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Expression of HIV-1 Gag and Env Genes Using the Vesicular Stomatitis Virus Vector System

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Abstract

Traditional vaccine methods have long been employed to control widespread infectious diseases, but so far, all commercially available vaccine strategies have been inadequate in efforts to develop an effective therapeutic HIV vaccine. However, recent advancements in immunological research have lead to the generation of novel vaccine strategies, one of which is the recombinant virus vaccine, a method of particular interest that has shown promise in the clearance of HIV infection within HIV-positive patients who have retained immunocompetence. This study examined the stability of the expression of HIV-1 genes, *gag* and *env,* through a recombinant virus vector, a recombinant vesicular stomatitis virus (VSV), a temperature-sensitive mutant genetically modified to contain the select HIV-1 genes (VSV_{Ind}(GML)HIV-1*gag-env*). For SDS-PAGE, incubation was at 31 °C and 37 °C, the permissible and semi-permissible growth conditions respectively for VSV_{Ind}(GML) temperature-sensitive mutants. Western blot analysis was used to quantitate levels of protein expression of full VSV proteins, Gag and Env, using a primary rabbit antibody of anti-VSV anti-serum and a secondary anti-IgG from rabbit, a primary antibody of anti-p24 anti-serum and a secondary anti-IgG from rabbit, and a primary goat antibody of anti-gp120, and a secondary anti-IgG from goat. Results indicated that the VSV_{Ind}(GML) vector system allowed for high levels of expression of HIV-1 *gag* and *env* genes. It is known that the expression of these genes induces the production of major neutralizing antibodies and the stimulation of cytotoxic T lymphocytes, revealing the potential to use a genetically modified recombinant VSV as a universal vector for the development of recombinant virus vaccines. Specifically, the VSV_{Ind}(GML) mutant vector is thus an attractive candidate for the viral vector of a therapeutic HIV vaccine system.

Key words: Recombinant virus vaccine/ vesicular stomatitis virus / HIV-1 gag gene / HIV-1 env gene/ HIV/AIDS

Background and Rationale

According to the World Health Organization report in 2013, HIV has infected over 70 million people and 36 million have died from AIDS-related illnesses since 1981. Additional reports from United Nations AIDS disclosed that 2.3 million were newly infected in 2012, adding to a total of 35.3 million people diagnosed with HIV infection as of 2013. HIV/AIDS is a rising world epidemic and our search for a method to control the spread of this disease has presented one of the most challenging problems of the past three decades.

Vaccines are one of the most effective methods of controlling infectious disease and thus a central focus for HIV treatment has been on efforts to develop an effective therapeutic vaccine that will clear HIV infection. There are three types of commercially available vaccine strategies that are used to prevent many different viral diseases. These include the live-attenuated virus vaccine, such as the measles and mumps vaccines, the killed whole-virus vaccine, which has been used to prevent influenza virus and hepatitis A virus infection, and subunit vaccines, such as human hepatitis B virus and human papilloma virus vaccines. However, these three conventional vaccine strategies have so far proved inadequate for use as therapeutic HIV vaccine systems, effectively demonstrating our limited and largely empirically-based knowledge of these technologies and our lack of a comprehensive understanding of the immunological principles underlying widespread infectious diseases such as HIV (Luo, Li, & Kang, 2003; Hanke, 2001; Nature, 2009). Recent advancements however, have resulted in the generation of novel vaccine strategies, one of which is the recombinant virus vaccine, a method that has shown to be

promising in clearance of HIV infection within HIV-positive patients who have retained immunocompetence.

Recombinant virus vaccines depend on the use of a viral vector to carry genes of interest, such as HIV genes, and express them in the body to activate CD8 positive cytotoxic T lymphocytes (CTLs). Development of a therapeutic HIV vaccine to clear persistently infected cells centers around the stimulation of adaptive host immune responses, in particular, the induction of antibody-dependent cellular cytotoxicity and the activation of viral-specific CTLs to destroy and clear HIV-infected cells (Kang, Luo, Wainberg, & Li, 1999). In particular, CTL response is considered a major immune defense mechanism required for recovery from nearly all viral infections (Luo *et al.*, 1998). It is not desirable to use live HIV for CTL activation, but through a recombinant virus vaccine, it is possible to express selected HIV genes, which will activate HIV-specific CTLs.

