

**Characterization and Use of Replication Competent HIV-1 Genomes
Carrying Fluorescent Reporter Genes**

A Major Qualifying Project Report

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ABSTRACT

HIV envelop genes specify which types of host cells the virus can infect. HIV clones carrying five different patient envelopes and two kinds of fluorescent reporter genes were constructed to develop tools for investigating viral tropisms. The data demonstrated that the new constructs showed the expected macrophage tropic or non-macrophage tropic phenotype and were detectable by FACS analysis. In future experiments, these clones will be extremely useful in evaluating whether highly macrophage-tropic envelopes confer a broader tropism among CD4-positive T-cell populations in blood lymphocyte cultures, and whether they out compete viral clones carrying non-macrophage tropic envelopes.

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BACKGROUND

HIV Introduction

HIV is a retrovirus in the lentivirus family. Retroviruses are enveloped viruses that have a single strand, positive RNA genome. HIV is transmitted through blood, semen, vaginal fluid and breast milk. HIV infects cells that are part of the immune system, such as T-cells and macrophages. Infection of these cells impairs the immune system resulting in acquired immune deficiency syndrome (AIDS). A person with AIDS is therefore more susceptible to infection from a range of opportunistic infections such as tuberculosis, *Pneumocystis carinii*, and Hepatitis.

According to The World Health Organization more than 25 million deaths have occurred since AIDS was discovered in 1981. This is one of the worst pandemics in recent history. Currently there is no cure for HIV infection. However, “highly active anti-retroviral therapy” (HAART) introduced in the mid 90s has dramatically reduced deaths from AIDS in developed countries. HAART involves the combination of inhibitors that target the HIV Reverse Transcriptase and protease genes.

HIV Genome

The HIV genome is a single strand of RNA that is approximately 9 kb in size. Following infection, the viral RNA is transcribed by the viral reverse transcriptase into a cDNA copy which becomes integrated into the cell’s chromosomal DNA. HIV mutates frequently because the reverse transcriptase has no proofreading mechanism, and it makes an error approximately every 10,000 bases.

HIV Protein Functions

The HIV genome encodes nine different genes (Figure-1). These genes can be divided into structural, regulatory, and accessory.

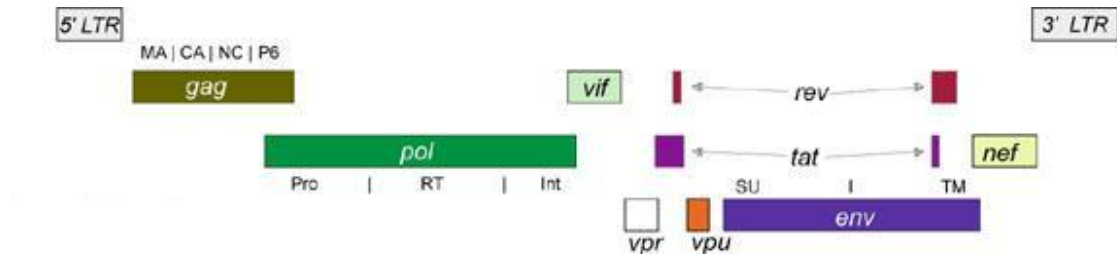


Figure 1: Diagram of the HIV-1 Genome (Harvard Images, 2006).

HIV Accessory Genes

The accessory genes and their proteins are Vpu, Vpr, Vif, and Nef. Nef is a 206 amino acid long protein that is expressed early in HIV infection. One function of Nef is the down regulation of CD4, and Class I MHC receptors (UCSF, 2006). Reducing Class I MHC receptors helps the infected cells avoid detection by the immune system. CD4 presents foreign peptides and is detected by cytotoxic T-cells, which functions to eliminate infected cells. The reduction of CD4 on the surface of the cell is also useful to the virus because the presence of CD4 has been shown to interfere with the production of virus particles. CD4 down regulation also prevents multiple infections by blocking more virus particles from entering the cell. Too many infections in one cell has been shown to induce apoptosis (Lama et al., 1999).

Vpr helps HIV infect cells that are not dividing. Vpr is present in the preintegration complex which carries the incoming viral cDNA to the nucleus and the

cell's chromosome (Heinzinger et al., 1994). Evidence indicates that Vpr accomplishes this by becoming tethered to nuclear pores (Vodicka et al., 1998).

Vpu is essential for release of virus particles after replication. This is partly accomplished by triggering the ubiquitin-mediated degradation of CD4 molecules complexed with HIV Env (Willey et al., 1992).

Vif induces degradation of a host protein called APOBEC3g, a cytidine deaminase. In the absence of Vif, host APOBEC3g is incorporated into virus particles and acts to stop viral replication by mutating dC into dU in the first minus strand of cDNA synthesized (Conticello et al., 2003).

The regulatory proteins are Tat and Rev. Tat is a factor that transactivates transcription of HIV mRNAs from integrated proviral DNA. Tat binds to TAR a secondary RNA structure at the start of all HIV mRNA. Without the Tat only short truncated mRNAs are made. Tat functions by recruiting a host serine kinase CDK9 which results in the phosphorylation of RNA polymerase II, allowing full length mRNA and genomic RNA to be synthesized (Southgate and Green, 1991).

Rev is involved in the transition from early to late phase HIV expression. Rev induces the expression of structural proteins. In the absence of rev, HIV-1 mRNAs are fully spliced into small mRNAs that encode rev, tat, and ref. When sufficient rev is produced, rev induces unspliced and singly spliced mRNAs that encode gag, pol, and env (Cullen, 1991).

HIV Structural Genes

The three structural genes are Gag, Pol and Env. The Gag gene produces four proteins: MA, CA, NC and p6 (Figure 2). Gag is made as a precursor protein (55 kd) that is responsible for budding virus particles from an infected cell. Gag recruits the viral RNA to the cell membrane (Welker et al., 2000). During budding, envelope glycoprotein spikes are recruited, and Gag is cleaved into MA, CA, NC and p6. MA (17 kd) stays attached to the cellular membrane which helps stabilize the virus particle. CA (24 kd) forms the core of the virus particle. NC (9 kd) surrounds the viral RNA. P6 recruits accessory proteins such as Vpr (Paxton et al., 1993) and also recruits cellular ESCRT proteins that confer the budding process.

