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Design of a Tetracycline Operon Inducible System for the Control of Vaccinia Virus Replication: Implications for Vaccine Development

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Design of a Tetracycline Operon Inducible System for the Control of Vaccinia Virus Replication: Implications for Vaccine Development

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ABSTRACT

The use of vaccinia virus (VACV) as a vaccine resulted in the eradication of smallpox in 1979. Characteristics that contribute to the effectiveness of VACV as a vaccine and viral vector include its ability to elicit strong, long-lived humoral and cell-mediated immune responses as a live-replicating virus and to accept large inserts of DNA into its genome. However, adverse events associated with its use as the smallpox vaccine have constrained it from being more widely utilized in vaccines and therapies. We propose to improve the safety of VACV as a live-replicating vector by using elements of the *tet* operon to control transcription of VACV genes essential for virus growth. This would allow viral replication to be regulated through the addition or removal of tetracyclines. Seven VACV genes were tested in an attempt to control viral replication. For each gene a different recombinant was generated in which the essential gene was placed under the control of *tet* operon elements. Of the seven VACV genes tested, recombinants utilizing the A6L, A7L, D6R, and F17R genes were successful in regulating viral replication with tetracyclines.

CHAPTER 1: INTRODUCTION

1.1. VACCINIA VIRUS AND SMALLPOX

Vaccinia virus (VACV) is the prototypical member of the family *Poxviridae*, genus Orthopoxvirus. This genus also includes cowpox virus, monkeypox virus, and most notably variola virus, the causative agent of smallpox. Smallpox was an extremely devastating disease that affected the world for thousands of years. Efforts to protect against this disease lead to the development of vaccination. The earliest reports of attempts to protect against smallpox come from China in the 10^{th} century, where variolation by insufflation was practiced (Fenner, 1988). People would be deliberately inoculated with smallpox from the pustules of patients that had the active disease. This practice decreased the fatality rate from 20-30% to 0.5%-2.0% (Fenner, 1988). It was not until after 1798, when Jenner discovered that cowpox was able to protect against smallpox, that modern vaccination practices began. Overtime VACV replaced cowpox virus as the vaccine agent; even though the origin of VACV is uncertain, it is genetically distinct from both variola and cowpox virus. The use of VACV as the smallpox vaccine was eventually responsible for the eradication of smallpox, declared in 1979 by the World Health Organization (WHO) (Wehrle, 1980).

1.2. VACCINIA VIRUS AS A VECTOR

VACV has been developed as an expression vector, allowing heterologous (foreign) genes to be expressed by the virus. Recombinant gene expression using VACV was first accomplished in 1982 when the herpesvirus thymidine kinase (TK) gene was inserted into a TK^- VACV strain, giving the recombinant a TK^+ phenotype (Mackett, et al., 1982, Panicali and Paoletti, 1982). The flexibility of VACV for accepting foreign

DNA was tested by inserting 25 kb of bacteriophage λ DNA into the VACV TK gene, thus determining that VACV is capable of forming stable, infectious, and replication competent recombinants with as much as 25 kb of foreign DNA (Smith and Moss, 1983). VACV expression vectors can be used to study protein structure, function, and processing, as well as to develop recombinant vaccines where the heterologous expressed gene is used to elicit immune responses (Carroll and Moss, 1997). The extensive characterization of VACV, along with its ability to elicit strong humoral and cell-mediated immune responses as a live replicating virus and accept large inserts of DNA into its genome, makes it an ideal viral vector (Bennink, et al., 1984). VACV has been successfully used in rabies and rinderpest vaccines, leading to better control of these diseases (Slate, et al., 2009, Verardi, et al., 2002). Another feature of VACV that makes it an appealing vector is its ability to infect a wide range of cells, including different tumors. The immunogenicity of VACV combined with its broad tumor tissue tropism can be used to enhance host immunity against tumor cells (Mullen and Tanabe, 2002). This allows VACV to be used as an oncolytic vector and to be used in immunotherapies for cancer treatment (Jager, et al., 2006, Kaufman, et al., 2004, Rochlitz, et al., 2003). For example, VACV has been engineered to express prostate-specific antigen (PSA), which is over expressed in prostate cancers. This VACV has proven to induce PSA-specific T cell responces and therapeutic activity. In clinical trial the therapy was tolerated well and in 78.1% of patients the cancer did not progress (Kaufman, et al., 2004).

1.3. ADVERSE EVENTS ASSOCIATED WITH THE SMALLPOX VACCINE

Although VACV is an excellent viral vector, the adverse events associated with its use as the smallpox vaccine have constrained it from being more widely utilized as vaccines and therapies. Adverse events associated with the smallpox vaccine fall into three major categories according to their severity. Mild to moderate adverse events include feeling sick enough to miss work, fever, and mild rash that resolves without intervention. Moderate to severe adverse events include autoinoculation, generalized vaccinia (a rash that covers the entire body but resolves without intervention), and myopericarditis. Severe to life-threatening adverse events include eczema vaccinatum (a severe rash on an individual with atopic dermatitis), post-vaccinial encephalitis, and progressive vaccinia (vaccinia necrosum) which leads to skin and tissue destruction due to uncontrolled replication of the virus in immunocompromised individuals (Fulginiti, et al., 2003). A study of smallpox vaccine complications in 1968 surveyed 10 US states for complications. This study found 529.2 cases of accidental implantation per million, 241.5 of generalized vaccinia, 12.3 of post-vaccinial encephalitis, and 1.5 of vaccinia necrosum (Lane, et al., 1970). Despite these complications, vaccination efforts during the global eradication program led the WHO to declare smallpox eradicated in 1979, more than 30 years ago.

1.4. TREATMENT OF ADVERSE EVENTS

With the current smallpox vaccine, if adverse events occur, vaccinia immunoglobulin (VIG) and investigative new drugs such as cidofovir are approved to manage the complications. Although there is evidence from the smallpox eradication

period that suggests individuals with adverse events benefit from VIG treatment, there have been no controlled studies. Intravenous VIG (IGIV produced by Cangene and VIGIV produced by Dynport) was licensed by the Food and Drug Administration (FDA) for use in 2005 for the treatment of progressive vaccinia, eczema vaccinatum, severe generalized vaccinia and extensive body surface involvement or periocular implantation (Wittek, 2006). Side effects associated with VIG treatment, although typically mild, can include severe events such as hypotension, renal dysfunction and aseptic meningitis syndrome (Cono, et al., 2003). Cidofovir is only recommended to treat adverse events if VIG treatment fails or if the patient is close to death. Side effects associated with Cidofovir include renal toxicity, neutropenia, and metabolic acidosis. Animal studies have also shown cidofovir to be carcinogenic (Cono, et al., 2003).

1.5. CONTRAINDICATIONS FOR SMALLPOX VACCINATION

As smallpox is no longer present in the world as a natural infection, there are many contraindications for receiving the vaccine. These include current or past history of eczema, acute or chronic dermatitis, being immunocompromised or immunosuppressed (due to illness, cancer therapy, immunosuppressive treatment after transplantation, or HIV/AIDS), heart conditions, pregnancy, or having contacts with anyone who has a contraindication (Fulginiti, et al., 2003). The population at risk for developing adverse events has risen since the time of the smallpox eradication program. With medical advances in transplantation, cancer and HIV treatment there are now more people living with compromised immune systems, a major contraindication for smallpox vaccination. The number of people living with HIV has continued to increase as HIV treatments

extend life and as new infections continue to occur each year. The number of people living with HIV worldwide at the end of 2008 was estimated at 33.4 million (UNAIDS/WHO, 2009). The prevalence of eczema has also increased since the smallpox eradication era, being estimated to be as high as 20% in Europe, Australia, and the United States (Brown and Reynolds, 2006). It has been estimated that 25% of the United States population is contraindicated for smallpox vaccination (Kemper, et al., 2002). The increasing number of people with major contraindications would greatly complicate smallpox vaccination efforts with the standard smallpox vaccine if it needed to resume, and it hampers the widespread use of VACV as a vaccine and therapeutic vector.

1.6. CURRENT STATUS OF SMALLPOX VACCINATION IN THE UNITED STATES

Although smallpox is no longer a naturally occurring infection, there is still the threat of reemergence through a bioterrorist event. Due to that threat, military personal, first responders, and researchers working with orthopoxvirues are still vaccinated against smallpox using VACV. However, the vaccine is not available to the general public since it would provide little benefit and may actually cause harm (Bozzette, et al., 2003). The United States military vaccine program has shown that careful screening and education of the vaccine recipient lowers the occurrence of adverse events below the historical rates from the eradication program era (Grabenstein and Winkenwerder, 2003). If smallpox were to reemerge through a bioterrorism event, or if another orthopoxvirus emerged as a significant human pathogen (e.g., monkeypox), mass vaccination with VACV may need to resume either in isolated populations or entire geographical regions. Current guidelines

state that in an emergency situation (for example the intentional release of the smallpox virus), no absolute contraindications would exist for persons exposed to smallpox (Centers for Disease Control and Prevention, 2001). Contraindications would be disregarded in this setting because individuals that are at the greatest risk for serious adverse events are also at the most risk for death from smallpox (Centers for Disease Control and Prevention, 2001).

1.7. NEW GENERATION SMALLPOX VACCINES

As safety is the foremost priority for a vaccine, there is a need to develop safer, new generation vaccines for smallpox. While the focus of new smallpox vaccines is on improved safety, their efficacy must also be maintained or improved. Dryvax is a first-generation vaccine that was used during the United States eradication campaign of the 1970s. It is based on the New York City Board of Health (NYCBH) strain of VACV and was produced by Wyeth Laboratories (Handley, et al., 2009). This vaccine was propagated in the skin of calves and isolated by skin scraping (Artenstein, 2008). ACAM2000 recently replaced Dryvax as an improved second-generation vaccine. This vaccine was licensed for use in the United States in 2007, and is clonally derived from the Dryvax vaccine by plaque purification. ACAM2000 is propagated in tissue culture, decreasing the risk of contamination by microbial agents. It is also less neurovirulent than the Dryvax vaccine (Monath, et al., 2004). ACAM2000 was able to be licensed because it demonstrated equivalent immunogenicity to the Dryvax vaccine, which is a requirement for new smallpox vaccines (Artenstein, 2008). Vaccination with either Dryvax or ACAM2000 by scarification produces a scar, known as the vaccine “take”.

There is a direct relationship between the take and protection from smallpox (Artenstein, 2008), and the take is generally considered the only known correlate of protection for smallpox.

New generation smallpox vaccines all focus on increasing vaccine safety while maintaining efficacy. Many new generation vaccines have taken the approach of attenuating the vaccine virus. Such vaccines include MVA (modified vaccinia Ankara) and NYVAC. NYVAC was developed by deleting 18 VACV genes, leaving the virus unable to replicate in humans (Tartaglia, et al., 1992). MVA was generated by passing VACV in chick embryo fibroblasts (>570 times) until the virus lost the ability to replicate in most mammalian cells. This virus has been studied extensively as a safer alternative to replication competent VACV. As MVA cannot replicate in human cells, no serious adverse events have been associated with its use in humans. Although MVA was used during the eradication period, it was never utilized in an area where smallpox was endemic, and therefore the efficacy of MVA against variola has never been assessed. While MVA is undisputedly safer than replication competent smallpox vaccines, it has been shown to require multiple doses to achieve immunogenicity equivalent to Dryvax (Grandpre, et al., 2009, Parrino, et al., 2007, Wyatt, et al., 2004).

Unfortunately the use of a replication deficient smallpox vaccine requiring a multi-dose regimen is unfeasible in the event of a bioterrorist event involving smallpox. One suggested approach for the use of MVA or other replication deficient VACVs is to immunize before there is any risk of smallpox infection and boost with the standard smallpox vaccine or with MVA in the event of an immediate threat (Earl, et al., 2004).

This proposed vaccination scheme would require keeping the population vaccinated with MVA in preparation for a possible smallpox threat.

Even with the improvements provided by ACAM2000, there is still an urgent need to develop an even safer smallpox vaccine for the ever-increasing population with contraindications that at a minimum retains the ability to elicit the protective immunity that the first-generation vaccines provided.

1.8. HYPOTHESIS

The goal of increasing vaccine safety while maintaining efficacy has proven to be difficult. The approach of attenuating VACV, while successful at minimizing adverse events, has come at the cost of efficacy and the uncertainty of its ability to provide protection equivalent to first-generation vaccines. In an effort to maintain vaccine efficacy while increasing safety, I hypothesize that elements of the *tet* operon can be used to control the transcription of a VACV gene essential for virus growth, thereby regulating replication of the virus. These viruses would address the need for a safer, yet indisputably protective, smallpox vaccine.

Regulation of an essential gene can be achieved by constitutively expressing the TetR repressor protein within the VACV genome and incorporating a *tet* operator (*tetO*) into the promoter of an essential gene (Figure 1-1).

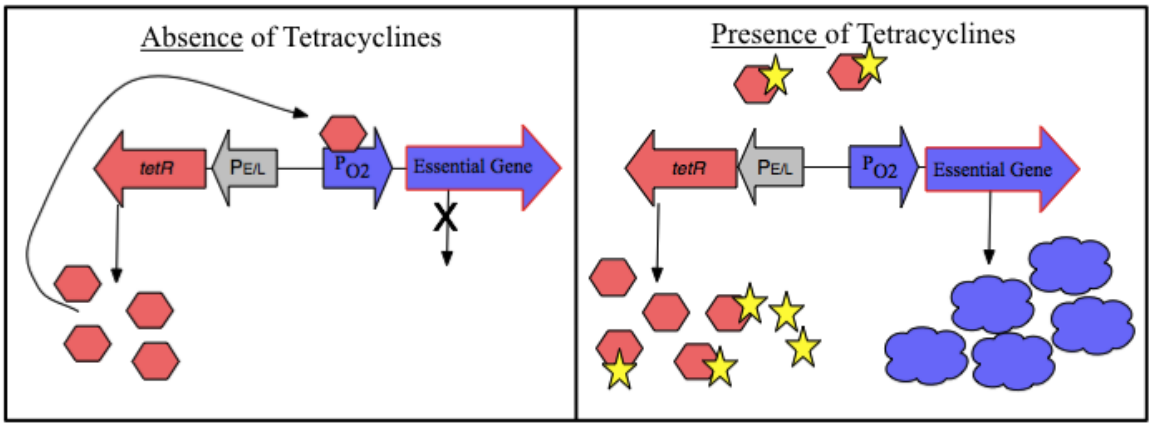

Figure 1-1. Tetracycline Induced Gene Expression in Vaccinia Virus. In the absence of tetracycline (denoted by yellow star) TetR protein is produced and binds to the operator sequence in the promoter for the essential VACV gene, preventing transcription. In the presence of tetracyclines, tetracycline binds TetR and prevents TetR from interacting with the operator, thus allowing transcription of the essential gene.

