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CREATION AND CHARACTERIZATION OF VZV RECOMBINANT STRAIN ORF7Δ IN A NOVEL RFP-ORF23 FUSION BAC CONSTRUCT

By

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ABSTRACT OF THE THESIS

Creation and characterization of VZV recombinant strain ORF7Δ in a novel RFP-ORF23 fusion BAC construct

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The Alphaherpesvirus varicella zoster virus (VZV), while widely vaccinated for in the United States, still remains an important human pathogen. The bacterial artificial chromosome method (BAC) was used to make a deletion mutant of the ORF7 protein, implicated in the observed tissue tropism exhibited by VZV, in an RFP-ORF23 fusion BAC. For this experiment, a novel double-selection cassette using galactokinase and zeocin as markers was developed to attempt to circumvent the shortcomings of single selection additions to a BAC. Red fluorescent protein coupled to ORF-23, a viral tegument protein, was used to confirm positive infection of ARPE cells, and should allow direct visualization of the viral life cycle with fluorescence microscopy illuminating where in VZV's life cycle ORF7 is required for viral release. The virus exhibited a slow-growing phenotype typical of ORF7 deletion mutants in relation to wild-type. Further research will aim to produce a suitable vaccine strain called ORF7S, and visualize this clone as it moves through differentiated neuronal cells.

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Introduction

Varicella-zoster virus (VZV) is a double stranded DNA herpes virus that is a member of the subfamily Alphaherpesviridae, along with herpes simplex viruses (HSV) one and two. The Alphaherpesviruses share homology in their virion structure as well as their genome organization, and are also characterized by their ability to remain latent and cause infection after initial acute infection. Many of the open reading frames of herpes simplex viruses have been used to infer the function of those present in VZV. VZV is also known by other names, such as herpes zoster, chickenpox/shingles virus, and human herpesvirus type three (1).

The history of VZV and humans goes back to ancient times, where multiple writings describe a chickenpox-like illness, caused likely by VZV or a related Herpesvirus (2). Arabic scholar Muhammad ibn Zakariya Razi, or simply Razi, set forth the first recorded comparison between the often confused measles and smallpox/chickenpox in his book al-Judari wa al-Hasbah (On Smallpox and Measles), noting: "Smallpox appears when blood 'boils' and is infected, resulting in vapours being expelled….The best thing to do during this first stage is to keep away from it, otherwise this disease might turn into an epidemic. (3)" It wasn't until 1767, however, that chickenpox was differentiated from smallpox by English physician William Heberden, who noted the mild disease chickenpox causes, while also observing that lifelong immunity is usually conferred after the initial infection (4). After it was found during the 19th century that chickenpox was caused by an infectious agent, the link between chickenpox and shingles was confirmed

during experiments in the 1920s and 1930s, in which children exposed to fluid from shingles blisters developed chickenpox. Jumping forward to 1986, after the important discoveries of early genetics and molecular biology, the full genome of VZV was sequenced (5). Since this time, VZV research has progressed rapidly.

Estimated from phylogenetic tree construction and analysis, the most recent common ancestor to VZV and HSV existed nearly 400 million years ago. Beginning with herpesviruses that infected ancient oysters and abalone, coevolution continued through amphibians and fish, to reptiles, birds, and mammals, which are the current hosts for the modern Herpesvirus family. It is theorized that VZV originated in an African primate 60 million years ago, and was carried out of Africa by ancient humans when they migrated elsewhere. The characteristic latency of VZV, which goes back to the virus that infected abalone, is thought to have evolved as a survival strategy in response to the small groups ancient humans were thought to travel in. Today, VZV is distributed into 5 clades, with clades 1, 3, and 4 corresponding to Europe and North America, clade 2 to Asia, and clade 5 to India. There are also 4 distinct genotypes that cannot be put into any of these clades. The homology within a clade is astonishing, with nearly 99.9% gene conservation within a clade, and 99.8% between clades (or a diversity of 1 base per 1,400) (6). The small amount of diversity is manifest through single nucleotide polymorphisms among VZV's open reading frames. Of these open reading frames, ORF62 is the most variable, and controls the regulation of immediate-early genes (7). The mutation rate for VZV is comparatively very slow, being estimated at $3.9 \times 10^{\wedge}{-9}$ substitutions per site per year

and a nucleotide diversity rating of 0.00063, nearly 10 times lower than HSV, and 40 times less than CMV (8).

Varicella-zoster virus has a double stranded linear DNA genome of close to 125,000 base pairs, which often circularizes upon infection. The genome is composed of 71 open reading frames, which have been characterized according to their dispensability in infecting human melanoma cells. About 44 of these open reading frames were found to be indispensable for growth in MeWo cells, 8 showed marked growth defects when absent, 4 contributed to tissue tropism, and 14 were found to be dispensable. Most of these open reading frames share sequence homology with those found in HSV, and their function in VZV has been inferred based on these homologies. The morphology of VZV is also similar to those of the other alphaherpesviruses (9). The nucleocapsid of the virus is 100 nm in diameter, icosahedral in shape, and consists of 162 capsomers (12 pentavalent and 150 hexavalent; T= 16). VZV also has a lipid envelope, consisting of the seven glycoproteins gB, gC, gE, gH, gI, gK, and gL. All of these glycoproteins are shared by HSV, but HSV has an additional glycoprotein not found in VZV (gD.) Between the nucleocapsid and envelope is an undefined layer called the tegument layer (1).

The virus initially enters a host through inhalation of virus particles shed from the skin lesions of infected people. Once the virus enters the new host's respiratory system, it is absorbed by the mucous membranes. The virus is carried to its first target cell, the skin, by T-cells, where the unenveloped virions spread from cell to cell. The VZV glycoprotein E shares structural similarities to nonviral receptors like Fc receptors and LDL receptors,

taking advantage of the trafficking systems they use to enter cells. Virions enter the cell via endocytosis into clathrin-coated vesicles. The naked nucleocapsids are transported to the nucleus, where the linear genomic DNA is injected and circularizes. The genes of VZV are separated by time of expression, and include the immediate-early genes (transcription factors) early genes (DNA replication), and late genes (structural/assembly associated). These genes are transcribed by the host RNA polymerase II, exported to the cytoplasm where they are translated, and brought back into the nucleus for nucleocapsid assembly. Nucleocapsids only acquire their glycoprotein envelope from epithelial cell endoplasmic reticulum, as skin cells lack the endosomal pathway that strips glycoproteins from the viral surface. Otherwise, the mature virions bud off the cell, causing lysis of the cell. After transport to epithelial cells, buildup of the enveloped virus causes the visible rash, which can act as a new source for aerosolized particles, which continue the infection cycle. While VZV lacks the latency associated transcripts (LAT's) produced by other herpesviruses, enveloped VZV virions are attracted to the mannose 6-phosphate receptors present on sensory neurons, due to the presence of mannose 6-phosphate in their lipid envelopes. From these cells, they travel to the dorsal root ganglia to establish latency. Normally these latent reservoirs are kept in check by the immune system, but can reestablish infection in at-risk populations (1, 10, 12).

The immune response the human body mounts to VZV was largely unknown until fairly recently, due to the highly cell-associated nature of VZV, and its difficulty infecting other species. However, the study of VZV's pathogenesis has taken advantage of the development of the SCID-hu mouse model, which attempts to replicate the actions of the

human immune system by grafting human immune cells into mice with severe combined immunodeficiency (11). Using this model, it has been demonstrated that upon initial infection, the innate immune system is the first to be mobilized. Natural Killer cells begin to produce the important antiviral cytokine interferon gamma, which induces the clonal expansion of antigen specific T-cells. NK cells can also directly lyse VZV infected cells by themselves. At the cellular level, VZV upregulates STAT3, a protein which induces production of the anti-apoptotic protein survivin. STAT3 also inhibits expression of interferon alpha and STAT1. Surrounding uninfected cells will conversely upregulate production of interferon alpha and STAT1, along with IFN-stimulated factors like PML. VZV also has host-suppression mechanisms that act to inhibit the action of MHC class 1 and 2 molecules in their antigen presentation of VZV proteins. In addition to the innate immune responses, perhaps the most important response is the reaction mounted by VZV-specific T-cells to prevent disseminated infection, demonstrated by the life-threatening Varicella infections in individuals with T-cell malignancies or abnormalities. These T-cells often recognize glycoprotein E or the immediate-early protein. The immunological memory tied to VZV, along with including the CD4+ and CD8+ T-cells, also includes B cells that can differentiate to secrete specific IgG and IgA antibodies targeting various VZV components (10).