The stimulation of HIV-specific CTLs also depends on the HIV genes chosen for expression through the recombinant viral vector. HIV is a retrovirus with a genome expressing three major structural proteins, Gag (capsid protein, matrix protein), Pol (reverse transcriptase, RNase H, Integrase, protease) and Env (gp120 and gp41) (Goff, 2006). Particularly relevant are HIV-1 *gag* and *env* genes of which can play critical roles in the induction of adaptive-host immune responses (Kang, Luo, Wainberg, & Li, 1999). Gag, group-specific antigen, facilitates viral assembly and maturation (Ono, Orenstein, & Freed, 2000). When *gag* is expressed without *pol* in recombinant virus-infected cells, the precursor Gag55 assembles virus-like particles (VLPs) that are non-infectious and mimic HIV virions (Luo, Li, Dales, &, Kang, 1994). *Env*, envelope, comprises the Gp120 and Gp41 glycoproteins present on the surface of HIV as a trimer (Wu *et al.*, 2009). Env precursor protein, Gp160, is synthesized in the endoplasmic reticulum and

then transported to the Golgi where it is cleaved into Gp120 and Gp41 by cellular proteases. The surface protein, Gp120, attaches to the cellular receptors, CD4 and chemokine receptors, of susceptible cells to initiate infection. In a study performed by Luo *et al.* (1998), it was found that HIV-1 Gag particles carrying multiple T-cell epitopes of Gp120 induced strong CTL responses. In a related study, Kang *et al.*, (1999) demonstrated that HIV-1 *gag-env* VLPs resulted in high CTL activity. HIV-1 *gag* and *env* genes have thus been identified as strong candidates for use in an HIV/AIDS vaccine system and are therefore the genes of interest for this experiment.

In this study, the viral vector selected for HIV-1 gene expression was an attenuated recombinant vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family. VSV infects cattle, horses, and swine and, in humans, infection results in asymptomatic or mild flu-like symptoms such as fever and malaise (Wu, Kim, & Kang, 2009). VSV has a negative-sense RNA genome containing 11,161 nucleotides, which encode five major viral structural proteins, the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the large protein (L) (Wagner, 1987; Lyles & Rupprecht, 2006; An, Kim, Wu, & Kang, 2013).

VSV presents an attractive viral vector system due to its wide host range, rapid replication processes, and mild pathogenicity in humans (An *et al.*, 2013). Kim and Kang (2007) found that mutations introduced into the *M* gene of VSV result in an attenuated virus that exhibits reduced inhibitory effects on host cell gene expression and results in significantly less cytopathic effects within infected cell lines, a finding allowing for the production of avirulent and temperature-sensitive VSV mutants. Most valuable is the existence of VSV in two serotypes; Indiana (VSV_{Ind}) and New Jersey (VSV_{NJ}). Wu *et al.* (2009) found that both serotypes have the capacity to stably express foreign genes and exhibit no cross-neutralization, revealing the potential for use of both VSV serotypes in a prime and boost method of vaccine delivery.

In order for the recombinant VSV vector to be used for an effective therapeutic HIV vaccine, the VSV vector system must be capable of stably expressing the HIV genes carried, an issue appraised in this study. In this research, I evaluated the expression of HIV-1 *gag* and *env* genes by employing VSV_{Ind}(GML), an avirulent and temperature-sensitive mutant vector system. High levels of HIV-1 *gag* and *env* gene expression by VSV_{Ind}(GML) indicate the potential for use of this viral vector system to activate CTLs and therefore confer immunity against infection through a therapeutic HIV vaccine. In this study, I describe the construction of the recombinant VSV_{Ind}(GML) vector, the recovery of the recombinant VSV_{Ind}(GML) mutant containing HIV-1 *gag* and *env* genes by reverse genetics, and the quantitation of levels of protein expression in cells infected with the recombinant VSV_{Ind}(GML) containing *gag* and *env* genes.