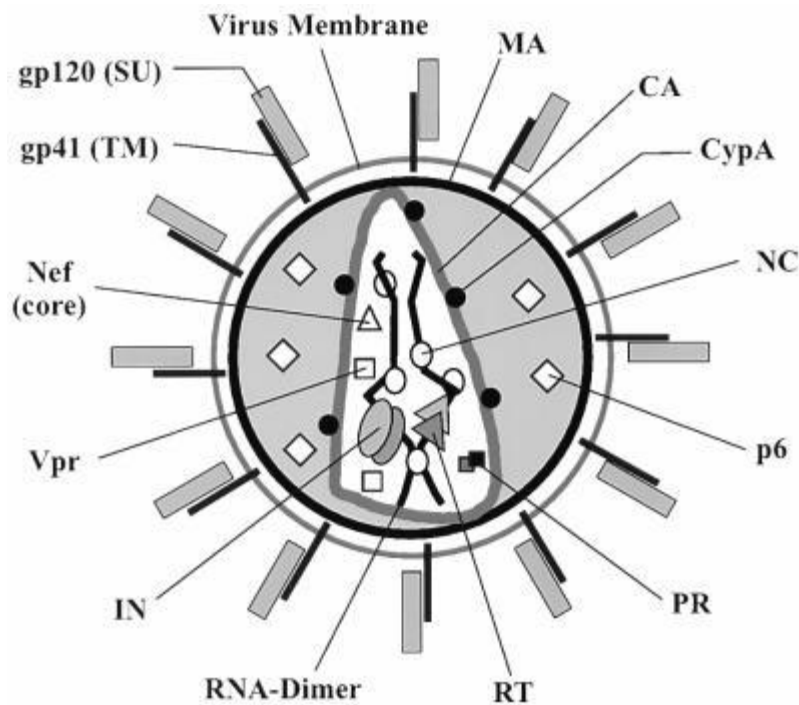


Figure 2: Model of the HIV Virus Particle (Welker et al., 2000).

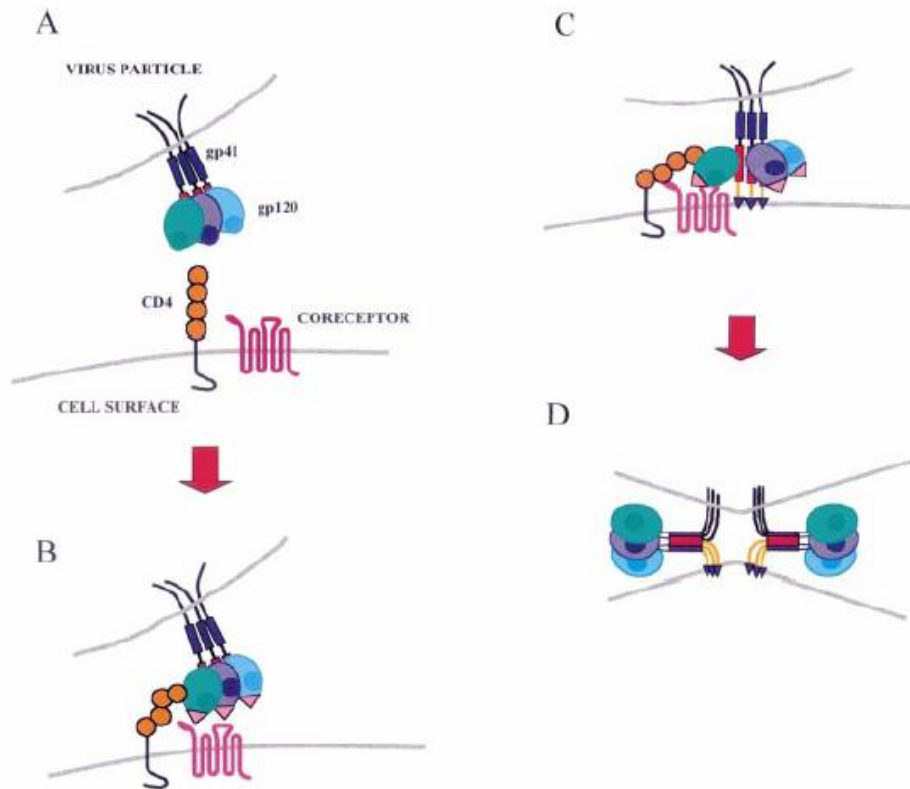
The gene Pol produces a protease (Pro), reverse transcriptase (RT), and an integrase (In). These three proteins are initially made as a large gag-pol precursor protein. The protease is responsible for cleaving gag and gag-pol precursor proteins into their constituent proteins in budding virions. RT's function is to make a dsDNA copy from the original ssRNA HIV genome. Once a dsDNA copy is made, the integrase integrates it into the host DNA. This is done by the integrase that first removes two nucleotides from viral DNA, then cuts the host DNA. Integrase then ligates the viral DNA into the host DNA (Yoshinaga and Fujiwara, 1995).

HIV Envelope Structure and Function/ Fusion

The HIV envelope proteins are made from a precursor protein gp160. This is cleaved by cellular proteases to form gp120 and gp41. These proteins form the envelope spikes. Gp120 is the viral surface protein, and gp41 is the transmembrane protein of the viral envelope spike. Three gp120 and three gp41 subunits combine to form a trimer which forms the functional envelope spike.

Fusion of the virus particle and host cell membrane begins with the interaction of gp120 and cellular receptor CD4 (Figure 3). This interaction signals the rearrangement of the envelope spike exposing a site that binds the coreceptor. Following coreceptor binding, gp41 is exposed to the cell membrane and initiates fusion (Clapham and McKnight, 2002). The major receptor in HIV infection is CD4. CD4 is found predominantly on T-helper cells and is responsible for interactions with MHC class II molecules. Some of the important amino acids on CD4 that interact with gp120 are F43,

R59, D368, E370, and W427. Gp120 interaction with CD4 causes a rearrangement of gp120 and exposes a site for coreceptor binding (Myszka et al., 2000).



Events in the attachment and fusion of HIV. (A) HIV virion binds CD4. (B) Conformation changes in the core of gp120 and movement of the variable loops cause exposure or formation of the coreceptor-binding site. Flexible regions in CD4 between domains D2 and D3 as well as domain D4 and the membrane allow orientation of the coreceptor-binding site for coreceptor binding. (C) Binding of gp120 to the coreceptor triggers further conformational changes, mainly in gp41, which trigger release of the fusion domain and result in it embedding in the host cell membrane. (D) Complexing of the leucine zipper domain (red) and α -helix (blue) repositions the gp41 TM region and fusion domains close together allowing a fusion pore to

Figure 3: Diagram of HIV fusion with the host cell (Clapham and McKnight, 2002).

The two major co-receptors for HIV infection are CCR5 and CXCR4. Coreceptor usage is used to categorize HIV infection, CCR5-using virus are designated R5, and CXCR4 are X4 virus. R5 viruses predominate in infection and are the viruses that are transmitted between people. X4 viruses are found in 50% of HIV infections but are not

found until the late stage of infection. CCR5 and CXCR4 are 7 transmembrane chemokine receptor proteins. Other 7TM chemokine receptors may also be used as coreceptors for HIV infection, however they are believed to insignificant for replication *in vivo* (Clapham and McKnight, 2002).