Even with the rapid development of VZV research, and the development of vaccines and antivirals to treat both chickenpox and shingles, VZV remains an important human pathogen. VZV usually causes a mild disease in healthy persons, but complications can arise in the immuno-compromised, pregnant women, the elderly, and the very young.

Nearly 90% of Americans will either experience chickenpox or have been exposed to it by the time they reach adulthood. After an initial acute infection, consisting of the namesake rash along with low-grade fevers and malaise, VZV can establish a reservoir in dorsal root ganglion cells of the nervous system, where reactivation occurs in around 10-20% of these individuals (12). The resulting reoccurrence can manifest more serious neurological symptoms, such as facial paralysis, encephalitis, aseptic meningitis, various neuropathies, and others. While these complications are otherwise rare in the immunocompetent, the disease can be very debilitating in the aforementioned susceptible populations. As of this writing, there are two vaccines for preventing chickenpox and shingles, respectively. An attenuated strain of VZV is distributed by Merck as Varivax in the United States. It is often incorporated with the measles-mumps-rubella combined vaccine and given to children between twelve and eighteen months of age (14). Zostavax is one of the only vaccines approved by the FDA for the prevention of the reactivation of VZV in older individuals. It is, in effect, a larger dose of Varivax to elicit an effective immune response in adults. While the vaccine is recognized as safe by the Food and Drug Administration, it has a relatively low efficacy and reduces the incidence of VZV reactivation by only 51.3% (15).

While a great deal is known about other members of the Alphaherpesvirus family, many of the specific infection and reproduction mechanisms of VZV still remain unknown. Some of the functions of VZV are inferred from its sequence homologies to HSV. Through bacterial artificial chromosome technology, some of the possible functions of the VZV open reading frames have been hypothesized. Bacterial artificial chromosome

(BAC) technology uses a DNA construct derived from an F plasmid present in *E. coli* that allow for the insertion, deletion, and editing of DNA within the bacterium. The BAC includes elements that allow for the replication, maintenance, and passage from bacterium to bacterium of DNA inserts varying in size from 100 to 300 kilobase pairs. While the technology is best known for its role in genome sequencing (particularly in the Human Genome Project), it has also been used to study the open reading frames of viruses such as VZV. The genome of VZV can be inserted into a permissive strain of E. coli such as DY380, which in this case includes a temperature sensitive repressor that is removed at high temperatures to activate a lambda prophage recombination system. These cells are first made electrocompetent, so that DNA can be inserted by passing an electric pulse between the cells to allow DNA entry. Fragments with flanking homology sequences to the target site can be inserted and removed via this method. Modifications are generally made using a double selection system, using a negative selection marker. Screening of these transformants on permissive media allows for the selection of cells which will then be transformed with the desired insert. After the desired modifications have been made to the genome, the BAC DNA can be purified from the bacteria and used to directly infect target cells and produce virus for further study (13).

Using the BAC method, four ORFs were identified in VZV that were shown to be required to some extent for the tissue-tropism displayed by VZV (9). Further experiments concluded that one of these ORFs, ORF7, is required for the infection of both skin cells and neurons (16). ORF7 itself is localized to the golgi apparatus of infected cells, where it is possibly involved in viral packaging, assembly, and release.

Besides its involvement in VZV's tissue tropism, little is known about the function of the ORF7 protein. It is predicted based on homology to HSV gene UL-51 to be a nonessential tegument protein, with a gene size of 780 base pairs and a protein size of 29 kilodaltons. Deletion of the functional ORF7 results in decreased plaque formation as well as a decrease in overall virus titer (9, 16). With these characteristics in mind, along with the fact that ORF7 is required for the effective infection of VZV's target cells, ORF7 deletion mutants are being considered as possible vaccine strains for use in new vaccines.

The system used by Zhang et al involved using the *E. coli* strain DY380 harboring the VZV BAC. The first selection was performed with a kanamycin resistance cassette, and then counterselection accomplished using a zeocin resistance cassette inserted into the pGem-lox-zeo plasmid. The insert cassette was produced from this plasmid, and used for the counterselection step. The addition of cre recombinase would remove the zeocin resistance cassette located between the lox recombination sites, leaving the desired open reading frame replacement in the BAC along with the lox sites (9, 16, 17). While this made it effective in the study of VZV's open reading frames, the system would be incompatible for producing vaccine strains of VZV, as foreign DNA is not allowed to be present in any virus used in vaccines. The selection system was further refined by Tang et al, where the *E. coli* strain SW102 is used instead of DY380, along with a selection marker for galactokinase (galK). Counterselection is then achieved by growing bacteria on 2-deoxy-galactose-1-phosphate, which forms a toxic metabolite in any cells that retain

the galK insert (18). This system allowed for the first forays into utilizing so called "seamless" recombination for suitable vaccine strain production.

The use of the galK homologous recombination system is advantageous in that it allows for the incorporation of large-sized DNA inserts with selectable markers, which would otherwise be limited by the small inserts characteristic of approaches using restriction enzymes or other previously described methods. Describing the process as "seamless" refers to the fact that there is no need for removal of non-viral DNA from recombination target sites, as recombination is achieved by the homology arms present in the inserts. However, the process is not without difficulties. Bacteria are constantly changing and evolving, and loss of the counter-selection marker during negative selection can cause background colonies to form. Great care must also be taken to preserve aseptic conditions as allowable to prevent contamination. In addition, while the BAC construct is itself very stable, deletions can occur during counter-selection, which can lead to background colony formation. All colonies must be confirmed to ensure that a background colony is not used by mistake. The two-step selection process can also introduce a considerable time delay in experiment progress, as the *E. coli* can take up to three days to grow, if they grow at all (18, 19, 20). Using galK alone as a metabolic selection method is effective, but can this be made more effective by adding another selection mechanism? The *E. coli* strains DY380, and the derivative SW102, have special homologous recombination systems supplemented by proteins from bacteriophage lambda, allowing the insertion of DNA as long as the insertion site has homologous flanking sequences to the target site. The inclusion of the temperature sensitive repressor also permits precise regulation of

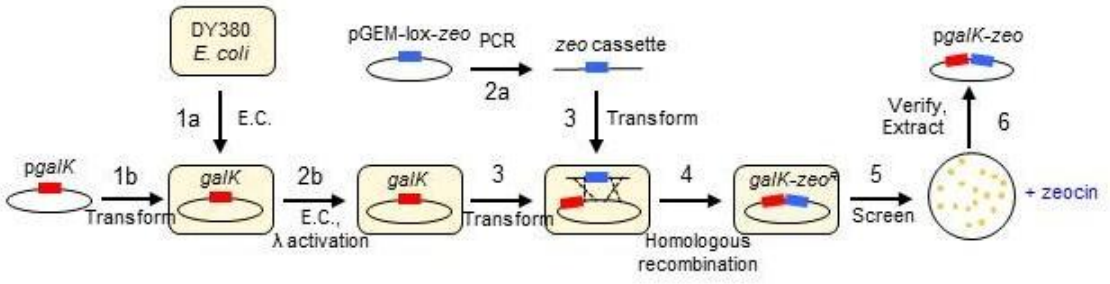
when recombination is activated in the bacteria. Using a derivative of this system, incorporating a newly developed galKzeo double selection marker, we aimed to resolve the difficulties that can be encountered using a single selection system to screen BAC alterations. In addition, the method can be used to achieve greater efficiency in creating vaccine-appropriate strains of VZV that integrate the ORF7 deletion mutant. For this experiment, the alterations were made to a VZV BAC which has RFP attached to ORF23, a virion component. ORF7 is thought to play a role in virion transport within the cell and release from it, thus attaching the locally expressed RFP to a virion component will allow direct visualization of the component by microscopy to confirm or refute the multiple theories of the role ORF7 plays in the VZV life cycle. Live video captured this way can provide an in-depth look at the movements ORF23 makes within the cell, up until the virus fails to mature and exit from the cell.