Hypothesis

It was hypothesized that the recombinant VSV_{Ind}(GML) vector would successfully express HIV-1 *gag* and *env* genes based on the reputation of the recombinant VSV as a tolerant viral vector that has already been identified as having the acute ability to effectively express a range of foreign genes, such as those of Marburg and Ebola viruses (Mire *et al.*, 2012). Quantitating the expression levels of HIV-1 Gag and Env proteins through the VSV_{Ind}(GML) vector system will indicate the potential for application of a recombinant VSV mutant as a major player in prospective therapeutic HIV vaccine development.

Experimental Methods

(1) Plasmid Construction

The recombinant VSV_{Ind}(GML)HIV-1*gag-env* vector was provided for this experiment; independent construction of the vector was not performed. However, the method of recombinant vector construction needs to be briefly addressed for clear understanding of the results. VSV_{Ind}(GML), an avirulent and temperature-sensitive mutant, was produced in the Kang Laboratory through the introduction of mutations at G21E, M51R, and L111A. Fusion of HIV-1 *gag* and *env* genes created a *gag-env* hybrid gene. The *gag* gene insert also contained 171 nucleotides that coded for T cell epitopes. The HIV-1 *gag-env* hybrid gene was inserted into the VSV_{Ind}(GML) genome between *G* and *L* genes of VSV by restriction enzyme Mlu1.

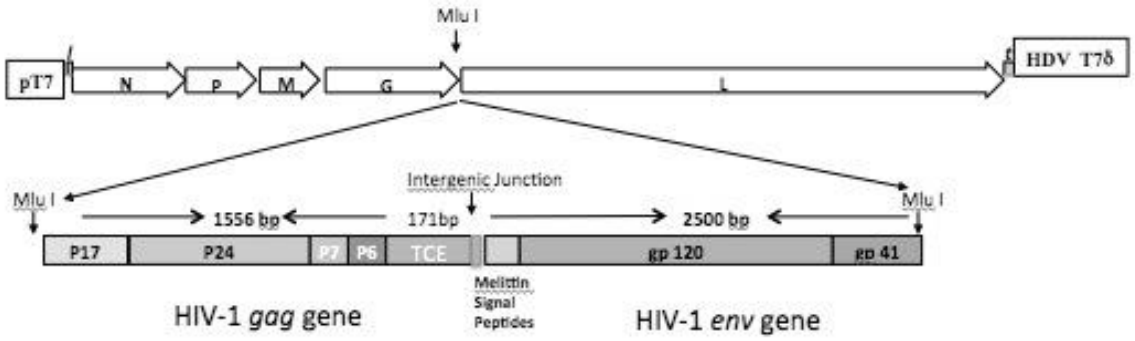

Figure 1. Construction of recombinant VSV_{Ind}(GML)HIV-1*gag-env*; insertion of HIV-1 *gag-env* hybrid gene into VSV_{Ind}(GML) mutant genome by restriction enzyme Mlu1. TCE (T cell epitopes from gp41, gp120, and Nef) was attached to the C-terminus of Gag protein. The *env* gene contains the honeybee melittin signal peptide gene. The total *gag-env* gene insert is approximately 4,300 nucleotides.

(2) Cell culturing

Baby Hamster Kidney21 (BHK21) cells were used to amplify recombinant VSV_{Ind}(GML)HIV-1*gag-env*. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5.0% fetal bovine serum (Gibco BRL), antibiotics penicillin (100 μg/mL), streptomycin (100 μg/mL), and kanamycin (100 μg/mL), and L-Glutamine (2 mM). The cells were incubated in a 5.0% CO_2 incubator at 37 °C for 17 hours and passaged when 100% confluent.

(3) Transformation of *E. coli* DH5α

Competent Cell Preparation

E. coli DH5α colonies were streaked on a SOB agar media without antibiotics and incubated overnight at 37 °C. Five isolated colonies were transferred into 100 mL of SOB liquid media without antibiotics and grown using a shaking incubator set to 210 rpm at 37 °C. Cells were recovered by centrifugation at 4500 rpm at 4 °C for 10 minutes.

Transformation

E. coli DH5α cultures were first transformed with the plasmids N, P, L, and VSV_{Ind}(GML)HIV-1*gag-env* according to the Molecular Cloning Manual (Sambrook & Russell, 2001). Plasmids diluted to 1 ng/μL were added into individual 200 μL aliquots of competent cells. A heat shock of 42 °C was then administered. Cells were shaken at 210 rpm and incubated at 37 °C for 1 hour. 200 μL of the transformed cells were spread onto lysogeny broth (LB) agar media with antibiotics and incubated at 37 °C.