HIV Tropism

R5 and X4 viruses show different cellular affinities or tropism. R5 virus infects both T-cells and macrophages. X4 virus infects macrophages less efficiently than R5 virus and instead targets T-cells when they emerge *in vivo*. X4 virus may have a broader tropism for different types of T-cells. R5 viruses infect memory T-cells, while X4 virus can also infect naïve T-cells. HIV can also infect non-immune tissue. For example the brain and liver can be infected by R5 viruses; X4 viruses are not usually found in these tissues (Clapham and McKnight, 2002). Although R5 viruses are considered macrophage tropic, not all R5 viruses infect macrophages. Evidence shows that R5 virus from brain tissue can infect macrophages very efficiently, while virus from blood, lymph node, and semen are often not able to infect macrophages (Peters et al., 2004).

PROJECT PURPOSE

The purpose of this project was to construct various clones carrying specific HIV envelop genes and fluorescence reporter genes to test the host cell tropism of specific HIV envelop genes. Patient envelopes were chosen in order to compare macrophage tropic and non-macrophage tropic viruses that arise in the same patient. Env genes were amplified from patient HIV isolates by PCR, then cloned into GFP and Ds2Red HIV-1 constructs. The clones were tested for phenotype conservation and for use in evaluating the cell types infected.

METHODS

Cloning of Patient-Derived Envelop Genes Into TN6R-NL and TN6G-NL

Preparation of HIV Envelope Genes

Plasmid PSVIII clones carrying five different patient-specific envelope DNAs (see below) were produced from previously transformed bacteria frozen at -80°C as *E. coli* glycerol stocks. The *E. coli* STBL2 cells containing the plasmid DNA were cultured in 4 mL of LB/AMP media at 30°C overnight. The plasmid DNA was isolated using a Qiagen DNA miniprep system.

Preparation of HIV-1 TN6R-NL and TN6G-NL Reporter Constructs

The TN6R-NL and TN6G-NL plasmid DNAs arrived dehydrated on a piece of filter paper (from Dr. Matthias T. Dittmar). This paper was rehydrated, and the DNA was transformed into competent STBL2 cells. The STBL2 cells were then grown at 30°C overnight, and the plasmid DNA was obtained using a Qiagen DNA miniprep system.

PCR Insertion of BstII and NcoI Restriction Sites into Patient Envelop Genes

BstII and NcoI restriction sites were introduced at either end of envelope sequences for patient envelopes NA420B33 (B33), NA420LN40 (LN40), NA20B59 (B59), NA20B501 (B501), and NA20LN8 (LN8) using limited PCR. The BstII restriction site was inserted in front of the start codon of the envelope gene, while the NcoI restriction site was inserted one base after the stop codon of the envelop gene (the

primer sequences are listed in Figure-4). The Taq polymerase used in the PCR was Expand High Fidelity (Roche) that contains proofreading capacity. The thermocycler protocol was: 95°C, 2:00 min; [95°C, 0:30 – 51.8°C, 0:30 – 72°C, 0:30] X 20; 72°C, 7:00; with a 4°C hold.

Figure 4: Primers Used for PCR Insertion of BstII and NcoI Restriction Sites

BstII site for all Envelops	TTGTGGGTCACCGTCTATTATGGG
NcoI site for B59	CTTACCATGGATTTTCTAGCAAATCCCTTTCCAAGCCCTG
NcoI site for B33 and LN40	CTTACCATGGATCTTATAGCAAAGCCCTTTCCGCGCCCTG
NcoI site for LN8	CTTACCATGGATCTTATACTAAAGCCCTTTCCAAGCCCTG

The BstII restriction site at the beginning of the envelope resides in a conserved sequence, while the NcoI restriction site is in a variable region. This is why there was only one BstII primer, and different NcoI primers for each patient envelope. The B33 and LN40 envelopes have the same NcoI primer because the LN40 envelope used is a chimera made of LN40 gp120 and B33 gp41.

Amplicon Extraction and Cloning

PCR products were run on a 1% agarose gel using crystal violet to visualize the amplified DNA. The DNA was extracted from the gel using a Qiagen gel extraction kit. The PCR product was then ligated in a TOPO-XL vector using Quick T4 DNA Ligase from New England Biolabs. Ligated DNA was transformed into TOP10F cells, plated on LB/KAN agar, and the plates were incubated overnight at 37°C. A stock of the transformed bacteria was created from selected colonies from the LB/KAN plate; this was done by growing the TOP10F cells in LB/KAN media at 37°C. This culture was

then centrifuged at 4000 rpm for 15 minutes to pellet the cells, and the cell pellet was resuspend in 1:1 solution of glycerol and PBS.

Ligation of Patient Envelops with Plasmids TN6R-NL and TN6G-NL

Plasmids TN6R-NL, TN6G-NL, and the TOPO cloned patient envelopes were each digested with BstII at 67°C for one hour, then NcoI was added and the DNA was incubated at 37°C for one hour. CIP (Alkaline Phosphatase, calf intestine) was added to reduce self ligation, and the DNA was incubated for another hour at 37°C. After the digestion, the DNA was run on a 1% agarose gel using crystal violet to visualize the DNA. The 9 kb bands from TN6R-NL and TN6G-NL, and the 2.5 kb envelop DNA was extracted from the gel. Each patient envelope gene was ligated with both TN6R-NL/-env and TN6G-NL/-env DNA fragments, using Quick T4 DNA Ligase. After the ligation the new constructs were transformed into STBL2 bacteria cells and grown on LB/AMP plates. Colonies were selected from this plate and grown at 30°C in LB/AMP media; the DNA was isolated using the Qiagen DNA miniprep kit. The new plasmids were screened by digesting with BstII and NcoI. This created 10 different constructs, TN6R-NL/NA420B33 (R33), TN6G-NL/NA420B33 (G33), TN6R-NL/NA420LN40 (R40), TN6G-NL/NA420LN40 (G40), TN6R-NL/NA20B59 (R59), TN6G-NL/NA20B59 (G59), TN6R-NL/NA20LN8 (R8), TN6G-NL/NA20LN8 (G8), TN6R-NL/NA20B501 (R501), and TN6G-NL/NA20B501 (G501). Each envelope gene was now in a virus genome with a Ds2red reporter and GFP reporter.

Phenotype Characterization of Fluorescent/Envelope Constructs

Preparation of Replication Competent TN6G-NL and TN6R-NL Viruses

Replication competent virus was prepared by transfecting each plasmid into 293T cells and harvesting supernatant 72 hours later. The virus containing supernatant was clarified by a low speed spin to remove cell debris and stored at -152°C . 293T cells were plated at a concentration of 1×10^6 cells/mL in DMEM 4% FBS Gentamicin media in a 6 well plate (2mL/well) and incubated overnight at 37°C . The Fugene6 Transfection system was used the next day. 6 μg of DNA was transfected per well using 15 μL of Fugene6.

