYSEWTL	LLE	D L K	NBAVED	F P	PWLSGL	G	IYBT	YG	DT	A	G -
8013	M	APSD	GPOR	PHNEWTL	LLE	L	AVE	FP	IWLHGL	GQH	IYET	YG	TI	A	G -
5002	ME	APEN	GPOR	EPYNEWAL	ILE	L	AV	F P	PWLSL	G Y	LYEI	YG	DTV	A	G -
02011	M	APED	GPQR	PHNEWTL	LLE	LK	NEAV	F P	IWLMGL	GOLH	IYBT	YG	DTV	A	G -
060011	ME	APED	GPOR	BPYNEWTL	PLE	L	NEAVEN	F P	EWLMSL	GQY	IYDT	YG	DTI	A	G -
04034	M	APED	GPOR	PHNEWTL	LLE	L	NDAVEN	FP	IWLEGL	G	IYET	YG	TI	A	G -
04064	MB	APED	GPQR	PUNEWTL	LSE	L	AVE	F P	IWLGL	G	IYET	YG	DTI	A	G -
consensus	ME	APED	GPOR	EP IN DWTL	LLE	L	NEAVRE	FPR	IWLBGL	GON	IYBT	YG	DTW	A	G



**Figure 13**: **Amino Acid Alignment of 8 Patient and 3 Control Vpr Alleles.** Sequence variability was observed in each patient sample. The LAI Vpr control (third sequence) can be used as a frame of reference when discussing certain amino acids changes in the Vpr gene product. Amino acids that are in the same types of colored boxes have similar properties. The Vpr mutated sample had a large amount of variability, which would explain its deficient gene product and Vpr- phenotype.

**Note:** White boxes denote non-polar amino acids, blue boxes are for polar amino acids, green boxes are specifically for Proline, and red boxes are for acidic amino acids. The orange boxes represent amino acids Tyrosine and Tryptophan, and yellow boxes represent Methionine and Cysteine, whereas pink boxes have only Threonine and Serine.

A large amount of variability was observed from patient to patient. Each patient had unique polymorphisms; however some regions were "hyper variable". To classify the types of mutation, Table 2 was constructed to organize the variability found in the patients examined.

		Drastic Change	
<b>Patient</b>	<u>AA#</u>	AA Change	(Yes or No)
060011	3	Q to R	No
5002	7	D to N	Yes
8009	15	R to Y	Yes
5002	15	R to Y	Yes
060011	15	R to Y	Yes
5002	19	T to A	Yes
060011	22	L to P	Yes
04064	23	L to S	Yes
8009	37	I to P	Yes
5002	37	I to P	Yes
060011	37	I to E	Yes
5002	41	G to S	Yes

### Table 2: HIV-1 Patient Vpr Variability Index

060011	41	G to S	Yes
5002	45	R to Y	Yes
060011	45	R to Y	Yes
5002	46	I to L	No
060011	48	E to D	No
060011	61	I to L	No
5002	71	I to T	Yes
02011	72	H to Y	Yes
04034	72	H to Y	Yes
04064	72	H to Y	Yes
8013	73	F to L	Yes
060011	73	F to V	Yes
5002	78	R to Q	No
02011	78	R to N	No
04034	82	I to M	Yes
8009	83	G to S	Yes
5002	84	V to I	No
06011	84	V to I	No
8009	85	T to V	Yes
8013	85	T to S	No
060011	85	T to I	Yes
8009	86	Q to P	Yes
060011	87	Q to R	No
8009	88	Q to R	No
5002	88	Q to R	No
8009	90	A to W	Yes
060011	90	A to T	Yes
8009	91	R to S	Yes
8009	93	G to I	No

**Table 2**: This table shows the AA variability the 8 ALT patient Vpr alleles exhibited in the sequencing done for this MQP. Multiple patients had variability in specific hyper variable regions. It is especially interesting that there is a Q3R mutation found in patient 060011, this mutation would definitely affect viral phenotype in a way that is remarkable similar to the lack of viral cytopathicity observed in the French ALT virus.

**Note:** A yes in the far right column in the table above denotes a substitution of an amino acid with different physical properties, for example a yes would be scored if a hydrophobic amino acid was substituted for a hydrophilic amino acid. This type of variability is more likely to change protein function, but may not necessarily impact phenotype.

To determine whether the ALT Vpr mutations found in this study correlate with

highly variable Vpr regions found in the Los Alamos HIV database, the sites of the ALT

patient Vpr mutations were compared to the Vpr gene for HIV-1 subtype B. Some

typical sequences were examined from this database; they are represented below in

Figure 14.