(4) DNA extraction by maxi-preparation

E. coli DH5α colonies transformed with plasmids N, P, L, and VSV_{Ind}(GML)HIV-1*gag-env* were screened for successful recombination. Positive clones were confirmed using restriction enzyme mapping with digestion by enzyme, Mlu1. Three transformed colonies for each plasmid were subcultured individually in 2 mL of LB with ampicillin and shaken at 210 rpm at 37 °C for 17 hours. The subcultures were recovered by centrifugation at 5000 rpm at 4 °C for 10 minutes. The plasmid DNAs were purified and amplified to large quantities by the MAXIprep protocol (Sambrook & Russell, 2001) using Solution I [50 mM glucose, 25 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0) with the addition of lysozyme [1 mg/1 mL], Solution II [1% SDS, 0.2 M

NaOH], and Solution III [7.5 M ammonium acetate]. DNA gel electrophoresis was performed to run extracted plasmid DNA alongside control plasmid DNA to verify successful extraction from bacterial cultures.

(5) Recovery of VSV_{Ind}(GML)HIV-1*gag-env* by reverse genetics

Transfection was performed using BHK_{T7} cells and Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications in order to recover the recombinant VSV_{Ind}(GML)HIV-1*gag-env* by the VSV recovery protocol (Kim & Kang, 2007). Confluent BHK_{T7} cells were transfected with 19.4 μg of the plasmid containing VSV_{Ind}(GML)HIV-1*gag-env* and co-transfected with 10 μg of pKS-HP, 10 μg of pKS-HN, and 5 μg of pKS-HL plasmid DNA. VSV-specific RNA polymerase was expressed through transcription of N, P and L plasmid DNA by T7 RNA polymerase and N, P, and L mRNAs were translated by Internal Ribosome Entry Sites (IRES). VSV_{Ind}(GML)HIV-1*gag-env* was transcribed and replicated by VSV-specific RNA polymerase (N, P, L), trimmed by HDV ribozyme, and assembled as the recombinant virus. Incubation was at 31 °C in a 5% CO_2 incubator because the recombinant VSV_{Ind}(GML)HIV-1*gag-env* is a temperature-sensitive mutant. The culture media was harvested when 80% of cells showed cytopathic effects (CPE), observed 96 hours post-transfection.

(6) Purification and determination of viral titer by plaque assay

Recovered virus, VSV_{Ind}(GML)HIV-1*gag-env*, was purified by three times plaque picking on BHK21 cells. The viral titer was then determined by a plaque assay in order to administer an infection at multiplicity of infection (MOI) of 0.1 to propagate the recovered

VSV_{Ind}(GML)HIV-1*gag-env* and attain a viral stock. Recovered VSV_{Ind}(GML)HIV-1*gag-env* was first diluted 10^{-4}, 10^{-5} and 10^{-6} times. A 6-well plate of confluent Vero cells was inoculated with 100 μL of each dilution and incubated at 37 °C for 17 hours. The resulting plaque numbers were counted and determined the virus titer. Confluent BHK21 cells were infected at an MOI of 0.1 with serum-free DMEM for propagation to attain a viral stock. A second plaque assay titration was performed repeating the same procedure in order to determine the viral titer of the viral stock for infection at an MOI of 6 to prepare the cell lysate for SDS-PAGE and Western blot analysis.

(7) SDS-PAGE and Western blot analysis

Infections at an MOI of 6 of confluent BHK21 cells were performed with VSV_{Ind}(GML)HIV-1*gag-env* and VSV_{Ind}(GML) without an insert as a negative control. Incubation was at 31 °C and 37 °C, the permissible and semi-permissible growth conditions for VSV_{Ind}(GML) temperature-sensitive mutants. The protein in the cell lysate obtained from these infections was quantitated by UV spectrophotometry. The infected cell lysates were used to perform an SDS-PAGE followed by Western blot analysis. Blotting was performed using TBS-T buffer (0.1% Tween). Detection during Western blotting was performed to quantitate the expression of VSV proteins, Gag, and Env consecutively. For detection of VSV protein expression, a primary rabbit antibody of anti-VSV anti-serum was used with a secondary anti-IgG from rabbit. Detection of Gag expression was performed with a primary rabbit antibody, anti-p24 anti-serum, and a secondary anti-IgG from rabbit. Detection of Env was then performed with primary goat antibody, anti-gp120, and a secondary anti-IgG from goat.