1.9. SIGNIFICANCE OF PROPOSED WORK

The inducible VACVs generated will have many potential uses. The new recombinant VACVs could serve as new generation smallpox vaccines that are safer and as efficacious as ACAM2000. They could also act as new generation viral vectors for both human and animal vaccines, and as oncolytic vectors. They would be safer not only for the recipients of the vaccine or therapy, but also for the personnel administering the vectors (e.g., vaccinators) and contacts of the vaccine/therapy recipients.

The inducible VACVs are excellent candidates for a new generation smallpox vaccine. As the inducible viruses can be made utilizing the ACAM2000 vaccine strain or another first-generation vaccine strain, the immune response that will be generated by the inducible VACVs should be extremely similar (if not identical) to the current vaccine and

produce a “take”. These similarities would allow the vaccine to fulfill the non-inferiority to ACAM2000 requirement for FDA licensure. If utilized as the smallpox vaccines, the inducible VACVs could be administered to individuals whom have contacts with contraindications. This would prevent the vaccinee from transmitting the virus to contacts (as long as the contacts were not receiving tetracycline treatment).

This inducible form of vaccination would have been particularly useful in a case of inadvertent inoculation that occurred in Indiana in 2007. A United States service member received smallpox vaccination in preparation for deployment, when his deployment was delayed he made an unplanned visit to his family. His son, who had eczema, developed a life-threatening case of eczema vaccinatum which required investigational antivirals (cidofovir), VIGIV, and 48 days of hospitalization to recover (Centers for Disease Control and Prevention, 2007). If the inducible VACV had been used, inadvertent inoculation could have been avoided, as tetracyclines would need to be taken with the vaccine for viral replication to occur. As long as the child was not taking tetracyclines the virus would not have been able to replicate and eczema vaccinatum would not have developed. Since VACV is also used in cancer immunotherapy and as an oncolytic vector in patients that can be mildly to severely immunocompromised, use of inducible VACV vectors would greatly enhance the safety of these therapies.

1.10. INDUCIBLE VACCINIA VIRUS SYSTEMS

Two operon systems have been adapted for use in VACV, the *lac* operon system and more recently the *tet* operon system. The *lac* operon system was first adapted to VACV in 1989, when it was used to regulate the expression of an inserted β-

galactosidase gene. The *lac* repressor (*lacI)* was inserted into the VACV genome under a constitutive promoter and the *lac* operator (*lacO)* was inserted after a strong VACV late promoter initiator sequence prior to the translational start site of the β-galactosidase gene. This genetic setup made the expression of β-galactosidase dependent on the inducer IPTG (isopropyl-β-D-thiogalactoside) (Fuerst, et al., 1989). This same *lac* operon setup was later used to control the expression of the VACV p11 (F17R) gene (Zhang and Moss, 1991a). The ability to control gene expression through the addition of inducer is a powerful method to investigate the function of individual VACV genes. The *lac* operon has been utilized to investigate the function of numerous VACV genes, including p11, early transcription factors, RNA polymerase associated protein, and membrane proteins.

The most common genetic setup using the *lac* operon involves the use of the bacteriophage T7 RNA polymerase and is commonly referred to as vIT7LacO. For this genetic arrangement, elements are typically inserted into the TK (or another nonessential) region of the virus. The P7.5 promoter expresses the *lacI* gene constitutively and the inserted bacteriophage T7 RNA polymerase gene is under the control of a strong late promoter (e.g, P_{11}) with a *lac* operator sequence inserted between the start site of the gene and the initiator of the promoter. This causes the expression of the T7 RNA polymerase to be inducible by IPTG. To regulate the gene of interest (originally tested with the β-galactosidase gene), it was placed under the control of a T7 promoter (sometime also incorporating a *lacO* sequence), making the expression of β-galactosidase inducible by IPTG (Alexander, et al., 1992). More recently the *tet* operon system has been adapted to VACV. The *tet* operon was first used in VACV as an alternative to the *lac* operon system to regulate the expression of the A14L gene (Traktman, et al., 2000).

1.11. THE TETRACYCLINE OPERON

In an effort to improve the safety of VACV vectors while maintaining efficacy, elements of the *tet* operon will be used to control the transcription of VACV genes essential for virus growth, thereby regulating replication of the virus. The *tet* operon is carried on *transposon* 10 in *E. coli*, conferring resistance to tetracyclines. It consists of two genes, the resistance gene (*tetA*) and the repressor gene (*tetR*) (Postle, et al., 1984). The *tetR* gene produces a repressor protein (TetR) that binds to the *tet* operator sequences (*tetO*) that overlap *tet* operon promoters, thus inhibiting the transcription of the *tet* operon genes. TetR binds to tetracyclines (e.g., tetracycline, doxycycline, and anhydrotetracycline), altering its conformation so that it is unable to bind to the operator sequences, thus allowing transcription of the operon genes (Hillen and Berens, 1994). The two binding sites (operators) for TetR in the *tet* operon (O_1 and O_2) consist of 19 bp sequences that bind two homodimeric molecules of TetR as a dimer. Even though O_1 is a perfect palindrome around a T/A center of symmetry and O_2 is an imperfect palindrome, TetR binds to operator O_2 with three- to five-fold higher affinity than to operator O_1 (Hillen and Berens, 1994).

The *tet* operon has been adapted to VACV where it was used to investigate the function of A14, a membrane protein, during VACV life cycle. The O_2 sequence was inserted between the transcriptional and translational start sites of the A14L gene, and tetracycline was able to regulate the expression of the A14L gene (Traktman, et al., 2000). More recently, the expression of reporter genes and cytokines both *in vitro* and *in*

vivo were controlled using several different promoters and operator sequence combinations (P. Oliveira-Weber et al., unpublished data; Weber, et al., 2007).

1.12. TETRACYCLINES

Tetracyclines, a group of antibacterials first used in the 1950s, includes tetracycline, doxycycline, minocycline, and numerous others. Tetracycline antibiotic activity results from the molecule binding to ribosomes, causing the inhibition of bacterial protein synthesis. Tetracycline (a group 1 tetracycline) was introduced in 1950 and is less lipophilic than group 2 tetracyclines, which includes doxycycline. Tetracycline is absorbed at 77-88% and the peak concentration in the serum (1-5 mg/l), is reached between 2-4 h when a 250-500 mg dose is given. The serum half-life of tetracyclines is 6-10 h. Doxycycline is thought to be almost completely absorbed when taken orally, reaches peak concentrations by 2-3 h, and has a long half-life of approximately 20 h (Agwuh and MacGowan, 2006). The typical tetracycline dose for adults is 500 mg twice daily. Complications associated with treatment using tetracyclines are generally mild and can include: photosensitivity (not typically associated with doxycycline or minocycline), discoloration to teeth (infancy to the age of 8), nausea, and diarrhea. Treatment with tetracyclines is not indicated for pregnant women and children less than 8 years of age (Cunha, 2001). Tetracycline treatment is generally safe and is utilized to treat many infections (Table 1-1).

Table 1-1: Uses and Doses of Different Tetracyclines.

Antibiotic	Treatment	Dose	Duration	Reference
Doxycyline	Adult Early Lyme Disease	100 mg, twice daily	14-21 days	(Wormser, et al., 2000)
Doxycycline	Lyme Disease in Children >8 yrs	1-2 mg/kg, twice daily (max. dose 500 mg)	14-21 days	(Wormser, et al., 2000)
Doxycycline	Moderate Acne	20-100 mg, twice daily	-	(Haider and Shaw, 2004)
Tetracycline	Moderate Acne	500 mg, twice daily	-	(Haider and Shaw, 2004)
Tetracycline	Cholera	200-400 mg, daily	3-5 days	(Wallace, et al., 1968)

1.13. VACCINIA VIRUS REPLICATION

Unlike most DNA viruses, poxviruses are unique in that they replicate in the cell cytoplasm, instead of the nucleus. Replication can occur in the cytoplasm because the virus encodes all proteins necessary for its replication, such as DNA and RNA polymerases (Moss, 2007). Transcription of VACV genes occurs in three stages: early, intermediate, and late. During each stage, proteins that allow transcription to progress to the next stage (e.g., transcription factors) are produced. VACV promoters determine the stage at which each gene will be transcribed. After VACV attaches to a cell, it releases its core into the cytoplasm. The core contains all of the factors necessary for early transcription; this allows early mRNA to be synthesized and early proteins to be expressed. After early transcription, uncoating occurs and the viral DNA is replicated within viral factories (granular foci within the cell that do not contain normal cellular

organelles). Intermediate transcription then takes place using the replicated DNA as a template. This is followed by late transcription, which produces abundant amounts of structural proteins, as well as all of the proteins required for early transcription. Virion morphogenesis follows, in which these proteins and the replicated DNA are packaged into new virions. Virion morphogenesis is first evidenced by the appearance of crescent-shaped membrane structures; these crescents turn into immature virions (IV), which may contain nucleoids of electron-dense DNA (INV, immature virion with nucleoid). The IVs continue to mature into infectious particles, either intracellular mature virions (IMVs) or extracellular enveloped virions (EEVs). The majority of the virus particles remain as IMVs within the cell and are thought to mediate spread between hosts, while EEVs are released from the cell by budding and are responsible for dissemination of the virus within the host (Moss, 2007).

CHAPTER 2: USE OF VACCINIA VIRUS EARLY D1R GENE TO CONTROL VIRAL REPLICATION

2.1. INTRODUCTION

To control the replication of VACV with tetracyclines, the VACV *D1R* gene was chosen to be placed under the control of *tet* operon elements. A comprehensive investigation of VACV genes directly involved in virus replication led to this decision. Candidate genes were selected based on their presumed function in the virus life cycle (e.g., RNA and DNA polymerases, transcription factors, etc.) and on published experimental work. A total of 16 essential candidate genes were initially identified. The positions of these genes in the VACV genome and their upstream intergenic regions (the region between the start site of the essential gene candidate and the end or start of the upstream gene) were then carefully inspected to determine if it would be possible to insert the *tet* operon elements without disrupting transcription of the surrounding genes. For example, the early transcription factor large subunit (*A7L*) was not considered an ideal essential gene candidate because its intergenic region contains both the promoter for the transcription factor and a promoter for the upstream gene. Likewise, the promoter for the RNA polymerase 22 kDa subunit gene (*J4R*) is located within the upstream gene. Inserting *tet* operon elements before either gene would cause the transcription of both the essential gene and the upstream gene to be affected. This initial analysis narrowed the number of candidate genes to seven.

All candidate essential genes with appropriate intergenic regions were then further researched to determine if they would be able to control VACV replication. B1R (a Ser-Thr kinase), E8R (a membrane protein) and D1R (the large subunit of the capping enzyme) were the three best essential candidate genes. Studies of E8R demonstrated only a reduction in plaques formed by temperature sensitive- (ts) mutants and studies of B1R

were only able to show that attempts to delete the gene were unsuccessful, suggesting that the gene is essential to viral replication (Kato, et al., 2007, Rempel and Traktman, 1992). A study of the *D1R* gene done by Shatzer et al. showed that ts-mutants were unable to generate any infectious progeny at non-permissive temperatures, indicating that the gene is essential for viral replication (Shatzer, et al., 2008). The *D1R* gene is expressed early (Lee-Chen, et al., 1988) and has been shown to have multiple roles in the VACV replication cycle. It functions not only as a capping enzyme, but also has roles in early gene transcription termination and in intermediate gene transcription initiation (Harris, et al., 1993, Hassett, et al., 1997, Shatzer, et al., 2008). After careful consideration, the *D1R* gene was chosen as the ideal essential gene because it was shown to be required for VACV replication and it is expressed under an early promoter (its repression should be able to stop replication of VACV at an early stage of the virus life cycle).

To utilize the *D1R* gene to generate an inducible recombinant VACV that replicates only in the presence of tetracyclines, *tetO* sequences were incorporated into the promoter for the D1R gene and by inserting the *tetR* gene (under a constitutive promoter) into the VACV genome. The resulting recombinant VACV (viD1R) was expected to be able to replicate only in the presence of tetracyclines.

Two recombinant VACVs were be designed: 1) viD1R (for VACV Inducible D1R) expressing TetR constitutively and having the *D1R* gene under the control of a *tet*-responsive early synthetic promoter (P_{iSE}). The replication of this virus was expected to occur only in the presence of tetracyclines. 2) viD1Rc (for VACV Inducible D1R control) also expressing TetR constitutively but having the *D1R* gene under the control of

its natural promoter (P_{D1R}). This virus was expected to replicate both in the presence and absence of tetracyclines.

The strategy aims to control the replication of VACV by inducing the essential gene through the addition of tetracyclines (e.g., doxycycline). This control over viral replication will increase the safety of VACV as a vaccine vector. If adverse events arise after vaccination with the recombinant VACV, the removal of doxycycline will stop or greatly attenuate the replication of the virus, giving the innate and adaptive immune systems the opportunity to clear the virus, stopping the adverse event.

2.2. MATERIALS AND METHODS

2.2.a. Research Design:

An inducible recombinant VACV was designed by incorporating *tetO* sequences into the promoter for the VACV D1R essential gene and inserting the *tetR* gene (under a constitutive promoter) into the D1R-H7R intergenic region of the VACV genome. The resulting recombinant VACV (viD1R) is expected to be able to replicate only in the presence of tetracyclines.

Recombinant viruses designed:

1. viD1R (for VACV Inducible D1R) expressing TetR constitutively and having the D1R gene under the control of a *tet*-responsive early synthetic promoter (P_{iSE}) -- replication of this recombinant should only occur in the presence of tetracyclines.

2. viD1Rc (for VACV Inducible D1R control) also expressing TetR constitutively but having the D1R gene under the control of its natural promoter (P_{D1R}) -- replication of this recombinant should occur both in the presence and absence of tetracyclines.

2.2.b. Early Tet-Responsive Promoter Design for viD1R:

Typical poxvirus promoters are about 30 bp in length with a core, spacer, and initiator regions (Moss, 2007). Early VACV promoters have a fairly conserved core region followed by an 11-16 bp spacer and a single purine initiator sequence (Broyles, 2003, Chakrabarti, et al., 1997, Davison and Moss, 1989, Moss, 2007). Extensive analysis of the early core sequences performed in the Verardi Laboratory suggested that the sequence, AAAAATAGAAACCATA, would serve as an optimal early core region (J. Sopronyi et al., unpublished data). To generate an early *tet*-responsive promoter for the *D1R* gene, the 19-bp *tet* operator sequence O_2 (TCCCTATCAGTGATAGAGA) was inserted between this designed core region and the translational start site of the *D1R* gene to originate the synthetic early promoter P_{iSE} (AAAAATAGAAACCATA**TCCCTATC-AGTGATAGAGA**). This new synthetic promoter would be expected to have the lowest levels of expression under repressible conditions, since the putative purine initiator sequences (underlined) are part of the operator sequence (in **bold**) (Table 2-1).