A previous study utilized an ORF23 gene with green fluorescent protein attached to its N-terminal end in an attempt to directly visualize wild-type VZV capsids. This was successful in showing that VZV virions travel in a retrograde fashion upon entry into the neuron, along the axon on its way to the nucleus. The experiment also showed the usefulness of using human embryonic stem-cell derived neurons for these types of studies. The study also elucidated what functions ORF7 does not contribute to in the infection cycle of VZV, by including in an ORF7 deletion BAC a separately encoded GFP, under an SV40 promoter. GFP encoded in this way causes the more general fluorescence of the entire cell instead of viral components upon infection. In this way, the experimenters were able to rule out two possible mechanisms ORF7 might affect. ORF7

deletion mutants were still able to infect axons, ruling out ORF7 as acting in initial entry, and these mutants were able to be transported to the nuclei of the cells along these axons. Retrograde motion to the nucleus seems not to be affected as well (21). The experiment narrowed the possibilities, but did not effectively pin down the phenotype caused by deleting ORF7. A similar experiment involving observing ORF7 cells in a similar way using RFP-ORF23 within an ORF7 deletion mutant should be useful in further elucidating the function of ORF7, and possibly lead to a better understanding of how exactly the ORF7 deletion mutant causes the observed phenotype in VZV, or point to experiments that can be done .

Materials and Methods

Creation of Plasmid Containing galKzeo Double Selection Marker



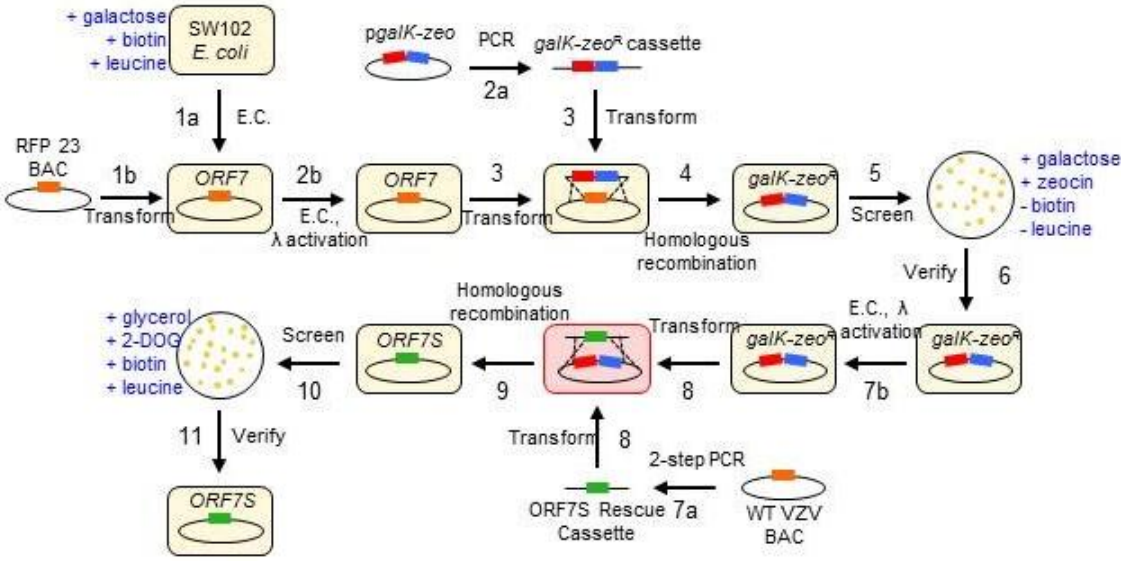
Starting with the pGEM-lox-zeo cassette, 1µg was digested with 1 µL of pvuII restriction endonuclease (New England Biolabs) at 37ºC for two hours. Then 4µL of EZ Vision III dye was added to the digestion tube and an electrophoresis was run on 0.5% agarose to separate the bands. The band ~1kb in size was extracted using the QIAquick Gel Extraction Kit (Qiagen, CA) and then further purified with the QIAquick PCR Purification Kit. The purified DNA fragment was then used as a PCR template that conferred sequences upstream and downstream of zeocin that are homologous to a region just downstream of galK in pgalK. The resulting cassette was purified by QIAquick PCR purification and 1µg was transformed into electrocompetent E. coli strain DY380 with its recombination system activated harboring pgalK. The transformants were screened by plating on LB agar containing zeocin (50µg/mL). Colonies were selected and plasmids were miniprepared using the QIAprep Plasmid MiniPrep Kit. The plasmids were confirmed by PCR using primers that anneal outside of the galK region on the pgalK backbone, in a fashion that allows for amplification of the region containing the zeocin resistant gene as well. DNA bands of ~1700 bp confirmed the successful cloning of zeocin resistant gene into the pgalK plasmid, whereas DNA bands of ~1200 bp in the pgalK PCR reaction served as a positive control for the primer pair used. One colony that

was confirmed was used to perform a maxipreparation of the plasmid DNA with the NucleoBond Xtra Maxi Kit (Macherey-Nagel).

Bacterial Strains

For preparation of the zeocin cassette from pGEM-lox-zeo, the plasmid was grown in *E. coli* strain DY380, provided by the Biological Resources Branch of NCI Frederick. Samples of *E. coli* strain GS1783 harboring the mred-23 BAC were received from Dr. Paul Kinchington of the University of Pittsburgh Medical Center; *E. coli* strain provided to him by Dr. Greg Smith of Northwestern University. These cells had their BACs extracted and transformed into *E. coli* strain SW102 according to Tang et al. SW102 is a modified DY380 strain which, including the lambda prophage recombination system, includes a defective galactokinase (galK) gene in the galactose operon, to effectively utilize galK selection. The resulting transformants were then prepared for electroporation as described below. The cells were also temperature induced to lift the temperature sensitive repressor, and thus activate the homologous recombination systems present in this bacteria. Cells were stored in a -80ºC freezer and thawed when ready for use.

Creation of ORF7S RFP-23 VZV BAC



Replacing ORF7 in RFP-23 VZV with galKzeo cassette

Preparation of Electrocompetent Cells/Recombination Induction

The following procedure was adapted from Tang, et al. with some alterations (18). A 5mL culture of SW102 containing the RFP-ORF23 BAC was first grown overnight in LB medium. The following day, the overnight culture was diluted 1:50 in a 50ml Erlenmeyer flask holding 25ml of LB. Some LB was saved as a blank for measuring OD600nm. This flask was incubated in a 32ºC shaking incubator until the OD600 was at least 0.6. A 10ml amount of the resulting solution was transferred to a 50mL flask, and heat-shocked at 42ºC for fifteen minutes to activate the recombination system of SW102. After fifteen minutes, the flask was cooled briefly on ice, the solution transferred to two 15mL Falcon tubes, and centrifuged at 4ºC and 5000 rpm for five minutes. The supernatant was poured off, and 10ml of cold ddH2O was used to resuspend the pellet by gently shaking the

Falcon tubes in the ice water bath. These tubes were then centrifuged again under the same conditions, the supernatant poured off, the pellet resuspended in another 10mL of cold ddH20, and centrifuged a third time. After this centrifugation, all of the supernatant must be removed by inverting the Falcon tubes on a paper towel. This pellet was then resuspended in 50µL of a 10% glycerol solution, and stored in a -80ºC freezer until ready for use.