Results

(1) Gel electrophoresis of extracted DNA by maxi-preparation

DNA gel electrophoresis was used to verify successful extraction of each plasmid. DNA gel electrophoresis was performed with DNA plasmids N, P, and L (Fig. 1) and the recombinant vector, VSV_{Ind}(GML)HIV-1*gag-env* (Fig. 2). Samples of extracted DNA plasmids were electrophoresed alongside corresponding stock control plasmids. Electrophoresis results showed that the fragment sizes of extracted plasmids with marker plasmids were identical, indicating the successful extraction of these plasmids from bacterial cultures.

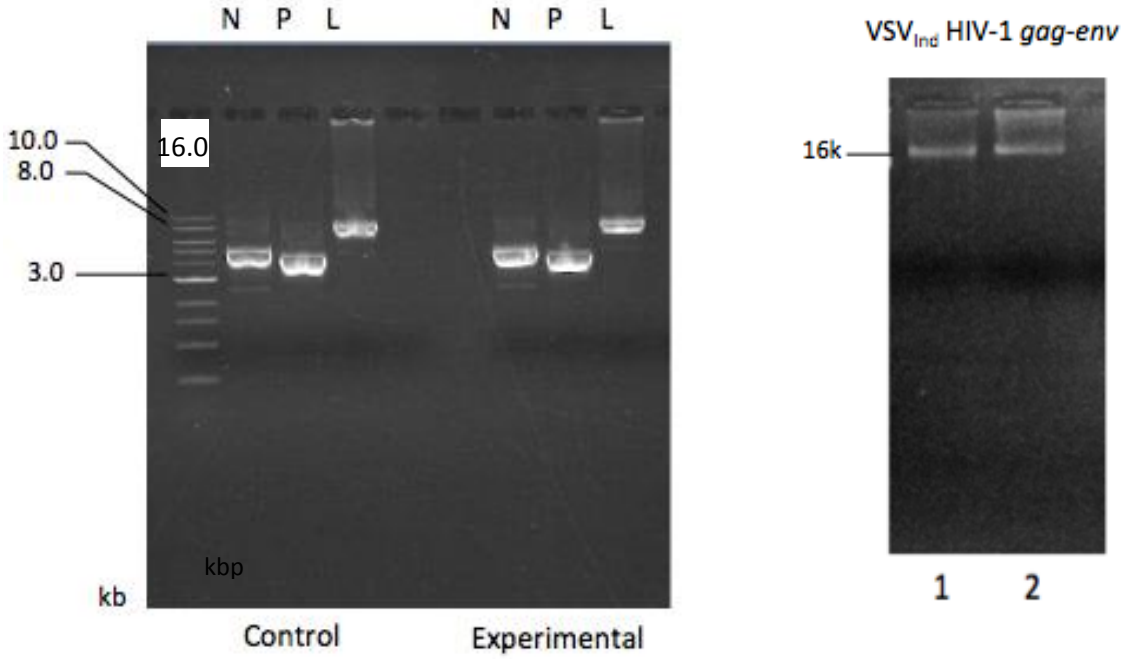

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Figure 2. DNA gel electrophoresis of control stock and extracted plasmid DNA of N, P, and L.

control stock and extracted plasmid DNA of VSV_{Ind}(GML)HIV-1*gag-env*; lane 1- control; lane 2- extracted.