2.2.c. Natural D1R Promoter Sequence for viD1Rc:

To generate an appropriate control virus expressing D1R constitutively, the intergenic sequence between the *D1R* and the upstream *H7R* gene coding sequences, plus

the last 9 bp from the *H7R* gene coding sequence, were used to design the natural *D1R* promoter sequence (P_{D1R}, **GCTTGTTAA**TAAGTAAATGAAAAAAAACTAGTCGTT-TATAATAAAACACGAT). *H7R*, the gene upstream from *D1R*, has a role in the formation of crescent membrane precursors and immature virions (Satheshkumar, et al., 2009). Since the *D1R* promoter has not been mapped, the 9 bp from the end of the *H7R* gene (in bold) were incorporated to ensure that the full natural *D1R* promoter is present (Table 2-1). In particular, any potential G nucleotides (required by early promoters around position -21) were included in the sequence (Broyles, 2003, Chakrabarti, et al., 1997, Davison and Moss, 1989, Moss, 2007).

Table 2-1: Recombinant Vaccinia Viruses and their Respective D1R Promoter Sequences.

Recombinant Vaccinia Virus	Inducible Promoter Name	Promoter Sequence[a]
viD1R	P_{iSE}	AAAAATAGAAACCATA**TCCCTATCAGTGATAGAGA**
viD1Rc	P_{D1R}	**GCTTGTTAA**TAAGTAAATGAAAAAAAACTAGTCGTTTAT-AATAAAACACGAT

[a] In the P_{iSE} promoter the *tetO*$_2$ sequence is in bold and underlined are potential early initiators of transcription. In the P_{D1R} promoter the last 9 bp of the upstream H7R gene are in bold.

2.2.d. Construction of Transfer Vectors for viD1R and viD1Rc:

A series of cloning steps was used to build the transfer vectors to generate the recombinant VACVs, based on existing plasmids and designed synthetic DNA

sequences. The final transfer vectors contain: (1) the selectable *E. coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene and the screening marker β-glucuronidase (*gusA*) gene, as a fusion gene (*gpt-gus*) under control of the synthetic early/late promoter Psel (Hammond, et al., 1997); (2) a second screening marker, the DsRed-Express gene (DsRed) encoding a red fluorescent protein under control of the natural late P_{11} promoter; (3) the repressor gene *tetR* under the synthetic early/late promoter $P_{E/L}$ (Chakrabarti, et al., 1997); and (4) either the P_{iSE} or P_{D1R} promoter to direct the expression of the D1R gene.

All of these elements were placed between the left border (LB) (the last 524 bp of the *H7R* gene, upstream of D1R) and right border (RB) (the first 600 bp of the *D1R* gene), which serve as recombination sequences for homologous recombination. Briefly, the *EcoRI-BglII* fragment (containing *gtp-gus* and DsRed genes) from pSMART10 (Weber, et al., 2007) was cloned into the *EcoRI-BclI* site of pCH001 (a synthetic plasmid containing the left and right recombination sequences for assembly of the final transfer vectors obtained from DNA2.0 (Menlo Park, CA), originating pCH003. Next the *SphI-XmaI* fragment of pSMART10 (containing *tetR*) was cloned into the same sites of plasmid pCH003, resulting in the final transfer vector (pCH004), which was used to generate viD1R (Figure 2-1). To generate the final transfer vector for viD1Rc, the *SphI-XhoI* fragment of plasmid pCH004 (containing P_{iSE} and the Right Border) was replaced with the *SphI-XhoI* fragment of the synthetic plasmid pCH002 (containing P_{D1R} and the Right Border, DNA2.0), generating plasmid pCH005 (Figure 2-1).

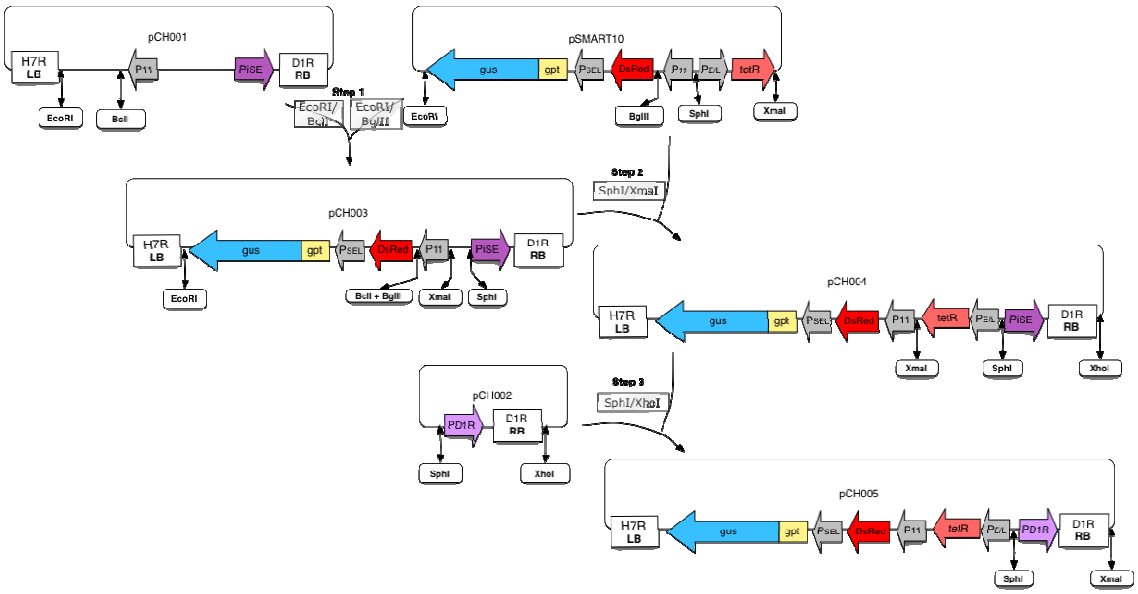

Figure 2-1. Construction of Final Transfer Vectors pCH004 and pCH005. The cloning steps used to generate the final transfer vectors are depicted. Both vectors express the *gpt-gus*, DsRed, and *tetR* genes under constitutive promoters. The transfer vector pCH004 was designed to express the *D1R* gene under the synthetic inducible early P_{iSE} promoter, while pCH005 expresses the *D1R* gene under its natural early promoter (P_{D1R}). All of these genetic elements for both plasmids are flanked by the Left Border (H7R LB) and Right Border (D1R RB) sequences, which direct homologous recombination with the VACV genome to generate viD1R and viD1Rc.

2.2.e. Generation of Recombinant Viruses:

Homologous recombination was used to precisely insert the genetic elements between the left and right borders of each of the final transfer vectors into the intergenic region between the essential and upstream genes, placing the inducible promoter in front of the essential gene. This step replaced the natural D1R-H7R intergenic region (43 bp)

with the desired genetic elements (for viD1R 3865 bp were inserted; for viD1Rc 3871 bp were inserted). The recombinant VACVs were generated by standard homologous recombination via transfection of the transfer vectors pCH004 and pCH005 into BS-C-1 cell monolayers infected 2 h earlier at 0.05 PFU per cell with VACV strain Western Reserve (WR) clone 9.2.4.8 (obtained from T. Yilma, University of California Davis). Recombinant *gpt*-positive VACVs were plaque purified on BS-C-1 cells from transfection lysates using *gpt* selection medium (25 µg/ml mycophenolic acid, 250 µg/ml xanthine, and 15 µg/ml hypoxanthine) (Legrand, et al., 2004). All recombinants were plaque-purified in the presence of inducer (1 µg/ml doxycyline). Expression of DsRed was detected via fluorescence microscopy (Carl Zeiss Axio Observer D1) to ensure that the recombinant viruses were free of parental virus.

2.3. RESULTS

During plaque purification of the recombinant viruses it was observed that many of the recombinant plaques that grew in gpt selective media were not expressing DsRed, but were expressing the *gus* gene indicating that the recombinants (both viD1R and viD1Rc) do not contain all of the inserted genetic elements (Figure 2-2). It was also observed that doxycycline had no effect on the replication of viD1R, the virus replicated both in the presence and absence of doxycycline.

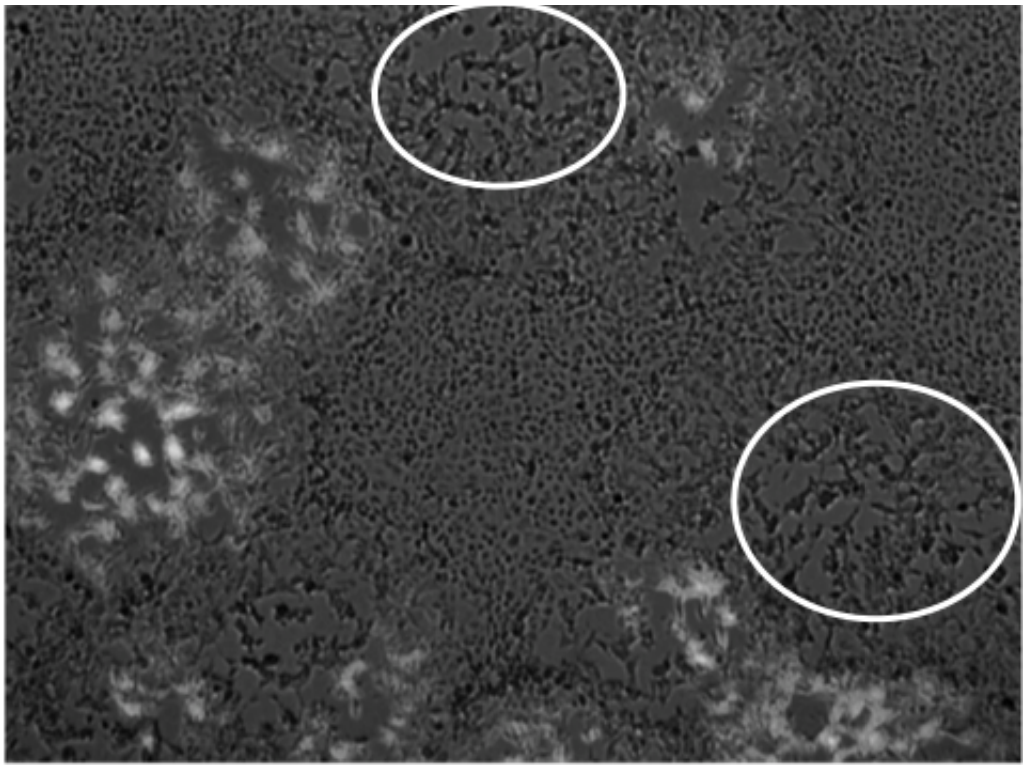

Figure 2-2. viD1R Grown in Selective Media Produces Fluorescent and Non-Fluorescent Plaques. BS-C-1 cells pre-incubated with gpt-selective media were infected with viD1R and plaques were observed at approximately 48 h post infection. Both DsRed fluorescent plaques and non-fluorescent plaques (shown inside the white circles) were observed.

2.4. DISCUSSION

The promoters used to express the *gpt-gus*, *DsRed*, and *TetR* genes are made up of very similar sequences that could promote recombination once inserted into the VACV genome. The variety of gpt positive recombinants that were observed could be the result of recombination between the promoters. In particular, recombination between the P_{11} promoter for *DsRed* and the P_{SEL} promoter for *gpt-gus* would cause the recombinants to lose the *DsRed* gene while maintaining *TetR* and *gpt-gus* expression. Recombination

between the $P_{E/L}$ promoter for *gpt-gus* and the $P_{E/L}$ promoter for *TetR* would cause the recombinants to lose both the *DsRed* and *TetR* genes while retaining the *gpt-gus* gene. If recombination occurred between the $P_{E/L}$ and P_{11} the *TetR* gene would be removed from the virus without altering the *gpt-gus* or *DsRed* genes. This recombination could explain why the viD1R virus was not responsive to doxycycline (Figure 2-3). Due to the recombination events occurring in the virus, the transfer vectors were re-designed to decrease the likelihood of recombination.

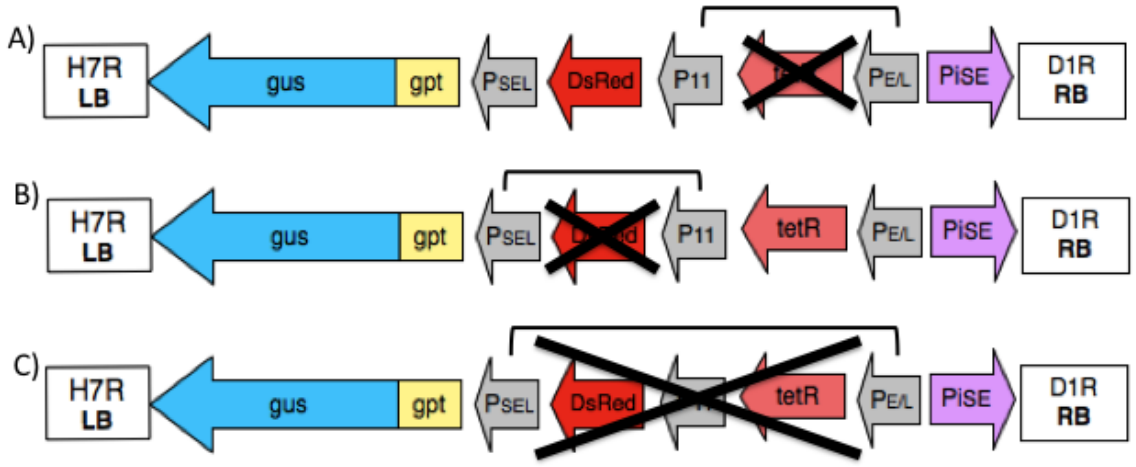

Figure 2-3. Possible Recombination among Promoters within viD1R and viD1Rc. Depicted are possible recombination events that could occur within the viD1R and viD1Rc recombinants. A) Recombination between P_{11} and $P_{E/L}$ would result in the removal of the *tetR* gene while maintaining *DsRed*. B) Recombination between P_{11} and P_{SEL} results in the loss of *DsRed* while maintaining *tetR*. C) Recombination between P_{SEL} and $P_{E/L}$ results in the loss of both *DsRed* and *tetR* expression.