Transformation of galKzeo Cassette by Electroporation

A 25µL amount of prepared electroporation-competent cells were thawed on ice, and added along with 10-30ng of either galKzeo cassette to a 0.1 cm cuvette (BioRad). Electroporation was carried out at 25mF, 1.8 kV, and 200 ohms, and pulsed for ~five milliseconds. After the reaction, 1 mL of LB medium was added to the cuvette to resuspend the cells, and the solution added to 10mL of LB in a 125mL Erlenmeyer flask and cultured for four and a half hours in a 32ºC shaking incubator. After incubation, 1mL of the culture was centrifuged at 13,200 RPM for fifteen seconds. The supernatant was removed by pipette, taking care not to disturb the pellet, and the pellet resuspended in M9 buffer and pelleted again in the same manner. The washing step was performed a second time, and then 100µL of the resuspended bacteria was plated onto plates containing galactokinase and zeocin. After being allowed to grow, sixteen colonies were chosen and re-streaked onto a plate with the addition of chloramphenicol. Six of these colonies were chosen for miniprep confirmation, of which a confirmed colony was then maxiprepped. Confirmation was performed by PCR amplification and gel electrophoresis screening using relevant primers.

Miniprep and Maxiprep of BAC DNA from SW102 Cells

Purified plasmid extraction and preparation were done according to the QIAprep Spin Miniprep protocal and Maxiprepped using the Nucleobond XtraMaxi protocol. BAC extraction and preparation was done according to either the Nucleobond XtraMaxi protocol, or miniprepared by the following method: 5mL of culture was first grown in a 32ºC shaking incubator overnight. The next day, 200µL of the culture was saved along with 50µL of 100% glycerol pending confirmation. The rest of the culture was centrifuged at 4500 RPM for ten minutes at 4ºC. The supernatant was removed, and the pellet resuspended in 1mL of RES buffer. Next, 1mL of LYS buffer was added, the tubes inverted four to five times to mix the contents, and were allowed to incubate at RT for less than five minutes. After waiting, 1mL of NEU buffer was added, the tubes inverted gently, and put on ice for a maximum of five minutes. This solution was then centrifuged for fifteen minutes under the above conditions. After filtering the resulting supernatant, an equal volume of phenol/chloroform was added under a fume hood, and the tubes were mixed by shaking. This solution was then centrifuged for four minutes under the same conditions, the upper layer transferred into a new tube, and two volumes of 100% ethanol added. This tube was allowed to sit at room temperature for five minutes before being centrifuged for thirty minutes. The supernatant was removed, the pellet dried, and the DNA resuspended in 20µL of H2O. The concentration could be taken at this time using a Nanodrop machine as well.

Generation of Virus from Confirmed galKzeo Clone

After purification of BAC DNA as described in the QIAprep Spin Maxiprep protocol, purified DNA was transfected into ARPE cells on six-well plates growing in Dulbecco's Modified Eagle Medium (DME), with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 0.9% amphotericin b, using the GeneExpresso DNA Transfection Reagent distributed by Excellgen, as per manufacturer's protocol. Infected cells were allowed to grow until sufficient plaque formation was observed, and were then transferred to a 20cm plate. Visualization was performed on an Olympus IX50 inverted microscope, and pictures acquired with an attached camera. One photo was taken with fluorescence, and one without. These photos were overlaid on each other using Photoshop. Once this culture becomes around 25% infected, a cell-free powder would be prepared of the resulting virus, and saved until generation of the ORF7S clone, or confirmation of ORF7 protein absence by IFA or Western blot.

Confirmation of ORF7 Protein Absence

Due to the ORF7 deletion phenotype producing both a decreased viral titer as well as decreased plaque area, confirmation of successful infection by the resulting virus can be confirmed qualitatively by direct observation, and quantitatively by growth curve analysis. While Selariu et al took advantage of BAC constructs that incorporated the luciferin protein to estimate virus number by photon count, the BAC used in this experiment does not have the luciferase reporting system (16). To perform a growth curve analysis, a known concentration of virus (usually 100pfu/well) is added to ARPE or SH-SY5Y diluted by an order of magnitude, down to 10^-5, in a six-well plate. Plaques must be manually counted, if possible, and recorded until viral growth levels out or

begins to drop. Standard deviations and error bars can also be approximated according to the estimated amount at higher titer levels vs. actual counted plaques.

An immunofluorescent assay will also be performed to confirm the absence of production of the ORF7 protein. Cells to be analyzed will have their media removed, their wells rinsed twice with PBS, and fixed with 4% paraformaldehyde in PBS at RT for 15 minutes. The cells will then be rinsed with PBST three times, and blocked in 1mL of 1% BSA PBST for thirty minutes at RT. The blocking solution is removed, first antibody added diluted 1:400, along with 0.1% BSA, and incubated for one hour at RT. Then, the wells are washed with PBST three times for five minutes each. The second antibody is added in a 1:4000 dilution along with PBST and 0.1% BSA and incubated for an hour again at RT. The wells are washed again for ten minutes three times, and a counterstain added to observe the reaction.

Additionally, a Western Blot will be executed to confirm the absence of expressed ORF7 protein. The reaction is run on a 10% SDS PAGE gel, and then transferred to a PVDF membrane and left overnight at 4ºC. The next day, the membrane is trimmed to the size of the gel, and incubated in 10% milk TBST for a minimum of one hour. The solution is then removed, and 5mL of 1% milk TBST along with Anti-ORF7 mouse antibody 8H3 diluted 1:200 added and incubated on a rocker for one hour. After the elapsed time, the membrane will be rinsed twice with TBST, then wash three times for five minutes each wash. Then, Anti-mouse IgG-HRP antibody will be added at 1:2000 dilution in 1% milk TBST and incubated on the rocker for forty five minutes. The membrane will then be

rinsed twice with TBST, five times for five minutes each wash, then once for fifteen minutes. All liquid is then removed and 1mL each of Western Lightning Chemiluminescence and Oxidizing Agent is added directly to the membrane. This liquid is removed and the membrane placed on saran wrap. The membrane is exposed for thirty seconds and film acquired.

Production of ORF7S Clone with Rescue Cassette

The ORF7S rescue cassette was purified from an ORF7S containing BAC by PCR and gel purified as previously described. ORF7S is ORF7 in which the 5^{th} codon is mutated from TGT to TGA, a stop codon (16). Before transformation, confirmed galKzeo clones must first be made electrocompetent again by the previously described method. These cells were subjected to electroporation with the ORF7S rescue cassette, incubated for four and a half hours, and plated onto plates containing DOG, which forms a toxic intermediate in any cells still harboring the galKzeo cassette. Colonies would be picked and confirmed by miniprep, one colony's DNA maxiprepped and transfected into cells, and virus produced for any of the other experiments, but mainly for use in vaccines due to the seamless recombination involved.

Infection and Imaging of SH-SY5Y Neuronal Cells

The SH-SY5Y cells must first be differentiated before they are transfected with the resulting virus. The method for differentiation is adapted here from Selariu et al (16). Cells were grown to 80-90% confluency and split to 6 well plates, with or without coverslips for IFA or growth curve analysis. The next day, the media is changed to

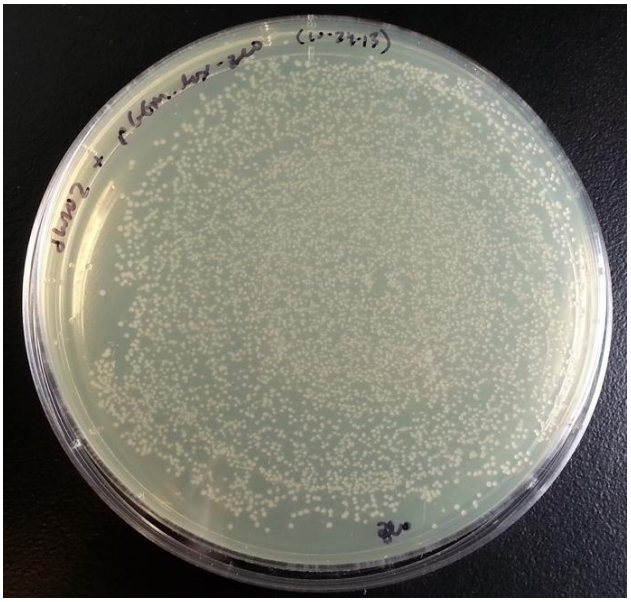
DME/F12, 5% FBS and 1% P/S. Two days later, the media is changed with the above formulation. In another two days, the media is removed, the wells washed with PBS, and DME/F12 w/o FBS, 1% P/S, nerve growth factor, and brain-derived neurotropic factor added. Three days later, the virus is introduced to the cells, and the media changed the next day.