B.AR.98.ARCH054	N
B.AU.95.LTS3-1A	H
B.BO.99.BOL0122	S
B.CN RL42	
B.GA. 88. OYI	C
B.GB.86.GB8	V
B. IN. 98. In14	
B. JP PT7 - 2	TV VN VV
B.KR. 97 WK	AN THE F E
B.NI. 91 17025-1	
B TW QA TWOYS	
B UC 83 CD2	FÇF
D 110 04 MN/00	Y
B.US. 84 MNCG	
D.03.04.N15CG	

**Figure 14: Typical HIV-1 Subtype-B Viral Protein R (Vpr) Amino Acid Alignment.** This figure shows the partial alignment of 14 typical HIV-1 subtype-B Vpr proteins found in the Los Alamos database. The sequences vary slightly from patient to patient, but extensive variability is observed at certain hyper variable positions. The locations with the most variability in this alignment are positions 15, 28, 37, 41, 72, 78, 85, and 86. (all Vpr sequences were obtained from Los Alamos).

A comparison of the hyper variable loci from the ALT patients versus the Los

Alamos isolates is represented by Table 3.

AA#	Number of samples with mutations	Drastic AA change (Yes or NO)
15	3	Yes (all 3)
28	0	-
37	3	Yes (all 3)
41	2	Yes (both)
72	3	Yes (all 3)
78	2	No (both)
85	3	Yes 2 No1
86	1	Yes

#### Table 3: Comparison of ALT and Los Alamos Vpr Hypervariable Loci.

Table 3: This table shows a comparison of the Vpr hypervariable loci for the 8 ALT patients examined in this MQP versus 14 randomly chosen Subtype-B isolates from the Los Alamos database.

Just to describe some of the hypervariable loci found in the ALT samples some statistics are given in Table 4 below. Each of the ALT hypervariable loci were also hypervariable in the majority of the wild-type Los Alamos samples.

Vpr AA Position	Percent (of 8) ALT Samples Variable at This Site	Percent of 14 Wild-Type HIV's Variable at This Site
15	3/8 37.5%	13 / 14 93 %
37	3/8 37.5%	9 / 14 64 %
85	3/8 37.5%	14 / 14 100 %
41	2/8 25%	7/ 14 50 %
72	2/8 25%	14 / 14 100 %
78	2/8 25%	11 / 14 79 %
86	1/8 12.5%	8 / 14 57 %

# Table 4: Vpr Hypervariable Loci.

### DISCUSSION

All of the 8 HIV Vpr hypervariable loci identified in this study for the ALT LTNP patients were also found to be hypervariable loci in 14 randomly chosen HIV subtype-B HIV sequences from the Los Alamos database. This fact implies that the hypervariable regions of the ALT sequences correlate with hypervariable regions of other HIV-1 subtype B Vpr isolates previously characterized, which suggests that sequencing done in this project was reasonably accurate, and the same Vpr loci change frequently. However many of the ALT mutations change the <u>type</u> of AA and so are predicted to affect Vpr function more than the wild-type polymorphisms which tend to be <u>silent</u>. It remains to be shown whether the ALT polymorphisms are responsible for the ALT LTNP phenotype.

The results shown in a study by (Di Marzio et al, 1995) imply that Vpr dependent cycle arrest is largely controlled by the carboxy-terminal domain of the protein. This was shown by comparing mutated Vpr genes, and their gene products. It was found that the amino-terminal, alpha-helical region of Vpr was required for nuclear localization and packaging into virions. Patients that have mutations in this region of HIV most likely have a deficient form of the virus. Vpr position 15 was found to be hypervariable in the ALT patients; DiMarzio also found mutations at Vpr position 15 to be hypervariable, which would possibly affect nuclear localization and packaging.

Another example of the effect of mutations in hypervariable regions of Vpr, is shown by a study completed by (Wang et al, 1996). Mutations found in positions 83-90, which encompasses hypervariable regions 85 an 86 identified in this MQP, confer a LTNP phenotype. In a set of 2 patients, a mother-child pair which has survived for > 13 years, these patients have mutations in this 85-86 Vpr region. Even though they are HIV

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positive they maintained stable CD4+ T-cell counts, so this suggests that mutations in this region of Vpr can confer the LTNP status.

Variability was also observed in ALT Vpr areas not considered hypervariable. For example, the Q3R mutation occurs in an area that is not considered hypervariable. There was also variability at position 84 for 2 out of the 8 samples sequenced (25%). Mutations in these areas may also have negatively impacted the ability of the French ALT HIV to cause its peculiar LTNP phenotype. Future experiments would involve mimicing these mutations in wild-type HIV's to determine whether they affect its ability to replicate in permissive cell lines.

Most of these mutations found in the regions listed in Table 2 above were drastic amino acid changes, or changes in the category of amino acid. These types of amino acid changes can drastically affect the capabilities of the protein being produced. Even substitutions of amino acids with similar properties can affect Vpr (Somasundaran et al, 2002), and in turn the viruses' ability to induce apoptosis. For example the Q3R mutation is substitution of two similar amino acids, yet it heavily alters viral phenotype (Somasundaran et al, 2002). Patient 060011 had this mutation along with several others, so would be predicted to also show diminished replication in cell lines.

The sequencing done here implies that mutations in Vpr may be responsible for the French ALT phenotype; however sequence data alone is not sufficient to truly determine whether these mutations are responsible for the LTNP status of the French ALT study group. Infectivity and apoptosis experiments using the constructs made in this MQP must be done to correlate specific polymorphisms in patient Vpr alleles with the virus's cytopathic ability.

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