CHAPTER 3: IMPROVEMENT OF TRANSFER VECTORS

3.1. INTRODUCTION

Due to the instability in the viD1R and viD1Rc viruses, the transfer vectors needed to be re-designed to decrease the likelihood of recombination events. Transfer vectors for viD1R and viD1Rc were re-designed to minimize the number of promoters utilized, thus preventing recombination from occurring within the inserted sequence (Figure 3-1). Placing similar/identical sequences (e.g, promoters) in very close proximity to each other (e.g, back-to-back) prevents recombination from occurring between the sequences. By reducing the number of promoters used to two, the two promoters may be identical and placed back-to-back to prevent recombination. If this setup is utilized, the only promoter in a location within the transfer vector that could undergo recombination is the synthetic early promoter (P_{iSE}). If recombination occurs with this promoter, the expression of the newly designed *gpt-EGFP* (enhanced green fluorescent protein) fusion protein would be lost, causing these viruses not to grow in gpt-selective media.

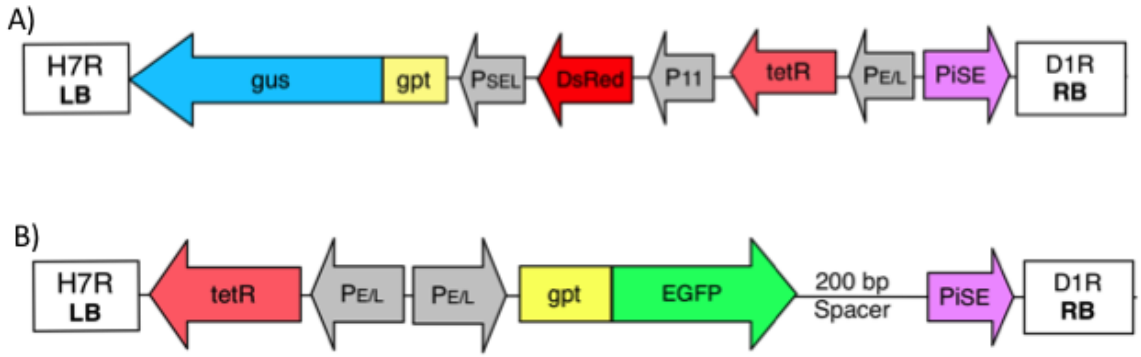

Figure 3-1. Re-design of Transfer Vectors. An overview of the design of the final transfer vectors is shown. The original transfer vector design is depicted in A and the new design is shown in B. The new design (B) minimizes the use of synthetic VACV promoters and arranges them in a way to minimize recombination.

3.2. MATERIALS AND METHODS

3.2.a. Re-design of Transfer Vectors for viD1R and viD1Rc:

A new *gpt-EGFP* fusion gene was generated. A synthetic plasmid containing the *EGFP* sequence with an NcoI site at the 5' end and an AatII-EcoRI site at the 3' end (pCH035) was used to generate the *gpt-EGFP* fusion gene. The synthetic *EGFP* gene was cloned into the pSMART10 plasmid (replacing the *GUS* gene) to generate pCH030 containing the *gpt-EGFP* fusion gene. The fusion gene was sequenced to confirm the sequence of *gpt-EGFP*.

A series of cloning steps was then used to build the transfer vectors to generate the recombinant VACVs (viD1R$_2$ and viD1Rc$_2$), based on existing plasmids and designed synthetic DNA sequences. The final transfer vectors contain: (1) the selectable *E. coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene and the screening marker *EGFP* gene, as a fusion gene (*gpt-EGFP*) under control of the synthetic early/late promoter $P_{E/L}$; (2) the repressor gene *tetR* under $P_{E/L}$ promoter (Chakrabarti, et al., 1997); and (4) either the P_{iSE} or P_{D1R} promoter to direct the expression of the *D1R* gene.

All of these elements were placed between the left border (the last 524 bp of the *H7R* gene, upstream of *D1R*) and right border (the first 600 bp of the *D1R* gene), which serve as recombination sequences for homologous recombination. Briefly, the *EcoRI-SphI* fragment (containing back-to-back $P_{E/L}$ promoters) from pCH029 (from DNA2.0) was cloned into the *EcoRI-SphI* site of pCH001 (a synthetic plasmid containing the left and right recombination sequences for assembly of the final transfer vectors obtained

from DNA2.0), originating pCH031. Next the *XmaI-NheI* fragment of pCH004 (containing *tetR*) was cloned into the same sites of plasmid pCH031, resulting in pCH032. Finally the *SacI/AatII* fragment from pCH030 (containing the *gpt-EGFP* gene) was inserted into the same site of pCH032 generating the final transfer vector pCH033, which was used to generate viD1R$_2$ (Figure 3-2). To generate the final transfer vector for viD1Rc$_2$, the *SphI-XhoI* fragment of plasmid pCH0033 (containing P_{iSE} and the Right Border) was replaced with the *SphI-XhoI* fragment of the synthetic plasmid pCH002 (containing P_{D1R} and the Right Border, DNA2.0), generating final transfer vector pCH034 (Figure 3-2).

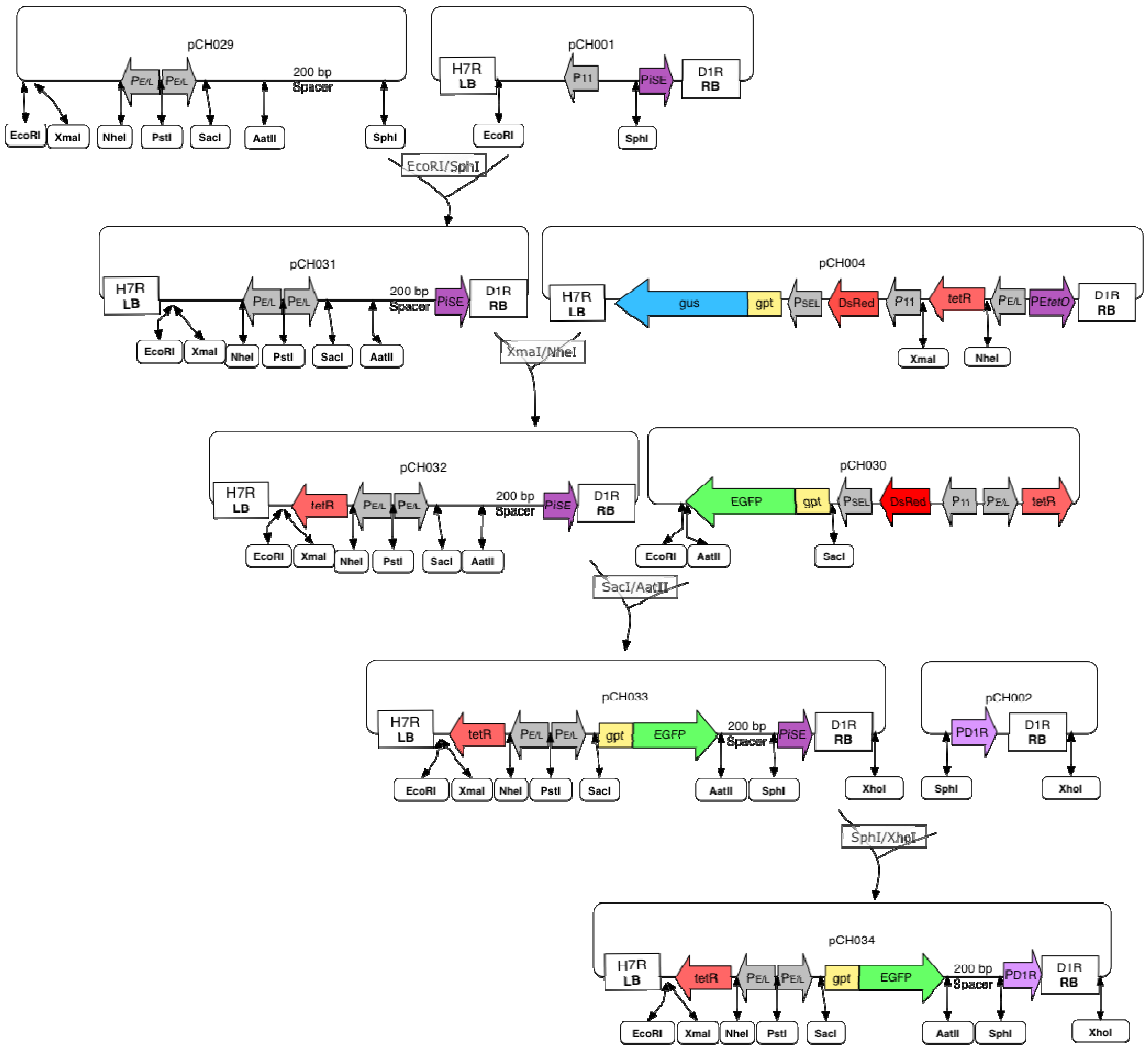

Figure 3-2. Construction of Final Transfer Vectors pCH033 and pCH034. The cloning steps used to generate the final transfer vectors are depicted. Both vectors express the *gpt-EGFP*, and *tetR* genes under constitutive promoters. The transfer vector pCH033 was designed to express the *D1R* gene under the synthetic inducible early P_{iSE} promoter, while pCH005 expresses the *D1R* gene under its natural early promoter (P_{D1R}). All of these genetic elements for both plasmids are flanked by the left border (H7R LB) and right border (D1R RB) sequences, which direct homologous recombination with the VACV genome to generate $viD1R_2$ and $viD1Rc_2$.

3.2.b. Generation of Recombinant Viruses:

Recombinant viruses were generated using the method previously described in Chapter 2, section 2.2.e.

3.3. RESULTS

Upon plaque purification, no recombination was observed in the recombinant VACVs, since all genetic screening/selective genetic elements appeared to be expressed. The recombinant viruses were able to grow in MPA selective media and to express EGFP (indicating that the viruses were gpt^+ and $EGFP^+$), and contained the *tetR* gene as determined by PCR (data not shown). The current genetic setup appears to prevent recombination from occurring among the transfer vector elements. Upon testing with doxycycline there was no observable effect on the replication of either $viD1R_2$ or $viD1Rc_2$. Plaques of similar size were formed both in the presence and in the absence of doxycycline, indicating that viral replication is not inducible (Figure 3-3).

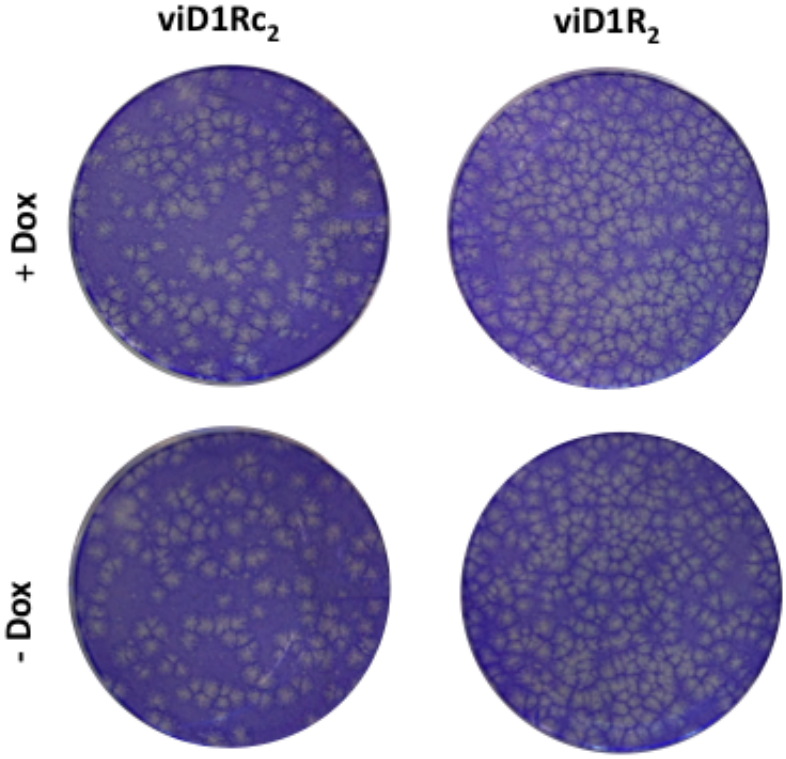

Figure 3-3. Effect of Doxycycline on the Replication of viD1R$_2$ and viD1Rc$_2$. BS-C-1 cells were infected with virus and allowed to grown for 48 h in either the presence of 1 µg/ml doxycycline or in the absence of doxycycline.

3.4. DISCUSSION

Minimizing the number of promoters used in the transfer vectors successfully prevented recombination from occurring among the inserted genetic elements. However, the recombinant viD1R$_2$ was not inducible; the virus was able to grow in the presence and absence of doxycycline. There are several possible reasons for why the *tet* operon elements were unable to control the replication of this recombinant VACV. First, as D1R is expressed early in infection there may not be enough TetR packaged into the virion to prevent this early transcription efficiently. Although early promoters are weak, the D1R

gene product (small subunit of the capping enzyme) is an enzyme and may not be required in high amounts, thus a low level of early transcription may produce a sufficient amount for viral replication. Secondly, as the structure of early promoters is not well understood, the operator sequence may not have been inserted in an ideal location causing the binding of TetR to be insufficient to prevent transcription of D1R.

To date, no early gene has ever been successfully controlled by an operon system while maintaining its early expression. Limited success has been achieved by changing the stage at which the early gene of interest is transcribed, as was done with the *L2R* gene. The *L2R* gene is expressed early in infection, but in an attempt to study its function using the *lac* operon, the promoter of the gene was changed to a T7 RNA polymerase promoter. Utilizing a genetic arrangement, in which the T7 RNA polymerase is under the control of a late promoter containing a *lacO* sequence, the transcription of *L2R* was successfully regulated by IPTG. Although gene expression was successfully regulated, a delay in virus replication of several hours was observed possibly due to the change in the time of expression of *L2R* (Maruri-Avidal, et al., 2011).

Due to the inherent difficulties in regulating early transcription using operon systems as observed in this system and by others as well, late VACV genes may be better candidates for the inducible control of VACV replication.

CHAPTER 4: USE OF VACCINIA VIRUS LATE GENES TO CONTROL VIRAL REPLICATION

4.1. INTRODUCTION

Controlling early gene expression through the use of operon systems has proven to be very challenging; controlling late gene expression may be a better alternative to generate inducible VACV recombinants. Several inducible VACVs were designed by placing late genes essential for VACV replication under the control of *tet* operon elements, allowing viral replication to occur only in the presence of tetracyclines. These inducible recombinant VACVs were designed by incorporating *tetO* sequences into the promoters for selected essential late genes (*A3L*, *A6L*, *A7L*, *D6R*, *E8R*, and *F17R*) and inserting the *tetR* gene (under a constitutive promoter) into the VACV genome. The resulting recombinant VACVs were expected to only be able to replicate in the presence of tetracyclines.