Observation by Microscopy of Resulting Virus

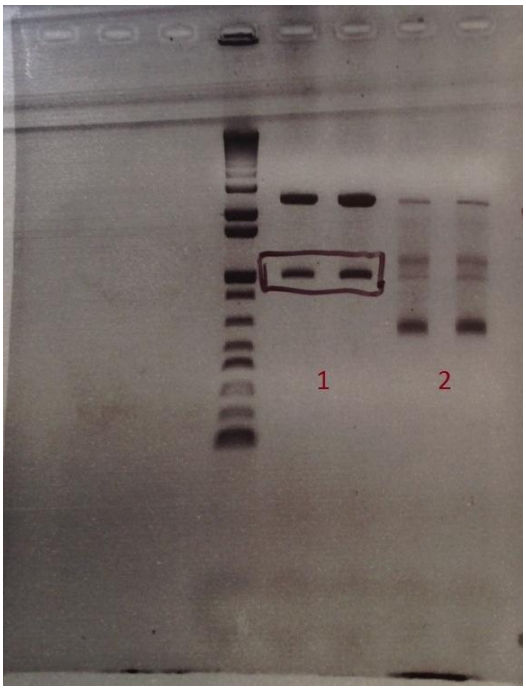
Live SH-SY5Y cultures would be observed and photographed using any microscope that has filters allowing the resolution of RFP (such as the Olympus IX70 inverted microscope used by Grigoryan et al). Digital images can be acquired with any camera that can be fitted to the aforementioned microscope. A microscope used in this manner would have a filter that allowed for the viewing of fluorescing virions. During recording, cultures would be maintained close to incubating conditions of 5% CO2, 37ºC, and 95% humidity. Kinetic analysis can be performed according to Grigoryan et al, analyzing acquired movies using the SpotTracker2D plugin for ImageJ, an image processor using Java. This allows greater contrast between separate virions. The software can also export kinetic data to programs such as Microsoft Excel for further analysis (21).

Results

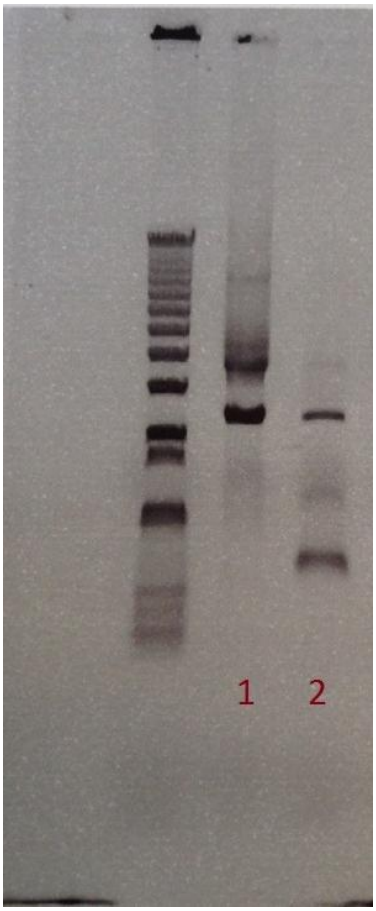
Confirmation and Production of the Zeocin Insert from pGem-lox-zeo



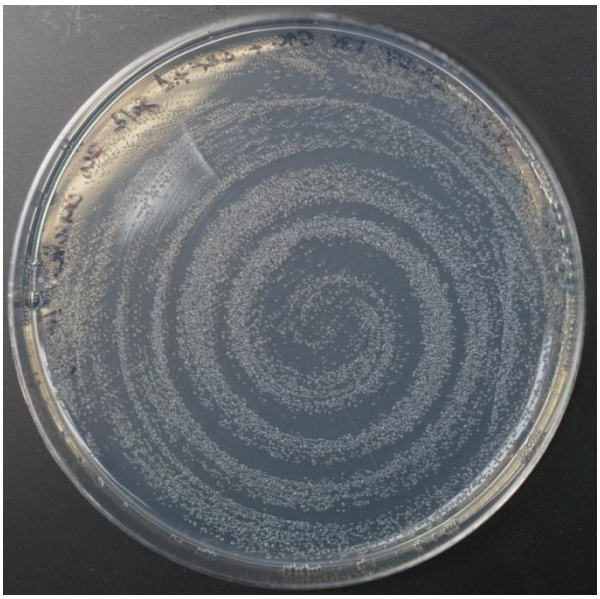
After confirmation, the zeocin fragment of the pGem-lox-zeo (shown as the circled fragments on the two lanes labeled “1” was excised, purified , and run on a gel to confirm the successful digestion down to zeocin (the lightest band on the bottom gel) and various plasmid fragments, shown as the two lanes labeled “2”.



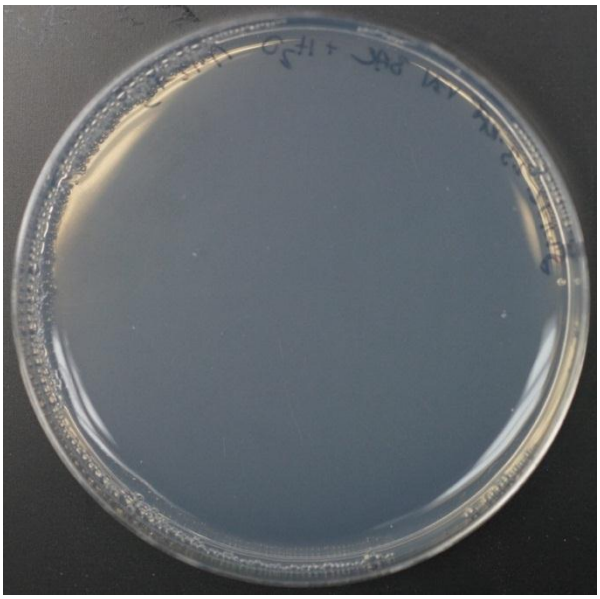
This fragment served as the template for the production of the cassette which was then inserted in front of galK to make the resulting galKzeo cassette. Successful ligation into the galK plasmid was also confirmed by gel, which is shown below.



The resulting galKzeo plasmid is shown here in lane “1”. This plasmid was given homology arms to ORF7 by using it as a PCR template in a similar fashion to the preparation of the zeocin cassette. After electroporation, incubation, and plating, along with a control, the following plates were obtained.