(2) Recovery of VSV_{Ind}(GML)HIV-1*gag-env* by reverse genetics

Bacteriophage T7 RNA polymerase is constitutively expressed in BHK_{T7} cells and this RNA polymerase transcribes the transfected pVSV_{Ind}(GML)HIV-1*gag-env* cDNA genome and co-transfected N, P and L plasmids. An Internal Ribosome Entry Site (IRES) allowed translation of VSV N, P, and L proteins, the VSV-specific RNA polymerase. After initial transcription of the recombinant VSV_{Ind}(GML)HIV-1*gag-env* genome by T7 RNA polymerase, it was replicated and transcribed by VSV-specific RNA polymerase (N, P, and L proteins) and the recombinant

VSV_{Ind}(GML)HIV-1*gag-env* was assembled and released as a recombinant virus. Culture media containing the recombinant virus was harvested after observation of CPE in 80% of transfected cells 96 hrs post-transfection.

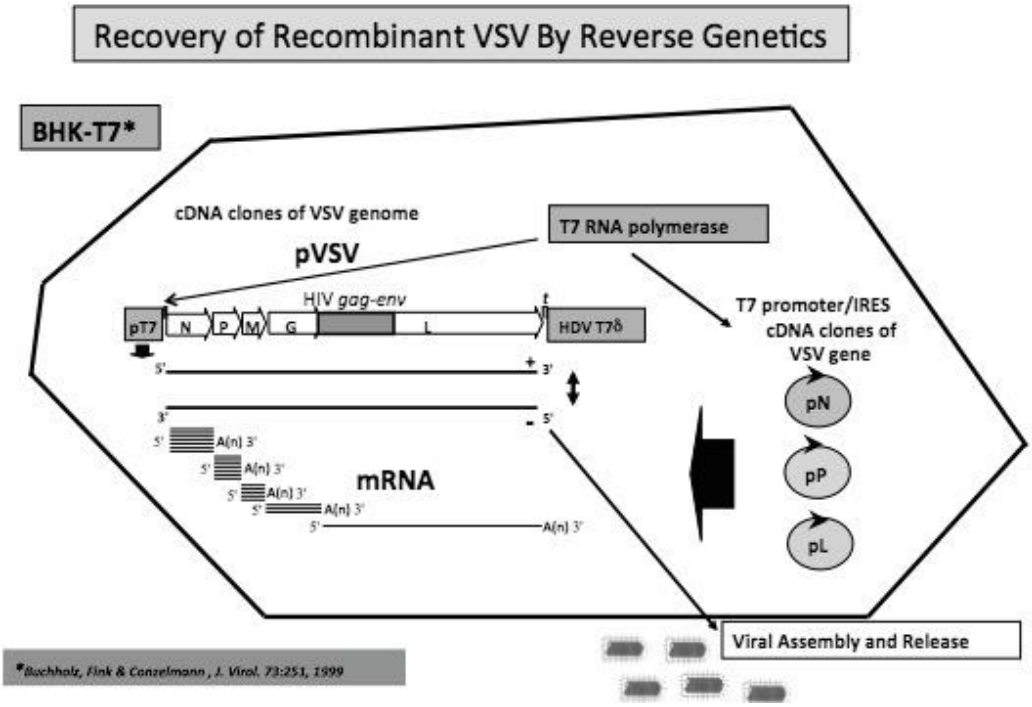

Figure 4. Recovery of VSV_{Ind}(GML)HIV-1*gag-env* using Reverse Genetics. BHK_{T7} cells are transfected with pVSV_{Ind}(GML)HIV-1*gag-env* and co-transfected with pN, pP, and pL. Culture media was harvested from transfected cells after observation of 80% CPE (Buchholz, Fink & Conzelmann, 1999).

(3) Determination of viral titer

The recombinant virus was propagated by infection at an MOI of 0.1. The viral titer of the recombinant virus was determined by plaque assay on Vero cells. This procedure was repeated to determine the viral titer of the amplified recombinant virus for a second infection of BHK21 cells at MOI of 6. Resulting plaques and calculations for the determination of the viral titer for the recovered virus and the viral stock are shown in Table 1.