The late essential genes were selected based on their presumed function in the virus life cycle (e.g., RNA and DNA polymerases, transcription factors, etc.) and on published experimental work. A total of 27 late essential candidate genes were initially identified. The promoters of each gene were then investigated to determine if a characteristic late transcription initiator (TAAAT) was present and the positions of these genes in the VACV genome and their upstream intergenic regions (the region between the start site of the essential gene candidate and the upstream gene) were carefully inspected to determine if it would be possible to insert the *tet* operon elements without disrupting transcription of the surrounding genes. For example, the RNA polymerase 132 kDa subunit (*A24R*) is not an ideal essential gene candidate because its promoter is located within the upstream gene (*A23R*). Inserting *tet* operon elements into this intergenic region would cause the transcription of the both the essential gene and the

upstream gene to be affected. Similarly, the *E10R* gene is not an ideal candidate because its promoter is back-to-back with the early promoter for the DNA polymerase gene (*E9L*). As the exact sequence of the early promoter for *E9L* could not be accurately determined and transcription of *E9L* would likely be disrupted by inserting the *tet* operon elements into this intergenic region, the *E10R* gene was not considered an ideal candidate. This analysis further narrowed the list of candidate genes; all remaining genes with appropriate intergenic regions were then further researched to determine if they would be able to control VACV replication. Based on this criteria the following late essential genes were chosen: *A3L* (precursor of core protein 4b), *A6L* (involved in the formation of mature virions), A7L (large subunit of the early transcription factor), *D6R* (small subunit of the early transcription factor), *E8R* (membrane protein), and *F17R* (virion core protein).

4.1.a. A3L Gene:

The *A3L* gene encodes the 72.5 kDa precursor to virion core protein 4b. Precursor 4b is proteolytically processed by the I7L gene product into 4b, a 60 kDa core protein. Core protein 4b accounts for 11% of the virion mass and has been localized to the surface of virion cores (Moss and Rosenblum, 1973, Sarov and Joklik, 1972, Wilton, et al., 1995). Temperature sensitive mutants that map to the *A3L* gene were used to investigate the role of A3 in virion morphogenesis. These mutants had normal patterns of gene expression and DNA replication, but were defective in the transition from immature virions containing nucleoids (IVN) to intracellular mature virus (IMV) (Kato, et al., 2004). Infections at the nonpermissive temperature formed normal IV (immature virions)

and IVN, but no normal IMVs were produced. The particles that were produced were abnormal in shape. The infectivity of these misshapen virions was reduced 30-100 fold and showed transcription levels as low as 2% of wild type levels (Kato, et al., 2004). Repression of the I7L gene product, which is responsible for cleaving the precursors of core protein 4b as well as core proteins 4a and 25k, also causes a defect in virion morphogenesis very similar to the *A3L* temperature sensitive mutants, further suggesting that core protein 4b is essential to VACV (Ansarah-Sobrinho and Moss, 2004, Byrd and Hruby, 2005, Kane and Shuman, 1993). An inducible *A3L* VACV mutant has not yet been generated.

4.1.b. A6L Gene:

VACV gene *A6L* is one of 91 open reading frames (ORFs) conserved among all chordopoxviruses (Upton, et al., 2003). A6 has been shown to be a minor virion component in VACV via mass spectrometry (Chung, et al., 2006). The A6 homolog of myxoma virus has also been identified as part of the virion core (Zachertowska, et al., 2006). Temperature sensitive mutants and a recombinant VACV encoding *A6L* with an epitope tag have been used to characterize the function of the *A6L* gene in the VACV lifecycle. The *A6L* gene product is expressed late in infection, tightly packaged into the virion core and appears to be essential in virion morphogenesis (Meng, et al., 2007). At the non-permissive temperature, ts-mutants were unable to produce infectious progeny and could not process the precursors of major virion proteins 4a and 4b (Meng, et al., 2007). At the non-permissive temperature, virion morphogenesis of the ts-mutants was blocked at the IV stage prior to IVN. Several proteins co-precipitate with A6 leading to

speculation that A6 may help with assembly of the virion core through its interaction with other viral proteins. An inducible recombinant for the *A6L* gene has not yet been successfully constructed. *Lac* operon inducible recombinants for the *A6L* gene have been attempted, but have not been successfully generated (Meng, et al., 2007).

4.1.c. A7L and D6R Genes:

The VACV early transcription factor (VETF) is composed of two subunits (a small 70 kDa subunit and a large 82 kDa subunit). The *D6R* gene encodes the small subunit and the large subunit is encoded by *A7L*. The VACV *A7L* gene is referred to as *A8L* in some literature; currently the A8 ORF is considered to be *A8R* and to encode a subunit of the intermediate transcription factor (Sanz and Moss, 1999). The VETF subunits are produced late in infection and packaged into the virus to be used in the next round of replication. VETF provides early promoter specificity to the RNA polymerase, by binding early promoters and recruiting the RNA polymerase for transcription of early genes (Baldick, et al., 1994, Li and Broyles, 1993b). The *H4L* gene product (RAP94) is also required for early gene transcription and is believed to have a role in docking the RNA polymerase to the VETF (Ahn, et al., 1994, Broyles, 2003). A7, the large subunit, interacts with the core region of the early promoter while D6, the small subunit, interacts with the promoter's downstream region (Cassetti and Moss, 1996). The small subunit of the VETF also contains the DNA-dependent ATPase activity of the transcription factor (Li and Broyles, 1993a).

An inducible *A7L* VACV mutant was created using the *lac* operon system. The T7 RNA polymerase gene, under the VACV late P_{11} promoter containing a *lac* operator,

was inserted into the VACV genome and the *A7L* gene was placed under the control of a bacteriophage T7 promoter. This inducible system used IPTG to regulate the production of the T7 RNA polymerase and thus the transcription of the *A7L* gene. The replication of this mutant was shown to be dependent on inducer and the repression of A7 interfered with virion morphogenesis. In the absence of inducer, one-step growth curves showed little or no increase in viral titer compared to a 2-log increase in the presence of inducer. The repression of A7 produced immature and dense intermediate particles that did not have the characteristic brick structure and very few mature particles were produced. Only 5.6% of all particles formed were mature virions compared to 33.4% in the presence of inducer (Hu, et al., 1998).

An inducible *D6R* mutant was also generated using the T7lacO system. The *D6R* gene was placed under the control of a T7 promoter and the inserted T7 RNA polymerase gene was regulated by the VACV late P11 promoter with a lac operator, causing the *D6R* gene to be inducible by IPTG. Plaque formation of this mutant was dependent on the presence of inducer. Similar to the repression of A7, the repression of D6 also interfered with morphogenesis of the virus. In the absence of inducer, immature virions were found next to large granular masses and were round rather than brick shaped, and mature virions were not frequently observed (Hu, et al., 1996).

The disruption of virion morphogenesis caused by the repression of the VETF subunits may indicate a direct role for the VETF in virion morphogenesis or VETF may be required for the transcription of a unique set of late genes (Hu, et al., 1996, Hu, et al., 1998). The possibility of the VETF being required for transcription late in infection

supports the transcriptional reactivation of certain early promoters at late times (Garces, et al., 1993). Neither prospective role for VETF has yet been confirmed.

4.1.d. E8R Gene:

The specific role of the VACV *E8R* gene has not yet been accurately determined although several studies have been conducted. *E8R* was predicted to contain two transmembrane domains and was first investigated as a potential membrane protein involved in ER wrapping (Tolonen, et al., 2001). E8R was shown to localize to DNA replication sites and to be concentrated in the ER surrounding the replication site. Based on these findings E8R was suggested to be an ER-resident membrane protein that may bind newly synthesized VACV DNA and aid in ER wrapping (Tolonen, et al., 2001). Due to the suggested role of aiding in ER wrapping, the *E8R* gene was further investigated leading to conflicting reports on the characteristics of *E8R* and its role in the VACV life cycle.

Doligo *et al*. further characterized the *E8R* gene product as being made early in infection and as being associated with the ER membrane as early as 1 hour post-infection. E8 was also shown to associate with the membranes of immature virions (IV), intracellular mature virus (IMV), and with viral cores. E8 is phosphorylated *in vitro* by F10 kinase, a process that was shown to reduce the binding of DNA to E8. Based on this information it was speculated that E8R might mediate the binding of DNA to ER membranes leading to the enclosure of the replication site by ER membranes (Doglio, et al., 2002).

The role of *E8R* was also investigated using ts-mutants; the results from these experiments are inconsistent with the characterization by Doligo et al. Kato et al. showed *E8R* to be expressed late in infection, contrary to previous reports. Upon investigation of the promoter and gene, sequences consistent with a late gene promoter were found. The coding sequence was also shown to contain two early transcription termination sequences further supporting the conclusion that E8 is a late protein (Doglio, et al., 2002, Kato, et al., 2007).

Ts-mutants in the *E8R* gene synthesized DNA and proteins at the same level for both permissive and non permissive temperatures, indicating that an absence or decrease of E8 protein did not affect the DNA factories, as would have been expected if E8 had a role in them. Ts-mutants and wild type viruses produced similar amounts of particles per cell at permissive and non-permissive temperatures, but the infectivity of the ts-mutant particles at the non-permissive temperature was greatly decreased. The infectivity of ts-mutants grown at non-permissive temperatures was reduced 35 fold compared to the same virus grown at permissive temperatures (Kato, et al., 2007).

Virions grown at non-permissive temperatures were able to enter cells; however, the virions were defective in early viral transcription, producing only 10% the amount of RNA compared to wild type. Although the virions were defective in early transcription, their extracts were able to synthesize RNA transcripts similar to wild type indicating that the mutant virions contain all the necessary factors for transcription. The E8R mutants resemble L3L ts-mutants in that both form virus particles indistinguishable from wild type, but that are deficient in early transcription within the core. Contrary to the conclusions of Dolgio et al. and Tolonen et al., Kato et al. concluded that E8 appears to

have a role in the virion core structure which impacts core transcription (Kato, et al., 2007). No definitive role for E8 has been established and an inducible *E8R* VACV mutant has not yet been generated.

4.1.e. F17R Gene:

F17R, also referred to as F18 or p11, is one of the most abundant core proteins, accounting for 11% of the virion mass (Sarov and Joklik, 1972). F17 binds strongly to DNA and has been characterized as a DNA-binding protein (Kao and Bauer, 1987, Kao, et al., 1981). In 1991, an inducible *F17R* recombinant was generated using the *lac* operon. *LacI* was constitutively expressed and the *lac* operator inserted between the gene's translational start site and its promoter. This recombinant was unable to replicate in the absence of inducer (IPTG) (Zhang and Moss, 1991a). The inducible *F17R* mutant was the first conditional-lethal VACV mutant generated using any operon system. In the absence of inducer, cleavage of the major virion protein precursors (p4a and p4b) was inhibited and morphogenesis was blocked at an intermediate stage (Zhang and Moss, 1991b). Further investigation determined that in the absence of F17 no typical mature virions are formed, and immature virions with unusual internal membranes and aberrant noninfectious mature virions are produced (Wickramasekera and Traktman, 2010).

4.2. MATERIALS AND METHODS

4.2.a. Research Design:

Inducible recombinant VACVs were designed by incorporating *tetO* sequences into the promoters for the selected essential late genes (*A3L*, *A6L*, *A7L*, *D6R*, *E8R*, and *F17R*) and inserting the *tetR* gene (under a constitutive promoter) into the VACV genome. The resulting recombinant VACVs were expected to only be able to replicate in the presence of tetracyclines.

Six recombinant VACVs have been designed:

1. viA3L (for VACV Inducible *A3L*) expressing TetR constitutively and having the A3L gene under the control of the *tet*-responsive *A3L* promoter (P_{iA3L}).
2. viA6L (for VACV Inducible *A6L*) expressing TetR constitutively and having the *A6L* gene under the control of the *tet*-responsive P_{11} (F17R) promoter (P_{i11}).
3. viA7L (for VACV Inducible *A7L*) expressing TetR constitutively and having the *A7L* gene under the control of the *tet*-responsive P_{11} (F17R) promoter (P_{i11}).
4. viD6R (for VACV Inducible *D6R*) expressing TetR constitutively and having the *D6R* gene under the control of the *tet*-responsive D6R promoter (P_{iD6R}).
5. viE8R (for VACV Inducible *E8R*) expressing TetR constitutively and having the *E8R* gene under the control of the *tet*-responsive E8R promoter (P_{iE8R}).
6. viF17R (for VACV Inducible *F17R*) expressing TetR constitutively and having the *F17R* gene under the control of the *tet*-responsive F17R promoter (P_{i11}).

4.2.b. Tet-Responsive Late Promoter Design:

Late VACV promoter sequences are less conserved than early promoters, but have a very distinct initiator sequence. Late VACV promoters commonly consist of a 20 bp long T-run, a 6 bp spacer region, and a highly conserved TAAAT initiator sequence (Davison and Moss, 1989). To make a promoter tet-responsive the operator sequence is added downstream of the initiator. The P_{11} promoter of VACV has previously been used as a lac-responsive and tet-responsive promoter (Fuerst, et al., 1989, Weber, et al., 2007) To make the P_{11} promoter tet-responsive, the *tetO*$_2$ sequence (TCCCTATCAGTGATAG-AGA) was inserted downstream of the initiator to generate P_{i11} (Weber, et al., 2007). The synthetic $P_{E/L}$ promoter has also successfully been made tet-responsive by inserting the *tet*O_2 sequence after the initiator. To transform the promoters of the chosen essential late genes into tet-responsive promoters, their promoters and intergenic regions were studied.

The *A3L* intergenic region is 52 bp and is expected to contain only the late promoter for the *A3L* gene. The entire intergenic sequence was used for the *A3L* promoter to ensure the entire promoter would be used in its natural state. The *tet* O_2 sequence was then inserted after the putative late initiator sequence (TAAATA) generating P_{iA3L} (Figure 4-1 and Table 4-1).

Figure 4-1. *A3L* Region of the Vaccinia Virus Genome. The *A3L* gene and surrounding genes are shown, with bp sizes of the genes (below) and intergenic regions (above).

The *A6L* intergenic region is only 23 bp, and since it is shorter than the typical poxvirus promoter length, it is likely that the start of the *A6L* promoter is located within the upstream gene. When the intergenic region was carefully inspected, the sequence contained several possible late initiator sequences and the start of the promoter could not be identified with confidence; therefore the intergenic region containing the natural *A6L* promoter was removed and the P_{i11} promoter was used to control transcription of the *A6L* gene (Figure 4-2 and Table 4-1).

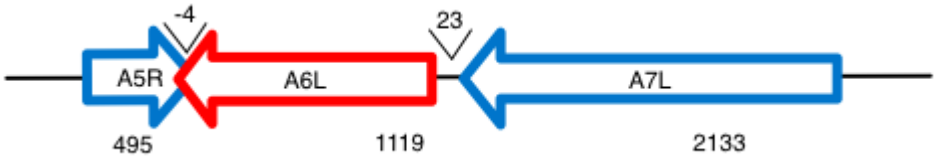

Figure 4-2. *A6L* Region of the Vaccinia Virus Genome. The *A6L* gene and surrounding genes are shown, with bp sizes of the genes (below) and intergenic regions (above). A negative size indicates an overlap between genes.