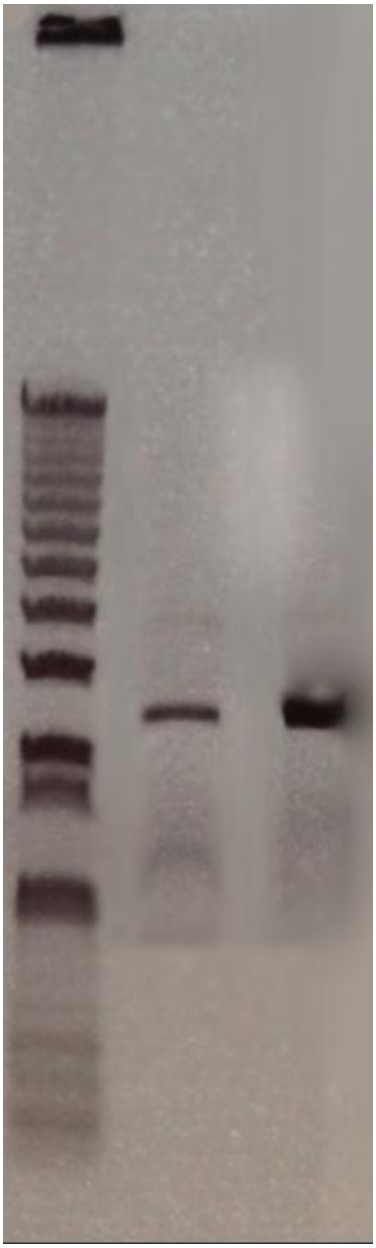


- Transformation plate

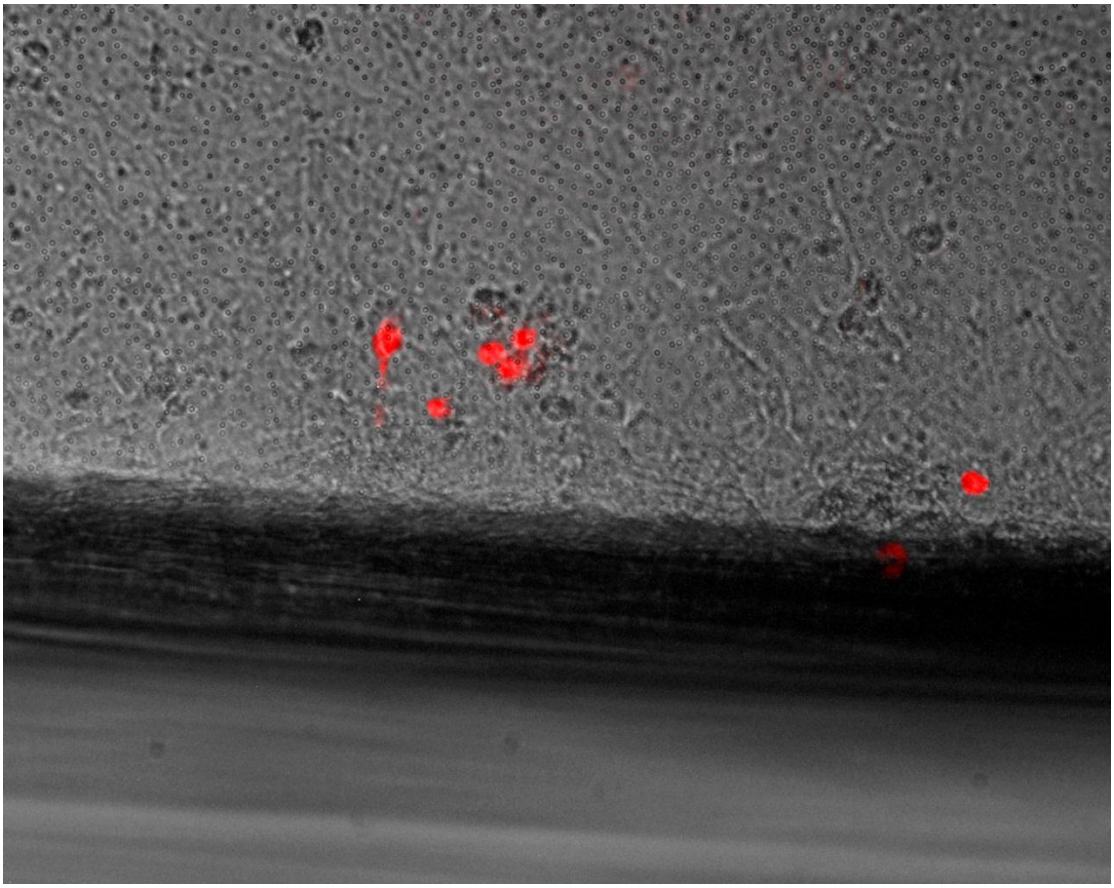


- Negative control

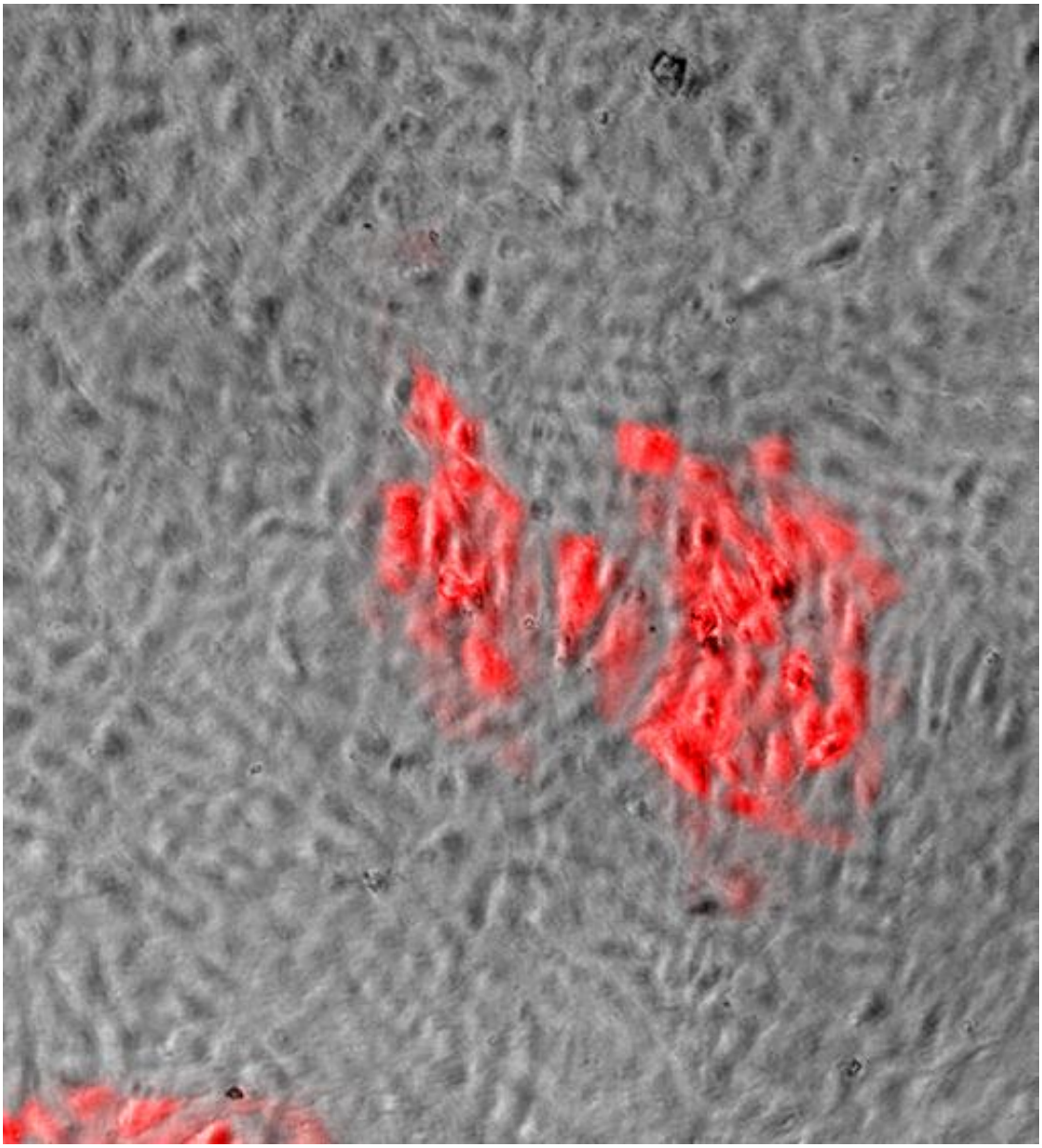
Unfortunately, this clone became contaminated before it could be further experimented with. As a result, a new clone was created the same way, with a further confirmation step involving re-streaking colonies acquired by an initial plating on galK and zeocin onto another plate containing galK, zeocin, and chloramphenicol. From this plate, six colonies were miniprepped, of which colonies 5 and 6 were confirmed. Colony 6 was maxiprepped, and the resulting DNA transfected onto ARPE cells in a 6-well plate, along with WT VZV maxiprep DNA and ORF23-RFP maxiprep DNA to compare.