Table 1

Determination of Viral Titer of VSV_{Ind}(GML)-HIV-1gag-env

For Infection	Counted Plaques	Averaged Plaques	Account for Dilution Factor	Account for Inoculation (100 μL)	Virus Titer (pfu/mL)
MOI 0.1	22, 23	22.5	$\times 10^6$	$\times 10$	2.25×10^8
MOI 6	22, 33	27.5	$\times 10^6$	$\times 10$	2.75×10^8

(4) Quantitation of levels of protein expression by SDS-PAGE and Western blot analysis

Virus-infected cell lysates were analyzed by SDS-PAGE followed by Western blot analysis for the quantitation of levels of viral protein expression. This was performed using cell lysate prepared from infections with VSV_{Ind}(GML) containing no insert and VSV_{Ind}(GML)HIV-1*gag-env* at 31 °C and 37 °C. Expression of HIV-1 Gag and Env proteins are marked by arrows in the following figures.

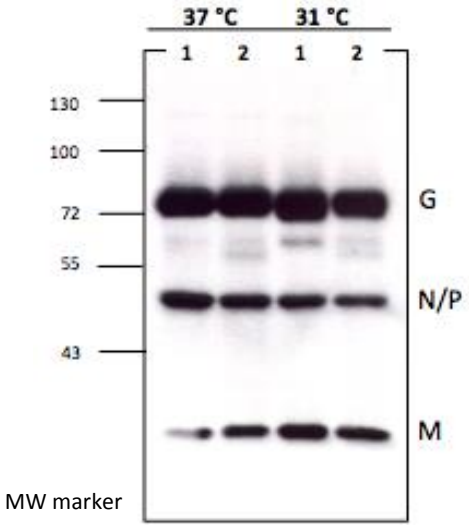

Figure 5. Expression of full VSV_{Ind} protein; lane 1- VSV_{Ind} no inserts, lane 2- VSV_{Ind}(GML)HIV-1*gag-env*

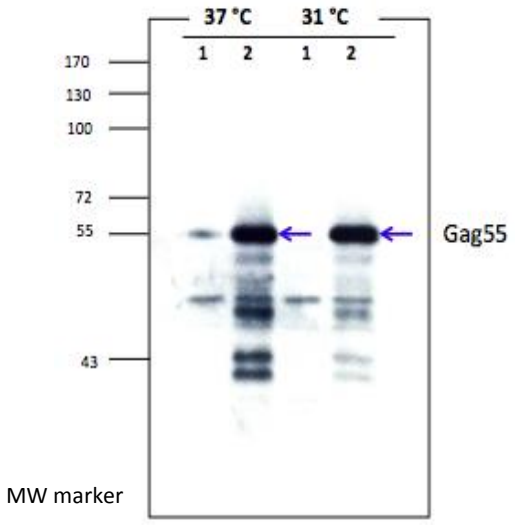

Figure 6. Expression of HIV-1 Gag55; lane 1- VSV_{Ind} no inserts, lane 2- VSV_{Ind}(GML)HIV-1*gag-env*

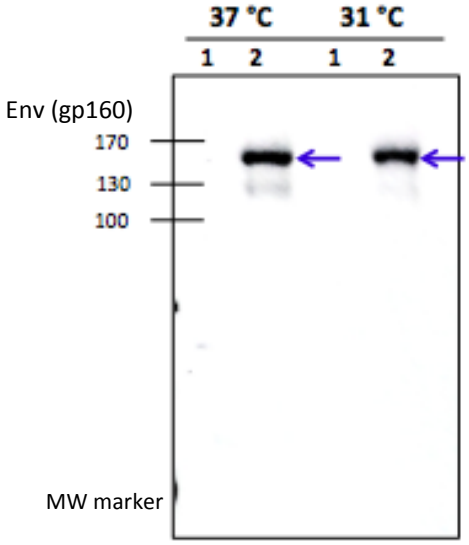

Figure 7. Expression of HIV-1 Env (gp160); lane 1- VSV_{Ind} no inserts, lane 2- VSV_{Ind}(GML)HIV-1 *gag-env*

Discussion

Results indicate that the VSV_{Ind}(GML) vector system allowed for high levels of expression of HIV-1 *gag* and *env* genes. This finding reveals the potential to use a genetically modified recombinant VSV as a universal vector for the development of recombinant virus vaccines. The VSV_{Ind}(GML) mutant vector is thus an attractive candidate for the viral vector of a therapeutic HIV vaccine. These major findings are concluded through the following analysis of results:

(1) Gel electrophoresis of DNA extracted through maxi-preparation confirmed successful extraction of pN, pP, pL, and VSV_{Ind}(GML)HIV-1*gag-env* from *E. coli* DH5α bacterial cultures. This was established from the results of gel electrophoresis shown in Figures 1 and 2, which depict identical fragment sizes for corresponding control and experimental plasmids run for each DNA isolate.