Virus Titers

Virus titers were evaluated using Hela TZM-BL cells which contain a β -galactosidase reporter gene under the control of an HIV-1 LTR promoter. 0.5 mL of TZM-BL cells at 2.5×10^5 cells/mL in DMEM 4%FBS Gentamicin were added to each well of a 48-well plate, and incubated overnight at 37°C . The next day, the medium was aspirated off, and 100 μL of virus at ten-fold serial dilutions was placed on the cells. After three hours at 37°C , 400 μL of media was added. Three days later the cells were fixed with 0.5% gluteraldehyde, washed twice with PBS, then stained with 100 μL of yellow PBS (40 mg/mL in N,N,-dimethyl formamide, diluted to 0.5 mg/mL in 1X PBS with 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, and 1 mM magnesium chloride) and X-gal (0.5 mg/ml). Infected cells stained blue due to the expression of β -

galactosidase . Blue focus forming unit were then counted and virus titers were calculated.

Reverse Transcriptase Assay

1x10⁶ PM1 cells were infected with 200 µL of virus, and incubated at 37°C for 3 hours. After three hours the cells were washed twice with PBS and then resuspended in DMEM 20% FBS gentimicin media. On days 0, 3, 7, and 10 cells were spun down at 1200 rpm for 5 minutes, and 100 µL of supernatant was removed.

Detection of reverse transcriptase was done using the *Lenti-RT*TM RT activity kit. RT makes a copy of template DNA using Bromo-deoxyuridine triphosphates (BrdUTP). After RT incorporates BrdUTP into a new DNA strand, antibody tracers are added that detect the BrdUTP DNA. This was quantified using a spectrophotometer.

DNA Sequencing

2 µl of plasmid DNA at a concentration of 0.2 µg/µL was used for the sequencing reaction. Sequencing was done using BigDye V3.0 DNA which contains AmpliTaq DNA Polymerase, dye terminators, deoxynucleoside triphosphates, magnesium chloride and a buffer. The thermocycler protocol was: [95°C, 1:00 min; 55°C, 1:00 min; 60°C, 6:00 min] x 28 with a 4°C hold at the end. After the PCR, 1 µL of 2% SDS was added and the tube was heated at 95°C for 5 min. The sequencing reaction was processed using a Qiagen DyeEx 2.0 spin kit to remove unincorporated nucleotides, then it was dehydrated in a vacuum centrifuge. The DNA was processed at the PMM/CFAR DNA sequence facility.

Infection of Adherent Cells

Hela cell clones JC53, JC37, JC10, RC23, RC33, HIJ, and primary Macrophages were plated in 48 well plates with 0.5 mL of cell suspension at 2.58×10^5 cells/mL, and incubated at 37°C overnight. Hela cells were grown in DMEM, 4%FBS, and gentamicin; the macrophage media was DMEM, 10% human plasma, and gentamicin. The next day the cells were infected with 100 μ L undiluted virus and serial dilutions in media at concentrations of 1:10, 1:100, 1:1000, and 1:10000. After a three hour incubation at 37°C, 400 μ L of media was added to the cells. Seven days later the cells were stained for the presence of HIV infection (see below).

Cells were washed once in PBS and fixed with methanol-acetone (1:1) at -20°C for ten minutes before being washed once with PBS/0.1% azide. Then the cells were washed once with PBS/0.1% azide/1%FBS. 100 μ L of two α -p24 monoclonal antibodies (38:96K and EF7 provided by the AIDS Reagent Program) was added and cells were incubated for 1 hour at room temperature. After one hour, the cells were washed twice with PBS/0.1% azide/1%FBS, and 100 μ L of secondary antibody (goat anti-mouse β -galactosidase conjugate from Southern Biotechnology Associates, Inc.) diluted in PBS/1%FBS at 1:400 dilution was added, then the solution was incubated for one hour at room temperature. The cells were then washed once with PBS/0.1% azide/1%FBS and once with PBS/0.1%azide, before staining with 150 μ L of yellow PBS and X-gal (as described above). The staining was left to develop at room temperature overnight, and the next day blue stained focus forming units were counted.

FACS Detection of Infected Cells

2 mL of 1×10^6 cells/mL of infected PBMC cells were centrifuged at 1200rpm for 5 minutes. The media was aspirated off and the cell pellet was resuspended in 200 μ L of G33, G8, G40, G59, or G501. The infected cells were incubated at 37°C for 3 hours. The cells were then placed in 2mL of RPMI 10% human plasma, IL-2, and gentamicin. A week later the infected cells were stained for presence of CD4, CD8, CD45RA, and CD45RO. The cells were centrifuged at 1200rpm for 5minutes, washed with PBS, and resuspended in 20 μ L of PE-conjugated antibody for CD4, or 10 μ L of PE conjugated antibody for CD8, CD45RA, or CD45RO, then incubated at 4°C for half an hour. The cells were centrifuged, washed twice with PBS, and then fixed in 3% paraformaldehyde.

Fixed cells were processed for flow cytometer. PM1 cells were used for preliminary FACs characterization. The same protocol was followed for staining PM1 cells.

RESULTS

Cloning of Patient Envelopes Into TN6R-NL and TN6G-NL HIV Genomes

Insertion of BstII and NcoI Restriction Sites in Env Genes Using PCR

Patient HIV envelopes NA420B33, NA420LN40, NA20B59, NA20B501, and NA20LN8 were each cloned into HIV genomes TN6R-NL and TN6G-NL. These patient envelopes were chosen in order to compare macrophage tropic and non-macrophage tropic virus envelopes that arise in the same patient. PCR was used to introduce BstII and NcoI restriction sites at the beginning and end (respectively) of the patient envelope genes amplified from previously cloned plasmids. Figure 5 shows the successful PCR amplification of the 2.5 kb fragment of the envelopes described above.

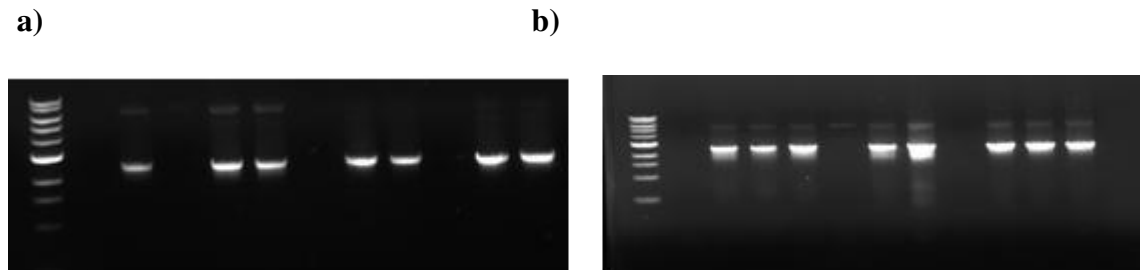


Figure 5: PCR Amplification of Patient Envelope Genes. The first lane is a 1 kb marker. Panel a) shows successful amplification of the envelope gene (~2.5 kb band in the center of the gel) for envelopes B33 (lane 3), LN40 (lanes 5-6), B59 (8-9), and LN40 (11-12). Panel B) shows successful amplifications of B59 (lanes 3-5), B501 (7-8), and B33 (10,11,12).