Confirmation of the maxiprepped DNA showing amplified galKzeo insert compared to galKzeo plasmid as a control.



ARPE cells visualized nine days post infection. Both the fluorescent and non-fluorescent images were merged to show which cells are expressing RFP (positive sign of infection). Most infected cells were located on the edges of the plate. The leftmost virus shows a dendritic protrusion, common of VZV infected cells which may be involved in cell-to-cell transition. Infection in ORF7 mutants causes a small-plaque phenotype, and also results in slower growth rates compared to WT.



Same plate visualized ten days post infection. This image shows the formation of a viral plaque, which will continue to spread radially and lyse cells to release virus. In addition to plaque formation and the generation of dendritic projections in cells, syncytia can also form, as the result of the fusion of cells to form a multinucleated mass. This has not occurred on this plate yet.

Discussion

While the galK recombination system for BAC alterations is effective and has been shown to work, it is unreliable in the amount of background that can result from the often unpredictable variables that act on *E. coli* to cause failed experiments. In theory, providing another selection mechanism along with galK should suppress escape mutations by the product of the effectiveness of galK and zeocin screening alone. The galKzeo clone has been successfully produced experimentally, but the ORF7S clone has not been produced yet. The success of the galK model alone should infer the validity of replacing galK with the double selection cassette galKzeo, while producing fewer adverse effects and failed reactions. However, great difficulty presented itself during the course of experimentation. Perhaps the precise nature of the procedures used contributed to the difficulty of acquiring results. To begin with, electrocompetent cell preparation must be performed strictly according to protocol, or the cells will not effectively take up extracellular DNA. Preparation of insert cassettes can also be very sensitive to mistakes or outside contamination. During electroporation, an optimal pulse time of around 5 milliseconds is more conducive to successful insertion of cassette DNA. Once the cells are grown and plated on selection plates, the *E. coli* bacteria can spontaneously acquire mutations that allow them to survive selection. This possibility necessitates the lengthy confirmation process that involves extracting the bacterial DNA, amplifying it via PCR, and then running the product on the gel to confirm the presence of the desired clone.

To alleviate the frustration of attempting to produce an ORF7S clone, the galKzeo clone was used to produce a virus that is in effect phenotypically identical to an ORF7S virus,

as neither produce ORF7. The galKzeo clone is not suitable for use as a vaccine strain, however, as no foreign DNA should be present in the strain being used. The ORF7S strain that would be created using this method would be suitable, as the seamless recombination ensures that only the cassette is inserted (which is itself derived from VZV). Since the main objective of this experiment is to study the movement of an ORF7 deletion mutant in neurons, this should not be a problem, as there are already published methods for producing deletion mutants in the vaccine strain of VZV. However, if a vaccine is to be created, the galKzeo clone must go through electrocompetent cell preparation, transformation, confirmation, and virus produced if vaccine studies are to be performed.

The BAC used for the experiment does not have the luciferase reporting system that most other research BACs have, incorporating instead RFP attached to the virion component ORF23. While this does not allow growth curve analysis using the luciferase reporting system, it will permit the observation of virions as they move through the cell, possibly illuminating where in the virion-production cycle ORF7 might be involved. According to Selariu et al, the ORF7 protein might be involved in viral packaging and egress, as the protein localizes in the Golgi apparatus. They dismiss the possibility of ORF7 serving a role in virus entry into the cell, citing the strict cell-associated nature of VZV, especially in epithelial cells. It is entirely possible that VZV can be spread by both viral release and directly between cells, due to the mannose-6-phosphate receptors of most of VZV's target cells stalling virions in late endosomes. Eventual maturation of suprabasal epidermis keratinocytes causes them to lose these receptors, possibly allowing the release

of these particles. The hypothesis supported by Selariu et al is that ORF7 plays an integral role in viral egress, as deleting the homologous HSV protein (UL51) results in similar defects in the secondary envelopement of virions, instead causing them to build up in the perinuclear space of cells (16).

Along with this research, the experiments carried out by Grigoryan et al show that a virus containing GFP attached to ORF23 can be used to study viral kinetics (21). They also further narrowed the possible functions of ORF7. Their research can be considered a proof-of-concept for the experiments performed by our team using a similar RFP-ORF23 BAC. Manipulation of the viral BAC using the galKzeo double selection marker can allow this process to be expedited as needed, and its use is not limited to the experimental design presented here. Any BAC addition/deletion can take advantage of the galKzeo selection system as opposed to galK alone or using galK in conjunction with other antibiotic selection systems such as kanamycin or others. Zeocin and ampicillin resistance are the most commonly used selection markers that are used in our lab, but zeocin was selected for primarily for its availability in the pGem-lox-zeo cassette, as well as it being cost effective and proven in its use as a selection marker for molecular biology purposes.

As mentioned previously, the ORF7 deletion clone is a great candidate for a vaccine strain, as it may be able to elicit an immune response while not producing an effective infection in epithelial or neuron cells. Along with trying to elucidate the function of ORF7 itself, research is being done to improve the existing vaccine, which is distributed

by Merck. One of the avenues being pursued is to improve viral titers by reformulating the solution that the virus particles are suspended in in a vaccine. The current Merck formulation causes the loss of around half of the virions that are exposed, so various tweaks to this formula are being explored to try to improve viral titer in this way. Merck's formula includes the inorganic compounds sucrose, porcine gelatin, sodium chloride, monosodium L-glutamate, anhydrous disodium phosphate, potassium dihydrogen phosphate, potassium chloride, and sodium hydroxide, along with at least 19,400 PFU of VZV. The organic and inorganic portions are combined when ready to be administered, and the administered dose is 0.65 mL. ORF7 deletion mutants have not undergone any kind of human testing, but have been subjected to the freeze-thaw process and preserved in the Merck formulation as well as other viral protection solution formulas. Unfortunately, ORF7 deletion mutants are more affected by the freeze-thaw process than the vaccine strain pOka, meaning more virus must be present before cryopreservation to account for titer loss. This research has been put on hold in favor of using what is called a Lentiviral vector.

Viral vectors were first utilized in the 1970s, and have since garnered a great deal of research interest due to their ability to deliver genetic material to target cells. This system has been researched for possible use in vaccines and gene therapy, as well as for basic uses in research. Transfection is usually preferred due to the difficulty associated with creating viral vectors, although viral vectors can infect nearly 100% of cells without a significant loss in cell viability. Viral vectors are designed so that they are unable to replicate, are stable, can be cell specific, and have markers such as antibiotic resistance to

select for infected cells. Currently, viral-vectored gene therapies have not done well in clinical trials, due to their ability to cause cancer or a debilitating immune response, but adenoviral vaccines are actively being pursued. Lentiviral vectors are also being considered as well. The goal of these experiments is to develop a cell line that can continuously produce ORF7 for further study. This will involve infecting 293T (human embryonic kidney) cells with three separate plasmids, which will form a virus that will deliver the ORF7 gene into the genome of these cells. These plasmids are pVSV-G (which produces the viral envelope), pCMV.DR8.91 (required for viral packaging), and pLDT.ORF7 (the plasmid which has the desired gene). Additionally, each plasmid has an ampicillin resistance gene for screening while in bacteria, and pLDT.ORF7 includes a puromycin resistance gene for selecting in 293T cells (24). This experiment is still in progress at the time of this writing.