The *A7L* intergenic region (53 bp) possibly contains promoters for both the *A7L* gene and the *A8R* gene. It was not possible to accurately identify and separate the sequences for the two promoters; therefore the P_{i11} promoter was used to control the transcription of the *A7L* gene (Figure 4-3 and Table 4-1). The intergenic region was kept in the viral genome to prevent interfering with transcription of the *A8R* gene and the *A7L* natural promoter initiator sequence was changed from TAAAT to TAAGG to prevent transcription initiator from that site (Table 4-1).

Figure 4-3. *A7L* Region of the Vaccinia Virus Genome. The *A7L* gene and surrounding genes are shown, with bp sizes of the genes (below) and intergenic regions (above).

D6R has an intergenic region of 40 bp that is expected to contain only the late promoter for the *D6R* gene. The entire *D6R* intergenic sequence was used as the natural promoter of *D6R* and the *tet* O_2 sequence was inserted after the putative late initiator sequence (TAAATA) to generate the *tet*-responsive *D6R* promoter P_{iD6R} (Figure 4-4 and Table 4-1).

Figure 4-4. *D6R* Region of the Vaccinia Virus Genome. The *D6R* gene and surrounding genes are shown, with bp sizes of the genes (below) and intergenic regions (above).

The entire intergenic sequence of *E8R* (124 bp) with the *tetO*$_2$ sequence inserted after the putative late initiator (TAAATA) was used to generate P$_{iE8R}$ (Figure 4-5 and Table 4-1). The entire intergenic region, which only contains the promoter for *E8R*, was used to ensure that the entire natural promoter for the gene was included.

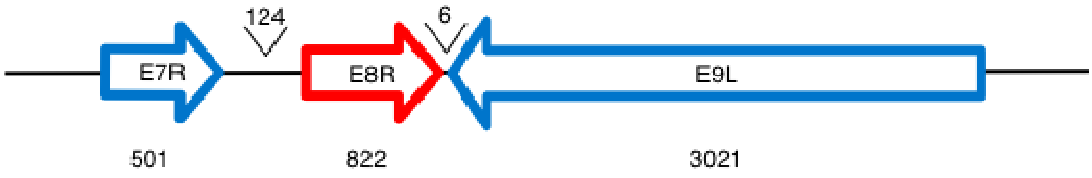

Figure 4-5. *E8R* Region of the Vaccinia Virus Genome. The *E8R* gene and surrounding genes are shown, with bp sizes of the genes (below) and intergenic regions (above).

The natural promoter for the *F17R* gene is the P$_{11}$ promoter. The intergenic region of the *F17R* gene is 62 bp and contains both the promoter for the *F17R* gene and for the *F16L* gene (Figure 4-6). The previously designed P$_{i11}$ promoter was used as the tet-responsive *F17R* promoter and 2 bp of the F17R promoter were repeated and included

as part of the F16L promoter to ensure the entire F16R promoter remains in its natural state.

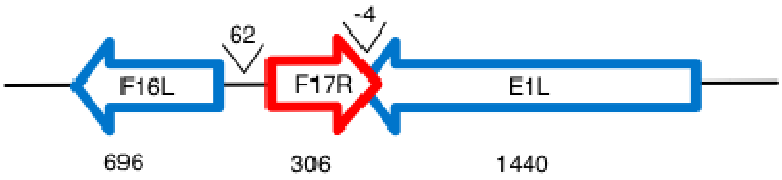

Figure 4-6. *F17R* Region of the Vaccinia Virus Genome. The *F17R* gene and surrounding genes are shown, with bp sizes of the genes (below) and intergenic regions (above). A negative size indicates an overlap between genes.

Table 4-1: Essential Gene Tet-Responsive Promoter Sequences.

Essential VACV Gene	Inducible Promoter Name	Promoter Sequence[a]
A3L	P_{iA3L}	ATAAGATTGGATATTAAAATCACGCTTTCGAGTAAAAAC TACGAATA**TAAATA**TCCCTATCAGTGATAGAGA
A6L	P_{i11}	ATATAGTAGAATTTCATTTTGTTTTTTTCTATGCTA**TAAAT A**TCCCTATCAGTGATAGAGA
A7L	P_{i11}	ATATAGTAGAATTTCATTTTGTTTTTTTCTATGCTA**TAAAT A**TCCCTATCAGTGATAGAGA
D6R	P_{iD6R}	ATATATGCTCATATATTTATAGAAGATATCACATATC**TAA ATA**TCCCTATCAGTGATAGAGA
E8R	P_{iE8R}	GTATAATCCCATTCTAATACTTTAACCTGATGTATTAGCA TCTTATTAGAATATTAACCTAACTAAAAGACATAACATA AAAACTCATTACATAGTTGATAAAAAGCGGTAGGATA**TA AATA**TCCCTATCAGTGATAGAGA
F17R	P_{i11} (P_{iF17R})	ATATAGTAGAATTTCATTTTGTTTTTTTCTATGCTA**TAAAT A**TCCCTATCAGTGATAGAGA

[a] Initiator sequences are in bold; *tetO*$_2$ sequences are underlined.

4.2.c. Construction of Transfer Vectors for viA3L, viA6L, viA7L, viD6R, viE8R, and viF17R:

A series of cloning steps was used to build the transfer vectors based on existing plasmids and designed synthetic DNA sequences. The final transfer vectors contain: (1) the selectable *E. coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene and the screening marker *EGFP* gene, as a fusion gene (*gpt-EGFP*) under control of the synthetic early/late promoter $P_{E/L}$; (2) the repressor gene *tetR* under the synthetic early/late $P_{E/L}$ promoter (Chakrabarti, et al., 1997); (3) a tet-responsive promoter to direct the expression of the essential gene; and (4) a left border sequence (600 bp of the gene upstream of the essential gene) and a light border sequence (the first 600 bp of the essential gene or intergenic region) to serve as recombination sequences for homologous recombination.

The *SphI-XmaI* fragment (containing the *gpt-EGPF* gene, *tetR* gene, and a spacer region) from pCH033 was cloned into the *SphI-XmaI* site of pCH051 (A3L), pCH052 (A6L), pCH053 (A7L), pCH054 (D6R), pCH055 (E8R), and pCH056 (F17L) (synthetic plasmids containing the essential gene tet-responsive promoter, and respective left and right recombination sequences (obtained from DNA2.0, Menlo Park, CA). This step generated the final transfer vectors: pCH057 (viA3L), pCH058 (viA6L), pCH059 (viA7L), pCH060 (viD6R), pCH061 (viE8R), and pCH062 (viF17R) (Figure 4-7).

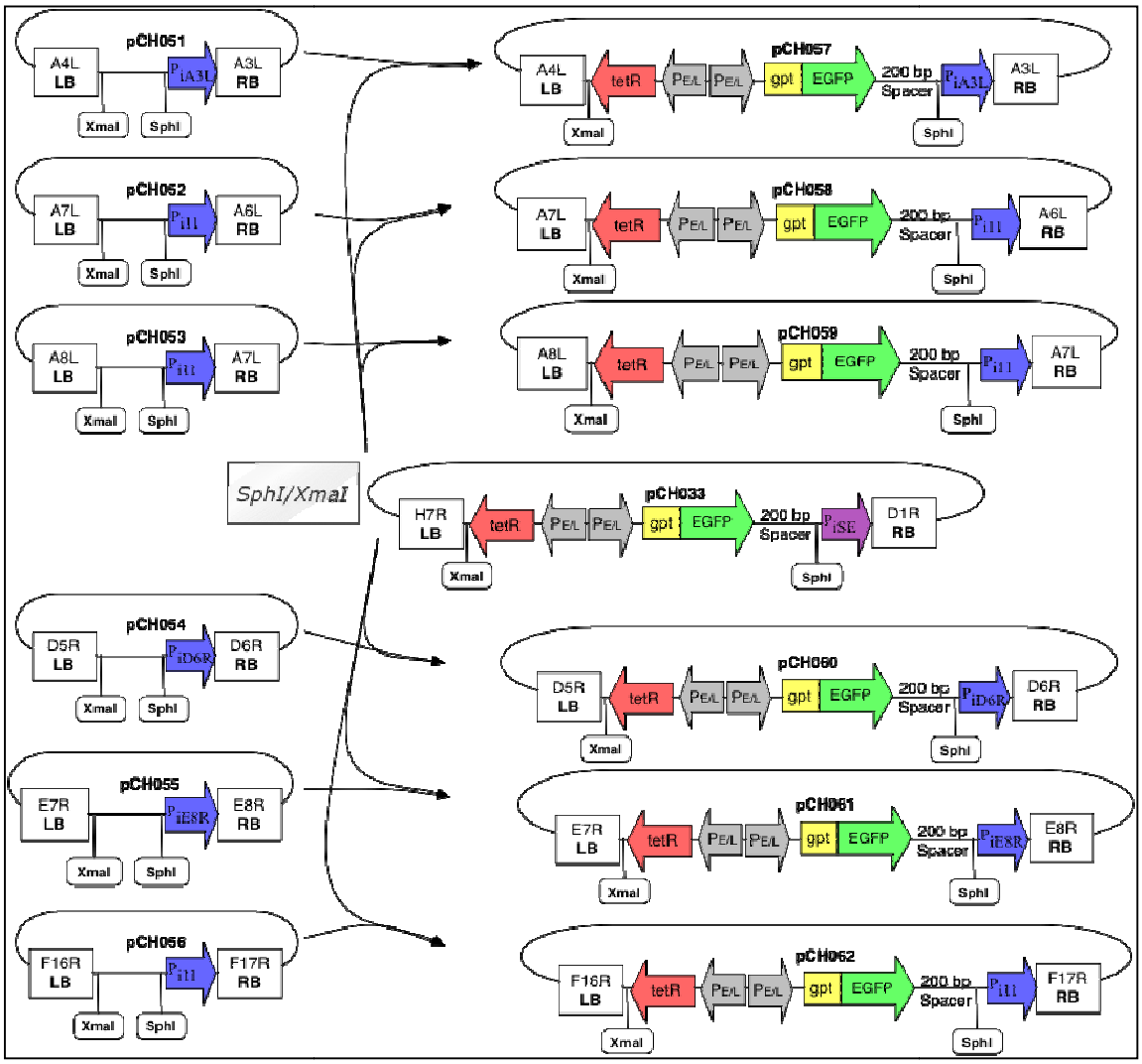

Figure 4-7. Construction of Final Transfer Vectors pCH057, pCH058, pCH059, pCH060, pCH061, and pCH062. The cloning step used to generate the final transfer vectors are depicted. All final transfer vectors express the *gpt-EGFP* and *tetR* genes under constitutive promoters. Each transfer vector was designed to express their essential gene under a tet-responsive late promoter. All of the genetic elements are flanked by left border (LB) and right border (RB) sequences, which direct homologous recombination with the VACV genome to generate their respective recombinants.

4.2.d. Generation of Recombinant Viruses:

Homologous recombination was used to precisely insert the genetic elements between the Left and Right Borders of each of the final transfer vectors into the intergenic region between the essential and upstream genes, placing the inducible promoter in front of the essential gene. The recombinant VACVs were generated by standard homologous recombination via transfection of the transfer vectors pCH057, pCH058, pCH059, pCH060, pCH061, or pCH062 into BS-C-1 cell monolayers infected 2 h earlier at 0.05 PFU per cell with VACV strain Western Reserve (WR) clone 9.2.4.8 (obtained from T. Yilma, University of California Davis). Recombinant *gpt*-positive VACVs were plaque purified on BS-C-1 cells from transfection lysates using *gpt* selection medium (25 µg/ml mycophenolic acid, 250 µg/ml xanthine, and 15 µg/ml hypoxanthine) (Legrand, et al., 2004). All recombinants were plaque-purified in the presence of inducer (1 µg/ml doxycycline). Expression of EGFP was detected via fluorescence microscopy (Carl Zeiss Axio Observer D1) to ensure that the recombinant viruses were free of parental virus. High-titer stocks were generated by infecting HeLa S3 cells with the recombinant VACVs at a multiplicity of infection (MOI) of 0.1. Infected cells were harvested 4 days post-infection by centrifugation at 200 × *g* for 10 min. Cells were then lysed by freezing and thawing, sonicated, and trypsinized. Finally, cell lysates were clarified to remove contaminating cell debris by a second round of sonication and centrifugation at 500 × *g* for 10 min. The overall genomic structure of each recombinant VACV was determined by restriction analysis and PCR analysis of viral DNA, which was purified using a small-scale method employing micrococcal nuclease (Lai and Chu, 1991).

4.2.e. The Effect of Doxycycline Concentration on Plaque Size:

The ability of the recombinant viruses to grow in the presence or absence of inducer (doxycycline) was first investigated by plaque assay. Cell monolayers in six-well plates were infected at 40 PFU/well, in the presence of 0, 1, 10, 100, or 1000 ng/ml doxycycline; photographs and measurements of isolated VACV plaques were taken 40 h postinfection with an inverted microscope. For plaque size measurements, cells were stained with crystal violet (0.5% in 20% ethanol), and the diameters of plaques were measured under an inverted microscope (Carl Zeiss Axio Observer D1) with measurement-capable software (AxioVision). Paired t-tests were used to determine the significance of doxycycline concentration on the plaque size of the different recombinant viruses and WR. Unpaired t-tests were used to determine the difference of plaque sizes between the viruses. All statistical tests were performed with the statistical software Prism (GraphPad Software Inc, San Diego, CA).

4.2.f. The Effect of Tetracycline, Doxycycline, and Anhydrotetracycline on Plaque Size:

The ability of the recombinant viruses to grow in the presence of different tetracyclines was investigated by plaque assay. BS-C-1 cell monolayers in 12-well plates were infected at 20 PFU/well, in the presence 1 µg/ml of doxycycline, tetracycline, or anhydrotetracycline. Photographs and measurements of isolated VACV plaques were taken 40 h postinfection with an inverted microscope. For plaque size measurements, cells were stained with crystal violet (0.5% in 20% ethanol), and the diameters of plaques

were measured under an inverted microscope (Carl Zeiss Axio Observer D1) with measurement-capable software (AxioVision).