After the PCR, the amplicons were directly ligated into the TOPO-XL vector, and then transformed in into competent TOP10F *E. coli* bacteria cells. Figure 6 shows the digestion of several positives cut with BstII and NcoI restriction enzymes. The gel shows

the envelope gene at 2.5 kb (in the center of the gel) and the TOPO vector (which contains an NcoI site) digested into two pieces underneath it.

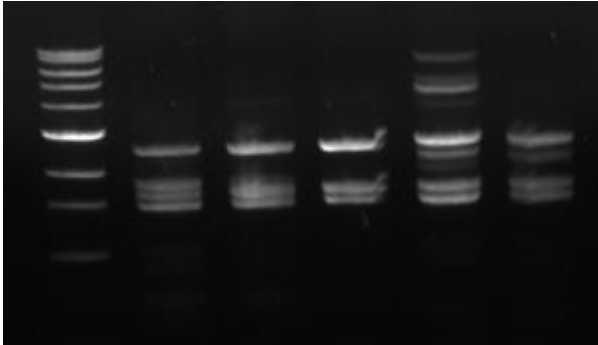


Figure 6: Digestion Screening of the TOPO Vector Containing Patient Envelope Genes. Lane 1, 1 kb marker; lane 2 B59, lane 3 B501, lane 4 B33, lane 5 LN8, lane 6 LN40).

Ligation of Envelope Genes into HIV Genome Plasmids TN6R-NL and TN6G-NL

After successfully cloning envelope genes containing BstII and NcoI restriction sites, these envelopes were digested out of the TOPO vector and ligated into the TN6R-NL and TN6G-NL HIV viral clones. Plasmids TN6R-NL and TN6G-NL have a BstII site at the beginning of their envelope and a NcoI site at the end of their envelope genes. Figure 7 shows the digestion of plasmids TN6R-NL and TN6G-NL with BstII and NcoI.

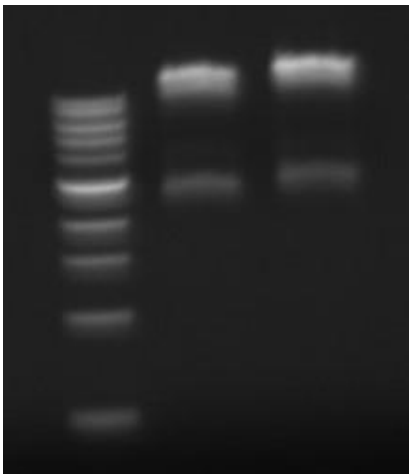
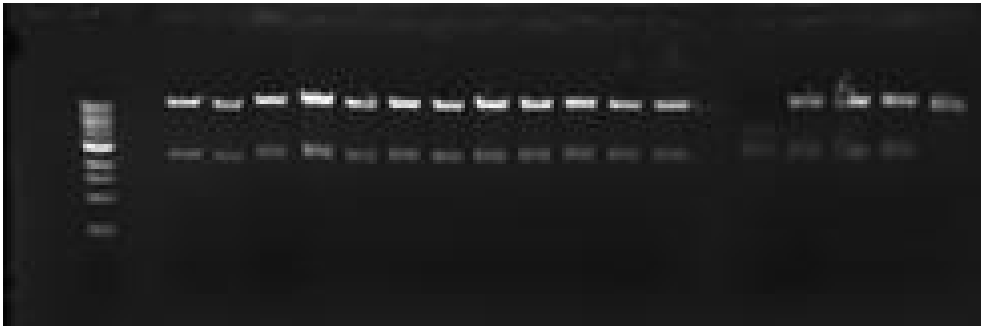


Figure 7: Digestion of Plasmids TN6R-NL and TN6G-NL with BstII and NcoI to Release the Env Gene. Lane 1, 10 kb marker, Lane 2 plasmid TN6G-NL; Lane 3 TN6R-NL plasmid. The 10 kb plasmid vector is seen as the upper band on the gel.

The 10 kb fragment missing the env gene was extracted from the gel and purified as described in Materials and Methods. The TOPO-XL clones containing the PCRed patient envelopes were also digested with BstII and NcoI, and the 2.5 kb Env bands were extracted and purified. Patient envelopes were then ligated with both TN6R-NL and TN6G-NL plasmids. Figure 8 shows the digestion screening of the recombinants.

a)



b)

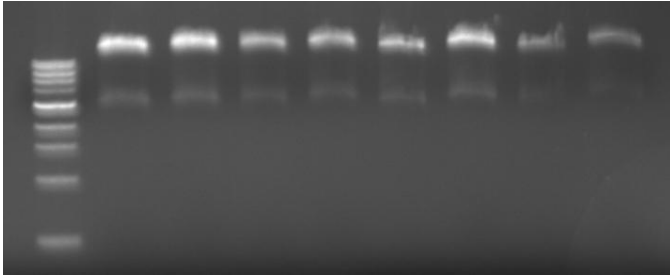


Figure 8: Digestion Screening of Recombinant Plasmids TN6R-NL and TN6G-NL Containing Patient Envelopes Using BstII and NcoI Restriction Enzymes. Panel a) shows G33 (lanes 2-4), R33 (lanes 5-7), G8 (lanes 8-10), R8 (lanes 11-13), G40 (lanes 14-16), R40 (lanes 17-19) lanes, and a 1kb marker (lane 1). Panel b) shows G59 (lanes 2-3), R59 (lanes 4-5), G501 (lanes 6-7), R501 (lanes 8-9) and a 1kb marker (lane 1).

To confirm that the envelope genes were cloned correctly into the new constructs the recombinant plasmids were sequenced. The construct sequences were aligned with a reference sequence for each patient envelope, and showed no errors (data not shown).

Testing for Functional Virus and Maintained Phenotypes for Constructs

Functional Virus and Fluorescent Markers

After constructing the plasmids containing the patient envelop genes in HIV genomes containing fluorescent reporters, I next tested to see if the new constructs had the same phenotype as the original envelopes, and evaluated whether the fluorescent markers were functional. Virus stocks were produced by transfection of 293T cell lines. The stocks were then titrated onto Hela TZM-BL cells that express CD4 and CCR5 receptors, and which also contain a β -galactosidase gene controlled by an HIV LTR promoter as a reporter for HIV infection. Two days after infection, the cells were fixed and evaluated for Beta-galactosidase activity as described in Materials and Methods, and blue infection foci were counted. Table 1 shows the infectivity titers for each construct. Beneath the table are micrographs of the infected TZM-BL cell line (Fig. 9).

Table 1: Virus Titers on the Hela TZM-BL Cell Line.