Another avenue being pursued is the use of small synthetic molecules to control VZV protein function. A system developed by Banaszynski et al chose a protein called FKBP12, which binds rapamycin and FK506, as a destabilizing domain that will cause any protein(s) attached to it to be unstable and targeted for degradation. This can be countered by the addition of a small ligand designed to bind to the protein and protect the fusion from degradation. Banaszynski et al developed mutants of the destabilizing domain that were specifically sensitive to a small ligand called Shield-1. It was shown that introducing Shield-1 to cells with multiple fusion protein constructs can stabilize a variety of proteins, as well as exhibit the ability to degrade relatively stable proteins like CDK1 and confer stability to short lived cell cycle regulators (25). With this in mind, it

was proposed that using this system in the BAC construct can create a virus that can be conditionally expressed by the addition of Shield-1. The idea is that by attaching a destabilizing domain to an essential gene required for VZV replication, the virus can be grown in cell culture with the addition of Shield-1, and then transferred to humanized mice (and eventually humans) to elicit an immune response. The virus would then be degraded in the absence of Shield-1. For reasons as yet unknown, the virus has still been able to grow, albeit at a diminished rate, after Shield-1 is removed or after transfer to living mice. Western blots also showed that the VZV protein attached to the destabilizing domain was still present in cells. New experiments are attempting to attach destabilizing domains to two or more of VZV's essential genes in an effort to destroy as much virus as possible when Shield-1 is removed .

Conclusions

There are countless avenues of research that can be undertaken in an effort to combat varicella-zoster virus, which remains an important human pathogen. Utilization of the BAC system has allowed for the clarification of the putative functions of the 71 open reading frames of VZV, which only had inferred functions based on homology to HSV. BAC studies also identified ORF7 as the main open reading frame involved in VZV's tissue tropism. Many of the continuing experiments progress on the assumption that ORF7 is required for tissue tropism, but experimenters do not know exactly where it is acting during the viral life cycle. As it has not been possible to elucidate the process by other means, live imaging of viral deletion mutants should shed more light onto how and why ORF7 works, and the resulting phenotype observed when ORF7 is deleted. The additional development of the galKzeo double selection marker in place of galK alone will also make the selection process much easier, reduce background and improve transformation efficiency, as well as making possible seamless recombination which does not leave exogenous DNA after replacing the galKzeo insert with the desired rescue cassette, allowing for production of vaccine suitable strains. Further research is of course required to confirm the unanswered questions this experiment brings up.

Literature Cited

1 - Shors T. 2011. Chapter 15: Herpesviruses. In: Understanding Viruses, 2 ed. p. 455-483, Jones & Bartlett Learning, Sudbury MA. Published October 3, 2011.

2 - Wood MJ, Whitley RJ, Grulich AE, Dore GJ, Brew BJ, Soto NE, Yao J. 2000. History of varicella zoster virus. Herpes, 7(3), 60-65.

3 - Rhazes (al-Razi). A Treatise on the Smallpox and Measles, translated by W A Greenhill. (London: Sydenham Society, 1848; reprinted in Baltimore: Williams & Wilkins, 1939)

4 - T. E. C. Jr. 1970. The first description of chickenpox as a disease, by William Heberden the Elder (1710-1801) in 1767. Pediatrics 45(4), pp588.

5 - Davidson AJ, Scott JE. 1986. The complete DNA sequence of varicella-zoster virus. Journal of General Virology 67(9):1759-1816.

6- Grose C. 2012. Pangaea and the out-of-Africa model of varicella-zoster virus evolution and phylogeography. Journal of Virology 86(18):9558-9565.

7 - Chowa VT, Tipples GA, Grose C. 2013. Bioinformatics of varicella-zoster virus: Single nucleotide polymorphisms define clades and attenuated vaccine genotypes. Infection, Genetics, and Evolution 18:351-356.

8 - Muir WB, Breuer J. 2002. Phylogenetic analysis of varicella-zoster virus: Evidence of intercontinental spread of genotypes and recombination. Journal of Virology 76(4):1971-1979.

9 - Zhang Z, Warden C, Huang G, Huang Y, Zaccheus O, Cheng T, Xia N, Zhu H. 2010. Genome-wide Mutagenesis Reveals That ORF7 Is a Novel VZV Skin-Tropic Factor. PLoS Pathogens 6(7):1-9.

10 - Moffat J, Ku CC, Zerboni L, et al. 2007. VZV: pathogenesis and the disease consequences of primary infection. In: Arvin A, Campadelli-Fiume G, Mocarski E, et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press; 2007. Chapter 37. Available from: http://www.ncbi.nlm.nih.gov/books/NBK47382/

11 - McCune JM, Namikawa R, Kaneshima H, Shultz LD, Lieberman M, Weissman IL. 1988. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science, 241(4873), 1632-1639.

12 - Dougherty M. 2002. From chickenpox to shingles: Researchers map infection pathway of varicella zoster. In Vivo: News from Columbia Health Sciences 1.

13 - Silver B, Lin V, Zhu H. 2013. Newly developed approaches for studying pathogenesis of varicella zoster virus. Journal of Antivirals and Antiretrovirals 5(3):062-071.

14 - Center for Disease Control and Prevention 2014. Recommended immunization schedule for persons age 0 through 18 years. Centers for Disease Control and Prevention [Online.]

15 - Oxman MN et al. 2005. A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. The New England Journal of Medicine 352(22):2271-2284.

16 - Selariu A, Cheng T, Tang Q, Silver B, Yang L, Liu C, Ye X, Markus A, Goldstein RS, Cruz-Cosme RS, Lin Y, Wen L, Qian H, Han J, Dulal K, Huang Y, Li Y, Xia N, Zhu H. 2012. ORF7 of varicella-zoster virus is a neurotropic factor. Journal of Virology 86(16):8614-8624.

17 - Zhang Z, Wang W, Sommer M, Arvin A, Moffat J, Zhu H. 2007. Genetic analysis of varicella-zoster virus ORF0 to ORF4 by use of a novel luciferase bacterial artificial chromosome system. Journal of Virology 81(17):9024-9033.

18 - Tang Q, Silver B, Zhu H. 2012. Protocol of a seamless recombination with specific selection cassette in PCR-based site-directed mutagenesis. Applied Biological Engineering - Principles and Practice, Dr. Ganesh R. Naik (Ed.), ISBN: 978-953-51-0412-4, InTech, DOI: 10.5772/31230.

19 - Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. 2005. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res. 33(4), e36. doi: 10.1093/nar/gni035

20 - Lee EC, Yu D, de Velasco MJ, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG. 2001. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics 73(1), 56-65.

21 - Grigoryan S., Kinchington PR, Goldstein RS. 2012. Retrograde axonal transport of VZV: kinetic studies in hESC-derived neurons. Journal of Neurovirology, 18(6): 462-47. doi: 10.1007/s13365-012-0124-z

22 - Markus A, Grigoryan S, Goldstein RS. 2011. Varicella-zoster virus (VZV) infection of neurons derived from human embryonic stem cells: Direct demonstration of axonal infection, transport of VZV, and productive neuronal infection. Journal of Virology, 85(13): 6220-6233. doi:10.1128/JVI.02396-10

23 - Mangeat B, Trono D. 2005. Lentiviral vectors and antiretroviral intrinsic immunity. Human gene therapy, 16(8), 913-920.

24 - Frecha C, Costa C, Nègre D, Amirache F, Trono D, Rio P, Verhoeyen E. 2012. A novel lentiviral vector targets gene transfer into human hematopoietic stem cells in marrow from patients with bone marrow failure syndrome and in vivo in humanized mice. Blood, 119(5), 1139-1150. doi:10.1182/blood-2011-04-346619

25 - Banaszynski LA, Ling-chun C, Maynard-Smith LA, Ooi AGL, Wandless TJ. 2006. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell, 126(5), 995-1004. doi:10.1016/j.cell.2006.07.025

Supplemental Information

Deletion cassette primer design:

galK Fw: (upstream flanking homology sequence) +
CCTGTTGACAATTAATCATCGGCATAGTATATC
Zeo Rv: (reverse complement of downstream flanking homology sequence) +
GGAACGGACCGTGTTGACAATTAATC

galK verify Fw: CCTGTTGACAATTAATCATCGGCATAGTATATC
galK verify Rv: CAGCACTGTCCTGCTCCTTGTG
zeo verify Fw: CAAGTTTCGAGGTCGAGTGTCAGTCC
zeo verify Rv: GGAACGGACCGTGTTGACAATTAATC