4.2.g. Viral Titers with Varying Doxycycline Concentrations:

BS-C-1 cells in 12 well plates were infected at an MOI of 0.01 with WR, viA3L, viA6L, viA7L, viD6R, viE8R, or viF17R. After 1 h, virus was aspirated and DME/2.5% FBS with 1000, 100, 10, 1, or 0 ng/ml doxycycline was added. Cells were collected either 0 h (immediately after the 1 h infection) or at 48 h post-infection. The intracellular fraction of virus was collected: cells were removed from the wells, centrifuged at 300 x g for 10 min to pellet the cells, supernatant (containing extracellular virus) was removed and the cells were resuspended in 500 µl of DMEM. The intracellular fraction was processed and titered on BS-C-1 cells as previously described (Verardi, et al., 2001), in the presence of 1 µg/ml of doxycycline.

4.3. RESULTS

4.3.a. Construction of Recombinant Viruses:

Recombinant viruses were successfully constructed and plaque purified. PCR of viral DNA, purified by small-scale miccrococcal nuclease method, showed all recombinants to have the genetic elements correctly inserted into the VACV genome (data not shown).

4.3.b. Plaque Assays and Plaque Size:

All of the recombinant viruses display unique growth characteristics in response to doxycycline. Overall, the recombinants viA6L, viA7L, viD6R, and viF17R all displayed a degree of dependence on doxycycline for viral plaque formation. Recombinant viA3L and viE8R did not (Figures 4-8 and 4-9).

Recombinants that were dependent on doxycycline for plaque production produced abortive infections in the absence of doxycycline. Infected cells could be detected by the expression of EGFP, but no plaques were formed after 48 h post-infection (Figure 4-10) or even after 1 week post-infection (data not shown).

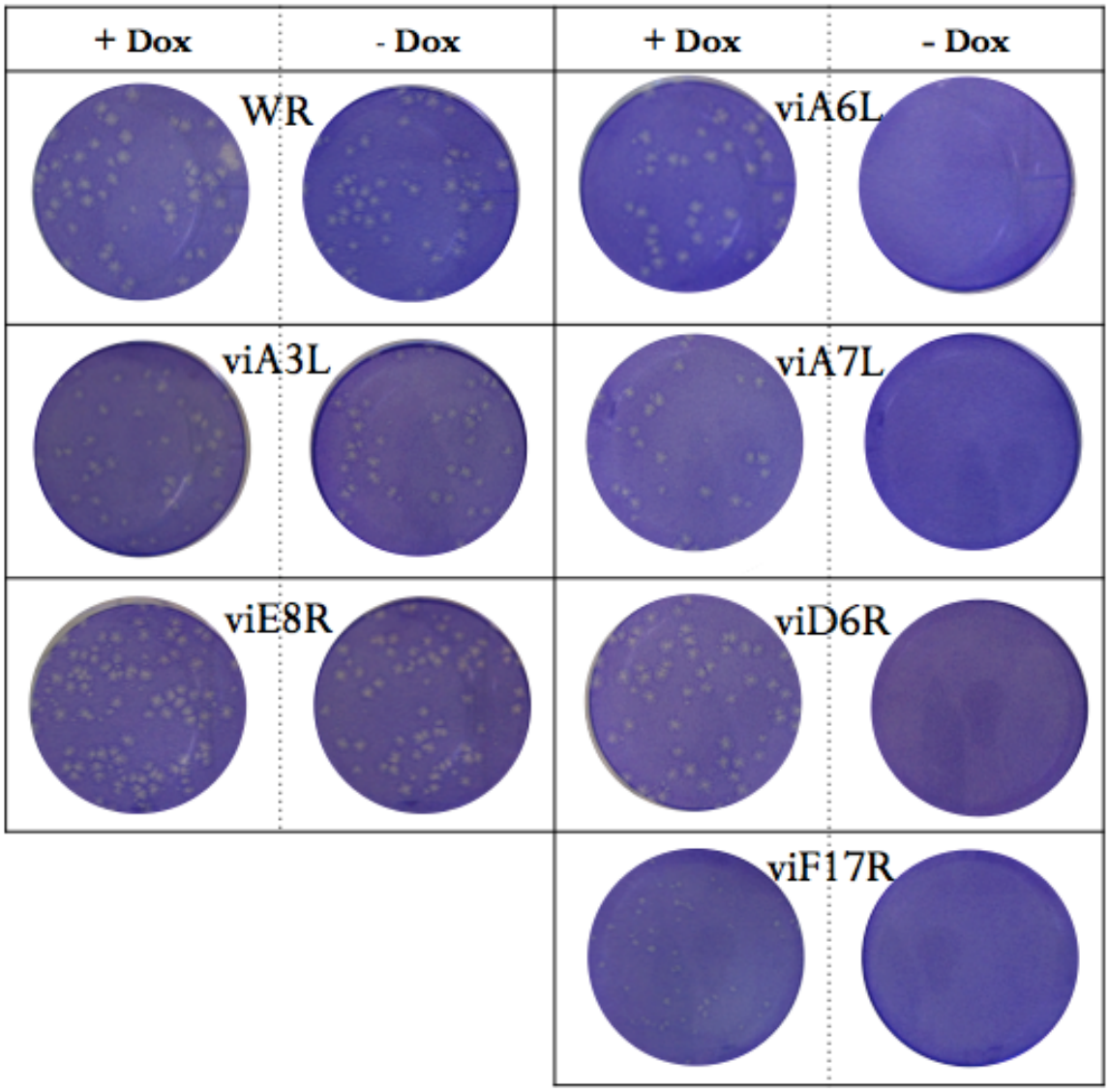

Figure 4-8. Viral Plaques in the Presence and Absence of Doxycycline. Wells were infected with 50 PFU of virus and grown the presence of absence of 1 µg/ml doxyxycline (Dox), after approximately 48 h cells were stained with crystal violet.

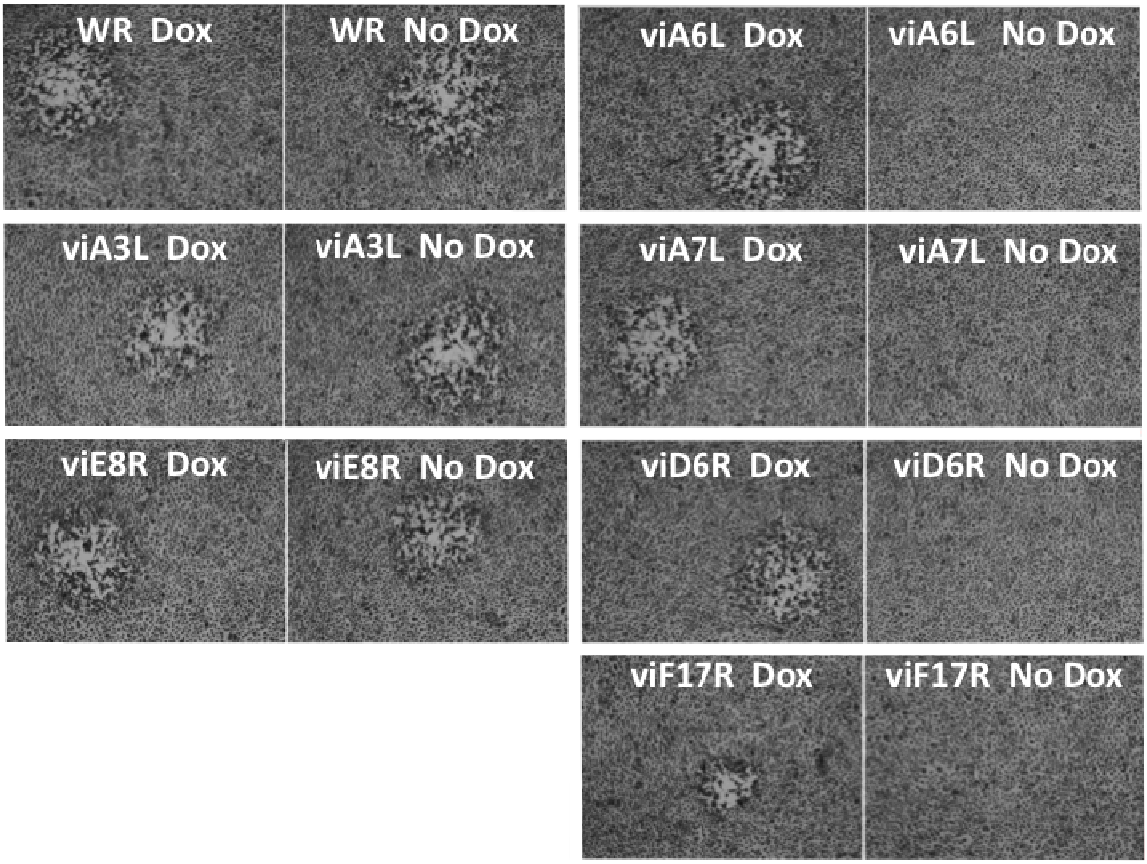

Figure 4-9. The Effect of Doxycycline on Plaque Size. Virus was grown in the presence or absence of 1 µg/ml doxycycline (Dox) for 40 h. After 40 h cells were stained with crystal violet, a representative image from each treatment is shown above.

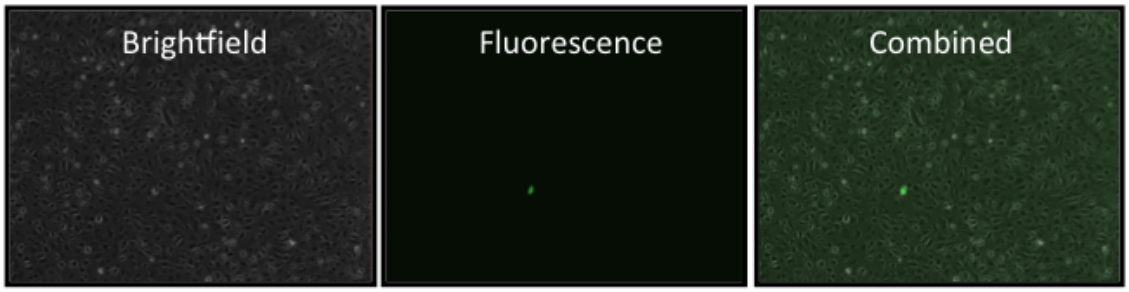

Figure 4-10. Abortive Infection in the Absence of Doxycycline. A single infected cell is shown in the images, the infected cell is detected by the expression of EGFP (seen in the fluorescence and combined images). The singe infected cell is not detectable in the brightfield image, as the expression of EGFP is the only indication that virus is present within the cell.

The recombinant viA3L is not dependent on doxycycline for replication, but at all doxycycline concentrations tested, viA3L produced slightly smaller plaques than WR. For example, at 1000 ng/ml, the average plaque radius for viA3L was 563.3 µm, compared to 645.6 µm for WR. However, this difference was observed even in the absence of doxycycline, where the average plaque radius for viA3L was 582.4.3 µm, compared to 653.1 µm for WR, perhaps indicating that viA3L does not express the A3 protein at wild type levels.

viE8R shows some dependence on doxycycline for replication, since plaque size decreased at 1 ng/ml and 0 ng/ml doxycycline concentration (Figure 4-11 and Table 4-2). However, even with no doxycycline present, viE8R was able to replicate and produce plaques. At 100 ng of doxycycline the plaque radius of WR and viE8R did not differ significantly.

Recombinants viA6L, viA7L, viD6R, and viF17R all were unable to form plaques in the absence of doxycycline (Figure 4-11 and Table 4-2). viA6L formed plaques at

1000, 100, and 10 ng/ml of doxycycline that were not significantly different in size. However, at 1 and 0 ng/ml doxycycline no plaques formed, only abortive infections occurred. At 1000 ng/ml doxycycline the average plaque radius of viA6L (628.2 µm) was not significantly different from WR (636.8 µm).

viA7L is also dependent on doxycycline for plaque formation; the virus was able to form plaques at 1000, 100,10, and 1 ng/ml of doxycycline, but only abortive infections were seen in the absence of doxycycline (Figure 4-11 and Table 4-2). Plaques produced in the presence of 1000, 100 or 10 ng/ml doxycycline did not differ in size, but plaques produced at 1 ng/ml doxycycline were significantly smaller at 216.6 µm average radius. Also, at 1000 ng/ml doxycycline the average plaque size produced by viA7L was significantly smaller (559.9 µm) than WR (636.8 µm).

viD6R has growth characteristics similar to viA7L, in that it produces plaques at 1000, 100, 10 and 1 ng/ml doxycycline, but does not produce any plaques in the absence of doxycycline (Figure 4-11 and Table 4-2). The plaque sizes produced by viD6R at 100 ng/ml are significantly larger than at 1000, 10 or 1 ng/ml. A doxycycline concentration of 1 ng/ml produces significantly smaller plaques (average of 320.1 µm) than at 1000, 100, or 10 ng/ml. The average plaque size of viD6R at 100 ng/ml doxycycline (628.4 µm) is not significantly different than the plaques produced by WR.

The recombinant viF17R responded to doxycycline differently than all other recombinants. The virus appeared to be attenuated; plaques were only produced at 1000 and 100 ng/ml of doxycycline, and the plaques were much smaller than plaques formed by any of the other viruses at these concentrations (Figure 4-11 and Table 4-2). The

average plaque sizes were 242.7 µm and 281.1 µm respectively. No plaques were formed at 10, 1 and 0 ng/ml doxycycline.

Table 4-2: Average Plaque Radius (µm) of Recombinant and Wild-type (WR) Vaccinia Viruses under Different Doxycycline Concentrations.

Doxycycline Concentration (ng/ml)	Average Plaque Radius (µm)[a]						
	WR	viA3L	viA6L	viA7L	viD6R	viE8R	viF17R
1000	645.4	563.3	628.2	559.9	566.4	650.4	242.7
100	616.9	591.9	645	570.9	628.4	626.1	281.1
10	627.1	579	593.5	590	559.5	627.3	AB
1	641.5	611.6	AB	216.2	320.1	603.8	AB
0	653.1	582.4	AB	AB	AB	554.2	AB

[a] AB = abortive infection.