Virus:	R33	R8	R40	G33	G8	G40
FFU/ml:	1.15×10^6	4.5×10^5	1.5×10^5	1.5×10^6	9×10^6	9×10^6

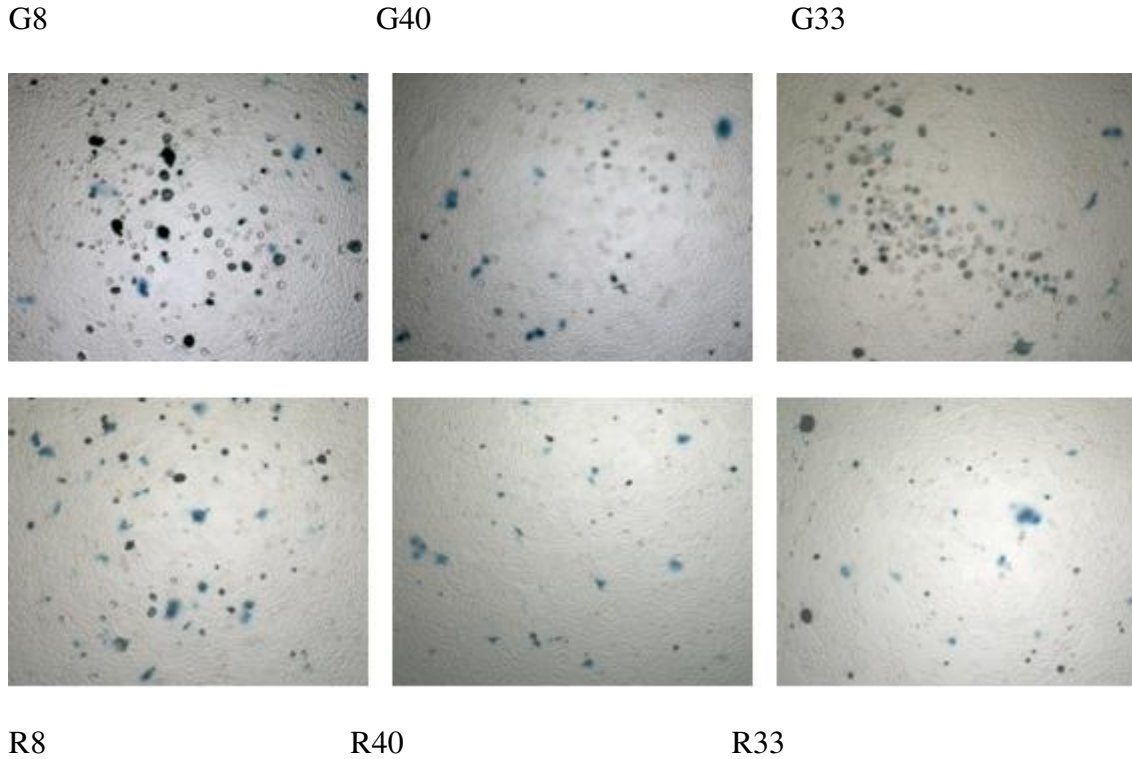


Figure 9: β -Gal Expression From Infected TZM-BL Cells. The blue color indicates the presence of replicating HIV in the cell.

The ability of the viruses to directly express their GFP and Ds2Red reporter genes was also monitored in PM1 cells. All constructs had functional fluorescence. Example micrographs for clones G33 and R33 are shown in Figure 10.

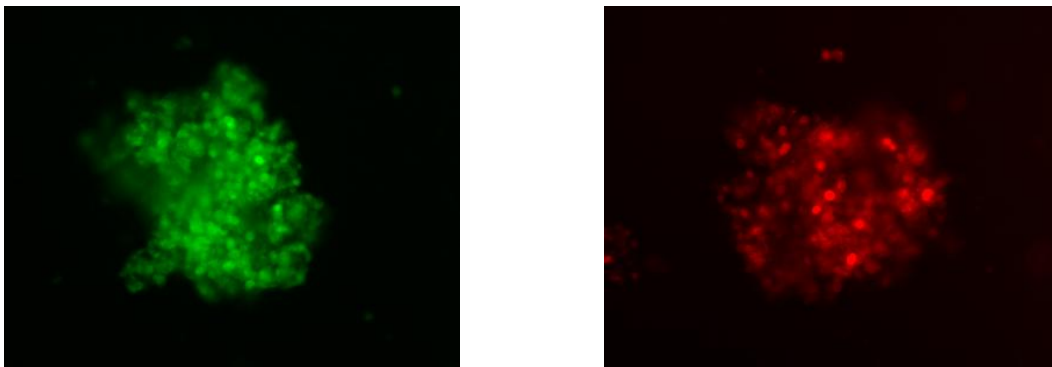


Figure 10: Expression of GFP and Ds2Red Reporter Fluorescent Activity from Viral Clones G33 (left) and R33 (right).

Figure 11 shows the production of virus particles into the cell supernatants for viruses G33 and R33 using PM1 cells. Virus production was evaluated by measuring RT activity. Both clones show approximately the same ability to produce HIV virions. Similar data was obtained for G8, R8, G40, and R40 (not shown).

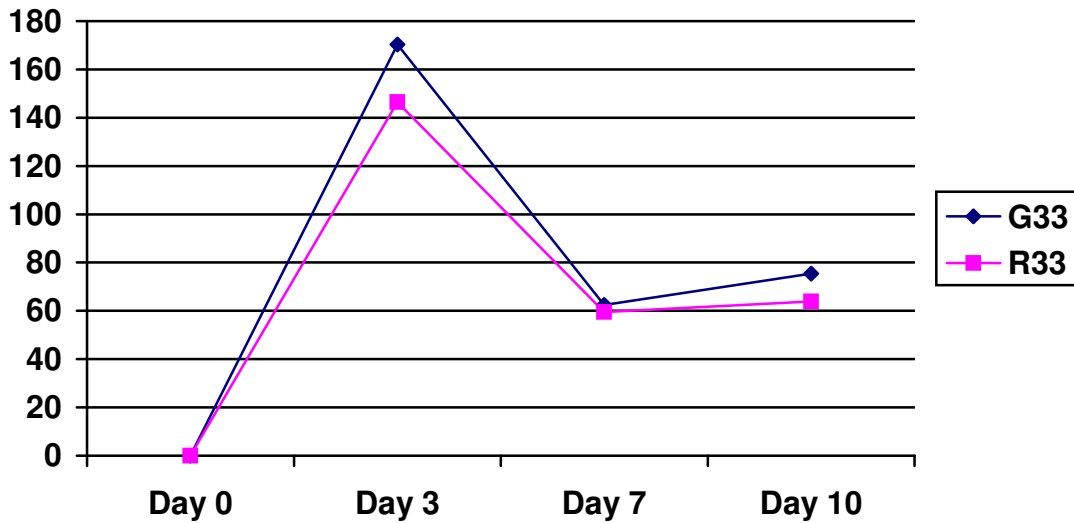


Figure 11: RT Activity in Cell Supernatants for Viruses G33 and R33 Using PM1 Cells. Y-axis shows picograms of RT per mL. The data for G8, R8, G40, and R40 are not shown.