(2) Recovery of VSV_{Ind}(GML)HIV-1*gag-env* using reverse genetics was accomplished with the harvest of culture media containing the recombinant virus after observation of CPE in 80% of transfected BHK_{T7} cells 96 hours post-transfection. Longer incubation time was required before infected cells exhibited CPE because VSV_{Ind}(GML) is an attenuated, avirulent mutant.

(3) The results acquired from SDS-page and Western blot analysis indicated that VSV_{Ind}(GML) containing no insert and VSV_{Ind}(GML)HIV-1*gag-env* both showed the same levels of VSV_{Ind} protein expression at both permissive and semi-permissive temperatures, indicating that the stability of VSV_{Ind} was not compromised by HIV-1 gene insertion. The high levels of expression of both HIV-1 Gag and Env proteins by VSV_{Ind}(GML)HIV-1*gag-env* demonstrates that the VSV_{Ind}(GML) vector system is capable of stably expressing HIV genes *gag* and *env*, revealing the potential of VSV_{Ind}(GML) for use in a recombinant virus vaccine system.

In summary of the experimental procedures performed, the major steps of this study comprised of vector construction with the insertion of an HIV-1 *gag-env* hybrid gene into the

VSV_{Ind}(GML) genome, recovery of the recombinant virus by reverse genetics, and quantitation of levels of protein expression in infected cell-lines. The procedures performed were successful for obtaining the results needed to make conclusive evaluations of the VSV_{Ind}(GML) vector system.

Similar results were shown in a study performed by Rose *et al.* (2000), who demonstrated the use of a recombinant VSV to effectively express HIV-1 genes through the sequential boosting of mice with recombinant VSV vectors encoding the HIV-1 Env protein. Results revealed a great increase in the production of HIV-specific neutralizing antibodies. CTL activation was not evaluated, but, a parallel study performed by Egan *et al.* (2004), demonstrated a significant increase in CTL response in macaques with vaccination using recombinant VSV-based vectors expressing HIV-1 Env and simian-human immunodeficiency virus (SIV) Gag proteins. The observations presented by these studies determined the same conclusions discussed, identifying the ability of a recombinant VSV vector to express and process HIV-1 Gag and Env proteins. Such results have also been expanded upon in a study performed by Wu, Kim, & Kang (2009) who, in addition to highlighting the capability of the VSV_{Ind}(GML) vector system to express HIV-1 genes, found that its counterpart, the VSV New Jersey serotype, also possesses the ability to express HIV-1 gene, *env* (gp160), a finding that can be used in conjunction with this study's results to conclude the capability of both serotypes of VSV to express inserted HIV-1 genes. This suggests the potential of employing both VSV vector systems in the development of priming and boosting vaccines, using the VSV expression system to activate CD8 positive CTLs.

To conclude, VSV_{Ind}(GML)HIV-1*gag-env* is capable of expressing HIV-1 Gag and Env proteins at both permissive and semi-permissive temperatures. For application to a recombinant virus vaccine system, it is desirable to develop a RNA virus vector capable of

accommodating large foreign genes, allowing high levels of foreign gene expression. Therefore, these positive results reveal the potential use of VSV_{Ind}(GML) as an efficient viral vector system for a candidate therapeutic HIV vaccine to clear chronic HIV infection. This presents optimistic prospects for the development of a vaccine system that can substitute the high prices of anti-retrovirus therapeutic drugs with a cost-effective immunotherapy that will help clear infection from millions of people diagnosed with HIV.

Future Work

Future research currently focuses on the application of recombinant virus vaccine systems for the treatment of other infectious diseases such as the hepatitis C virus and even bacterial diseases such as tuberculosis. The results from this experiment suggest that additional projected studies of protective immune responses in animal models and the evaluation of the side-effects of recombinant virus vaccine systems may be beneficial to expand our knowledge of the immunological mechanisms underlying viral infections so that it may be possible to develop more effective and economic anti-viral therapies.

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