Phenotype Testing

Viruses were titrated on Hela cell lines containing varying levels of CD4 and CCR5 receptors. Table 2 shows the different levels of CD and CCR5 expressed by each cell line, and also shows the viral titers obtained for each cell line.

Table 2: Titers of Hela Cell Lines with Varying CD4 and CCR5 Receptor Concentrations

	High CCR5	Med CCR5	Low CCR5	No CCR5
High CD4	JC53	JC37	JC10	HIJ
Low CD4		RC23	RC33	

FFU/mL

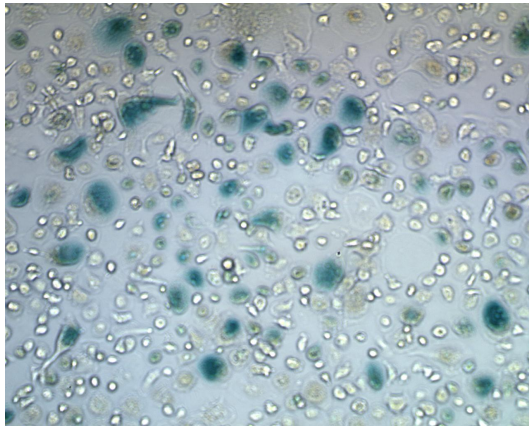
	G33	G8	G40	R33	R8	R40
JC53	4.2×10^5	1.6×10^5	4.5×10^4	8.3×10^5	1.8×10^5	1.0×10^5
JC37	5.6×10^5	2.1×10^5	1.0×10^5	1.6×10^4	1.3×10^4	8.5×10^3
JC10	4.0×10^4	2.6×10^4	1.0×10^2	2.6×10^4	1.0×10^2	1.0×10^2
RC23	2.5×10^3	0	0	5.5×10^3	0	0
RC33	2.0×10^2	0	0	1.2×10^3	0	0
HIJ	0	0	0	0	0	0

Figure 12 shows infectivity titers for macrophages; it contains micrographs of the successful titers and infection for G33 and R33. Only brain-derived virus was able to infect via low levels of CD4 as evaluated on Hela cells and infected macrophages.

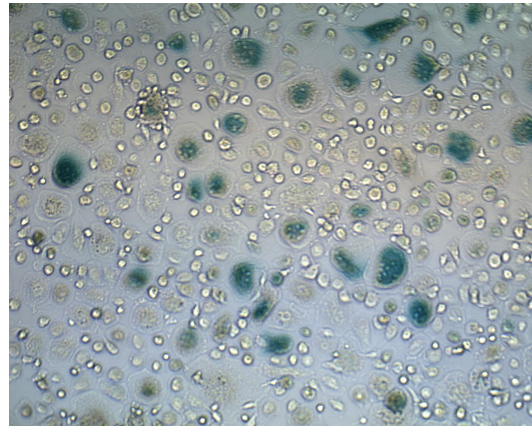
FFU/mL

G33	G8	G40	R33	R8	R40
1.25×10^5	0	0	5.5×10^4	0	0

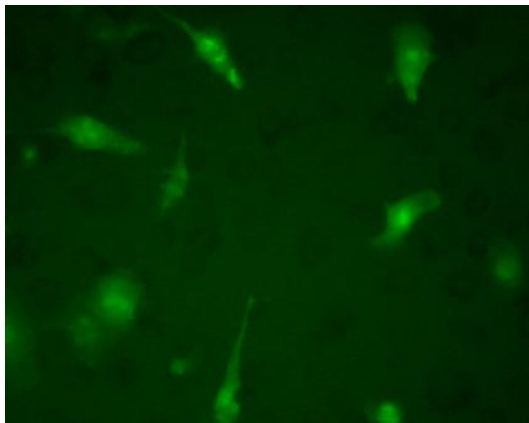
G33



R33



G33



R33

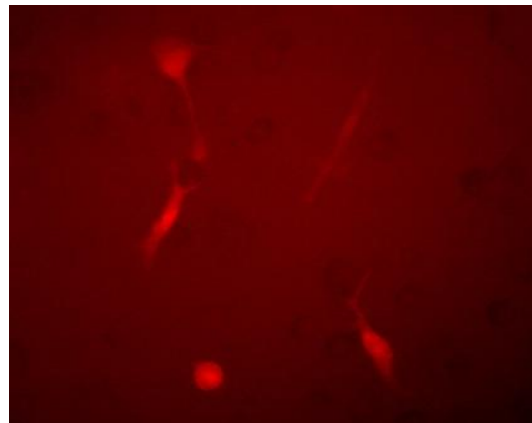


Figure 12: Viral Titers on Macrophages.

The results presented show that plasmids TN6R-NL and TN6G-NL containing Env B33 could infect macrophages and HeLa cell lines expressing low levels of CD4 and CCR5. The TN6R-NL and TN6G-NL constructs containing LN8 and LN40 envelopes could not infect macrophages or HeLa cell lines containing low levels of CD4 and low levels of CCR5. These results shows that envelopes B33, LN8, and LN40 retained the expected macrophage tropic or non-macrophage tropic phenotypes after being cloned into TN6R-NL and TN6G-NL reporter clones, thus the cloning of the env genes into the reporter constructs did not destroy the natural receptor usages of the HIVs.

Preliminary Experiments to Evaluate Cell Types Infected in PBMCs

The newly constructed HIV reporter clones were then tested to evaluate whether HIV infected cells could be detected by FACs. PBMCs infected with viruses G33 and R33 were processed in a flow cytometer. Preliminary data showed that PBMCs infected with R33 (expressing the Ds2Red fluorescent molecule) and fixed with 3% paraformaldehyde could not be detected by FACs, although G33 (expressing the GFP fluorescent molecule) were detectable. The next test done was to determine if PE-conjugated CD4 antibody staining could be detected by FACs, and to evaluate the ability of FACs to detect PE and GFP separately. This experiment was performed with PM1 cells that strongly express all known HIV co-receptors. Figure 13 shows the successful detection of both GFP fluorescence (HIV presence) and PE fluorescence (CD4 presence) in PM1 cells infected with G8. We conclude that the HIV fluorescence reporter constructs prepared in this project retain the receptor usage phenotypes of the parent

envelop genes, and are detectable by FACs analysis, so should be useful for future experiments to test HIV tropism.

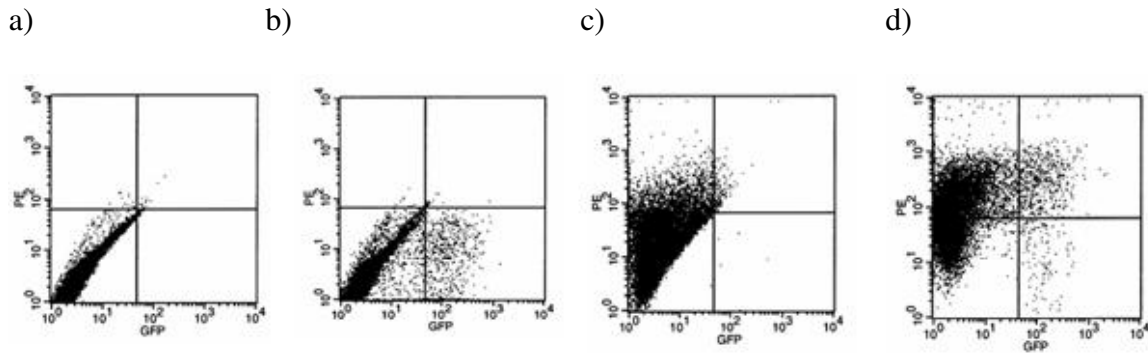


Figure 13: FACS Analysis of G8 for GFP and PE CD4 Staining. a) PM1 cells with no G8 or PE, b) PM1 cells with only GFP from G8, c) PM1 cells stained with PE CD4 antibodies, d) PM1 cells infected with G8 and stained with PE CD4 antibodies.

The two expected uses for the clones prepared here are to determine which cell types are infected in PBMCs, and to run viral competition assays. Unfortunately these experiments were not able to be finished. One problem that arose in determining which cell types are infected in PBMCs was the low amount of cells infected. Because PBMCs represent a variety of cells, not all of them contain CD4, this caused a large majority of them to be uninfected. Figure 14 shows a micrograph of the cells infected in PBMCs by virus G33. When this sample was run through the flow cytometer (Figure 14 right side) no GFP fluorescence was detectable.

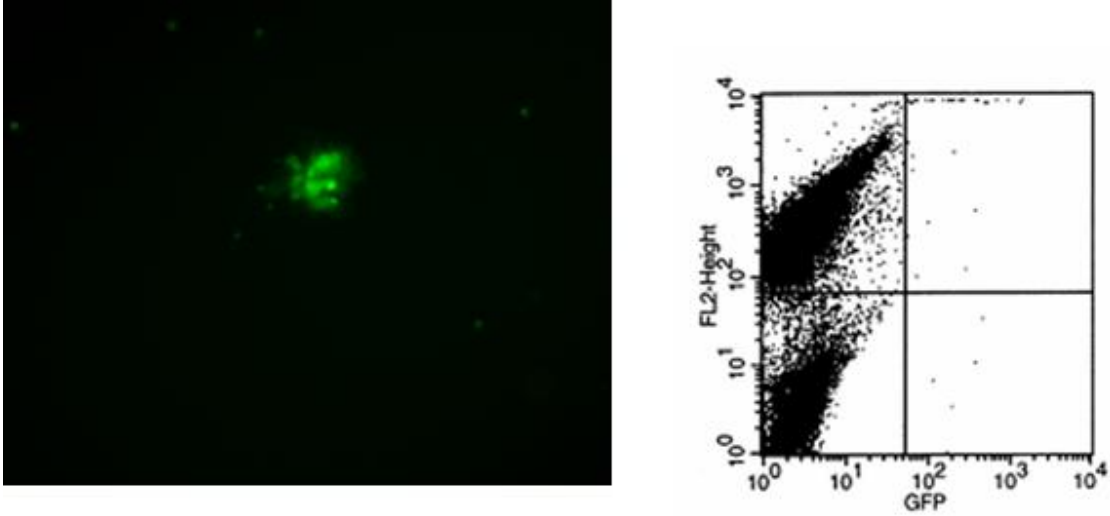


Figure 14: Analysis of PBMCs Infected with Virus G33. Few cells showing GFP fluorescence are seen in the micrograph (left side), and no GFP fluorescence was observed by FACS analysis (right side, x-axis).

The second experiment attempted with the newly constructed clones was to perform viral competition assays by infecting PM1 cells simultaneously with R33 and G40 viruses. Unfortunately the data from this experiment was not usable because the virus stocks used did not replicate well.

DISCUSSION

The data from this project shows that the HIV fluorescence reporter constructs prepared retain the expected receptor usage phenotypes of the parent envelop genes, and are detectable by FACs analysis. These constructs will be extremely useful for characterizing cell types infected in peripheral blood mononucleocyte cells and in viral competition assays. The fluorescent reporter gene provides a convenient direct way to observe infected cells.

The most important part of this project was to determine if the envelopes cloned into TN6R-NL and TN6G-NL retained their expected phenotypes. The patient envelopes cloned into TN6R-NL and TN6G-NL were originally characterized in previous papers. If the clones showed different phenotypes, then the data obtained using this detection system would not help to further the knowledge about HIV tropism, for example for viruses derived from the brain compared to lymph node. It was determined that G33, R33, G8, R8, G40, and R40 conferred the expected phenotypes. Unfortunately, B59 and B501 constructs were not evaluated on macrophages due to time restraints. Nonetheless, brain-derived G33 and R33 infected cells with low levels of CD4 included macrophages, whereas lymph node-derived clones G8, R8, G40, and R40 did not.

One key problem noted in the PBMC infection assay (Figure 14) was a low titer of CD4 presenting cells which was not sufficient for FACs detection of infected cells. One way to fix this problem would be to first separate CD4 positive cells from the original PBMC mixture to create a concentrated solution of CD4 positive cells, making

infection and replication more likely. This would still show which cells type are infected in PBMCs, but it would remove the cells that cannot be infected by HIV.

The second experiment attempted with the newly constructed clones was a viral competition assay in which both R33 and G40 viruses were used to infect PM1 cells. Viral competition assays are useful in determining how different tropisms may arise in one patient, by showing which virus grows best under competing conditions like those encountered *in vivo*. By infecting PM1 cells at the same time with two strains, it would show how the differences may occur. Also by adding antibodies against HIV this type of competition assay would be able to replicate the immune environment of different areas of the body. Unfortunately neither viral stock replicated well in the co-infection assay. Figure 11 shows that G33 and R33 viruses were able to replicate well on the PM1 cell line used for the RT assay, so the lack of replication was not due to a problem with the viral constructs. In addition, viruses LN40 and LN8 also did not show successful infection on this same cell line, but did previously as seen in figure 13 which shows G8 infection of PM1 cells. This shows that the problem likely arose from the cell line, not the viral constructs. Another reason it was probably the cell line and not the viral constructs was that all clones had the same genome from TN6R-NL and TN6G-NL, this is where the genes for replication are contained. Because G33 and R33 could replicate, it shows that this portion of the viral clones was functioning.

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