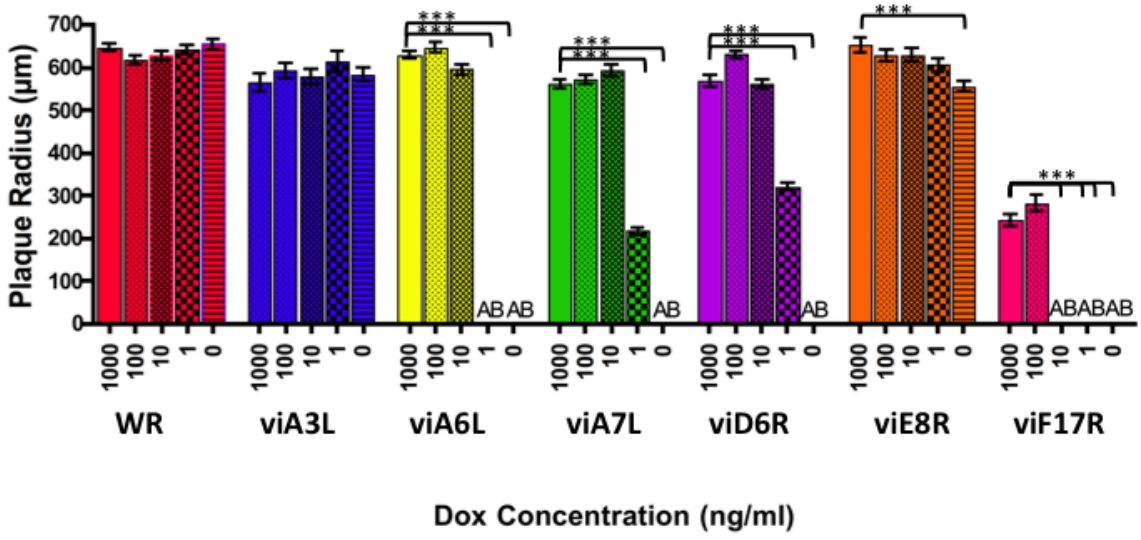

Figure 4-11. The Effect of Doxycycline Concentration on Plaque Size. The effect of doxycycline concentration on plaque radius is depicted. BS-C-1 cells were infected with 40 PFU/well of virus and allowed to grow in the presence of 0, 1, 10, 100, or 1000 ng/ml

doxycycline for 40 h. At 40 h plaque sizes were measured. Error bars = SEM. *** = p < 0.0001 (paired t-test).

4.3.c. Effect of Doxycycline, Tetracycline, and Anhydrotetracycline on Plaque Size:

No significant differences in the size of plaques were observed among the different recombinant viruses and WR grown in the presence of 1 µg/ml doxycycline, tetracycline, or anhydrotetracycline (Figure 4-12).

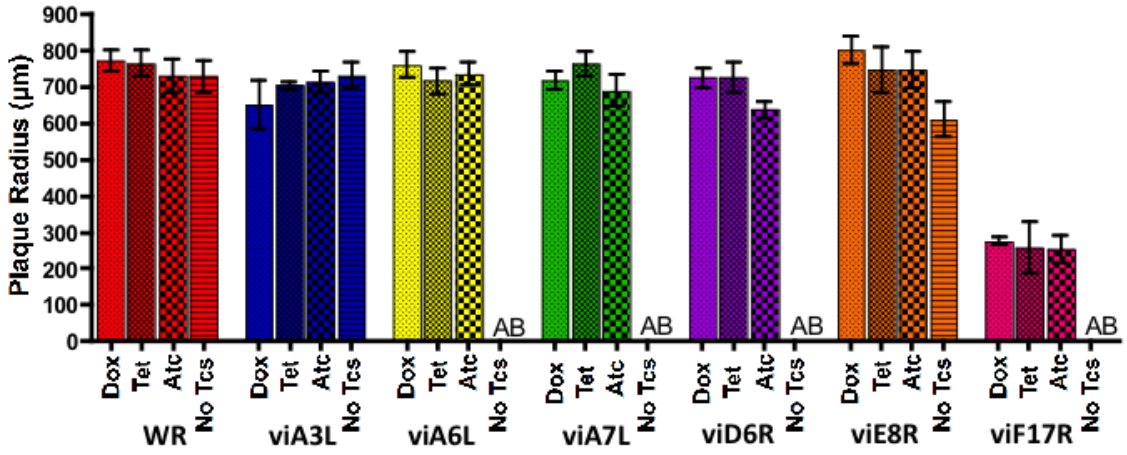

Figure 4-12. The Effect of Tetracyclines (1 µg/ml) on Plaque Size. The effect of different tetracyclines on plaque radius is depicted. BS-C-1 cells were infected with 20 PFU/well of the virus and allowed to grow in the presence of doxycycline (Dox), tetracycline (Tet), anhydrotetracycline (Atc) (1 µg/ml), or no tetracyclines (Tcs). No significant differences were observed among the different tetracyclines. Error bars = SEM.

4.3.d. Viral Titers with Varying Doxycycline Concentrations:

With the exception of viD6R, the viral titers obtained 48 h after infection of BS-C-1 cells at an MOI of 0.01 in the presence of varying doxycycline concentrations reflected the plaque sizes that were observed in the single plaque analysis (Figures 4-13 and 4-14). As expected, WR, viA3L, and viE8R showed no dependence on doxycycline for viral replication, while the titers of viA6L, viA7L, and viF17R were dependent on doxycycline. Recombinant viA6L had a high titer for 1000, 100, and 10 ng/ml doxycycline, which dropped rapidly from 8.5×10^6 PFU/ml at 10 ng/ml to 40 PFU/ml at 1 ng/ml doxycycline and remained close to that level at 0 ng/ml doxycycline. viA7L showed a more gradual decrease in titer. The titer dropped from 7.2×10^6 PFU/ml at 10 ng/ml doxycycline to 1.9×10^4 PFU/ml at 1 ng/ml and finally to 0 PFU/ml in the absence of doxycycline. The results for viA6L and viA7L mimic what was seen when observing plaque size.

The titers of viD6R did not follow the observation of plaque size. When measuring plaque size, no plaques were seen in the absence of doxycycline, however the titer of viD6R at 0 ng/ml was 1.05×10^5 PFU/ml, much higher than that observed for viA7L or viA6L (both of which also did not produce plaques in the absence of doxycycline). The attenuation of viF17R was also apparent in the titers. Although no viral plaques were observed at 10, 1, or 0 ng/ml doxycycline, the viral titers at 10 and 1 ng/ml doxycycline were increased in comparison to the absence of doxycycline.

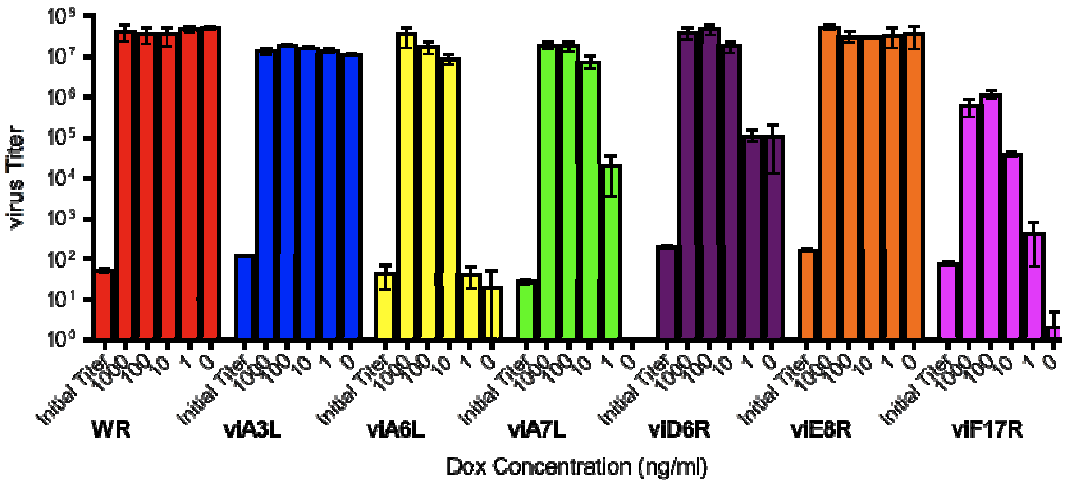

Figure 4-13. The Effect of Doxycycline Concentration on Viral Titers. BS-C-1 cells in 12 well plates were infected at an MOI of 0.01 for 1 h and either collected immediately (Initial Titer) or allowed to grow in the presence of 0, 1, 10, 100, or 1000 ng/ml doxycycline (Dox) for 48 h. Intracellular virus was processed and titered. Error Bars = SD.

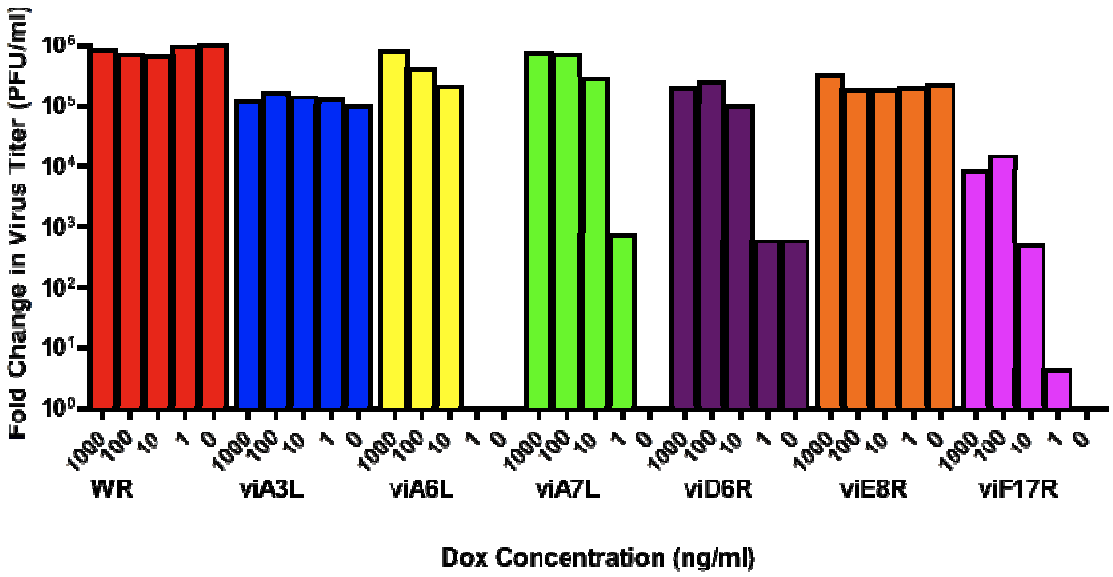

Figure 4-14. The Effect of Doxycycline Concentration on Viral Titers. BS-C-1 cells in 12 well plates were infected at an MOI of 0.01 for 1 h and either collected immediately

(Initial Titer) or allowed to grow in the presence of 0, 1, 10, 100, or 1000 ng/ml doxycycline (Dox) for 48 h. Intracellular virus was processed and titered. Fold change was calculated as the end titer (48 h) compared to the initial (input) titer (0 h).

4.4. DISCUSSION

Both viA3L and viE8R display little or no dependence on the presence of doxycyline for replication. This result was unexpected as there is evidence of both genes being critical to viral replication. Both viA3L and viE8R utilize their natural promoters with the operator sequence inserted prior to translational start site of the essential gene. The exact location of the promoter sequences for both *A3L* and *E8R* are unknown and the operators may have been inserted in a less than ideal location, causing the gene to be insufficiently repressed.

The recombinant viF17R was attenuated; replication even in the presence of 1 µg/ml doxycycline was very low compared to all other recombinants and WR. This effect was seen previously using the *lac* operon system to control F17R gene expression (Zhang and Moss, 1991a). In the *lac* inducible F17R virus the intergenic region of F17R was not modified except to insert the operator sequence. When creating the viF17R recombinant, the intergenic region was split at the end of the F17R promoter by the inserted genetic elements. This left only 34 bp of the intergenic region for the F16L promoter, if the entire F16L promoter was not located within the 34 bp, it may have affected the expression of F16L. In previous studies F16L was found to be nonessential to viral replication in cell culture and this modification to viF17R should not have decreased viral replication (Senkevich, et al., 2011). In addition, the viF17R gene

product, p11, may be required in such high amounts that any modification of this strong late promoter decreases transcription, causing viral replication to also be reduced.

Although viF17R plaque formation is prevented even when 10 ng/mL of doxycycline is present, the inability of the virus to replicate at levels comparable to WR even at high doxycycline concentrations makes this recombinant a less than ideal vaccine candidate. The low rate of replication would cause the vaccine to be attenuated and to produce an inferior immune response compared to a non-attenuated recombinant.

The recombinant viA6L replicated at levels similar to WR at 1000, 100, and 10 ng/ml of doxycycline. No replication was seen at 1 ng/ml doxycycline. These growth characteristics are highly desirable for a doxycycline dependent vaccine. This is the first successful VACV containing an inducible A6L gene. Thus, the viA6L recombinant may also be useful in clarifying the specific role of this gene. The arrangement of genomic elements used for this system is unique in that all elements were inserted into the intergenic region of the gene, this did not require moving the gene of interest to a different location within the genome, as is frequently done when using the *lac* operon system. Recombinant VACV encoding an inducible viA6L gene had previously been attempted using the *lac* operon. However, replacing the A6L promoter with an inducible promoter or inserting an inducible copy of the *A6L* gene and replacing the *A6L* gene with a GFP cassette both failed to generate an inducible A6L mutant (Meng, et al., 2007). The replacement of the *A6L* gene with a GFP cassette may have failed to produce a viable recombinant because removing the *A6L* gene also removes the last 4 bp of the *A5R* gene (RNA polymerase 19kDa subunit). It is unknown why replacing the A6L promoter with an inducible promoter also failed to generate an inducible mutant. It is possible that the

gene was unable to be induced to a high enough level to meet the requirement of A6L using the *lac* operon system (Meng, et al., 2007).

The recombinant viA7L is also dependent on doxycycline for viral replication. This virus was able to replicate at 1000, 100, 10, and 1 ng/ml doxycycline, but was unable to replicate in the absence of doxycycline. Although viA7L was able to replicate in the presence of only 1 ng/ml doxycycline, its replication was greatly reduced compared to the higher doxycycline concentrations. An inducible A7L mutant, previously generated using the *lac* operon system, was shown to replicate at levels lower than WR (Hu, et al., 1998). Our viA7L recombinant appears to replicate at levels much closer to WR than the *lac* operon mutant previously generated. This may be due to the different operon systems used and differences in the genetic setup of the viruses. In the *lac* operon mutant, the *A7L* gene is moved to the HA (non-essential) region of the VACV genome and placed under the control of a T7lacO promoter and the original *A7L* gene is replaced by a neomycin resistance gene. A T7 RNA polymerase gene is inserted into VACV in a non-essential region (TK region) under the control of a P11lacO promoter. This setup has two *lacO*-regulated steps, the IPTG dependent transcription of the T7 RNA polymerase and the IPTG/T7 RNA polymerase dependent transcription of A7L. The system used to make our recombinants only involves one *tetO*-regulated step and does not involve changing the location of the essential genes, the combination of which appears to allow the viA7L recombinant to replicate at levels close to WR.

Plaque size does not necessarily correlate to viral replication. While plaque size is a good correlate for some of the recombinants, it is not true for all recombinants. The recombinant viD6R, similar to viA7L, formed plaques in the presence of 1000, 100, 10,

or 1 ng/ml doxycycline, although at 1 ng/ml doxycycline plaques were significantly smaller. Interestingly, viD6R was unable to produce plaques in the absence of doxycycline, but the virus titer of viD6R in the absence of doxycycline did increase (compared to the initial or input titer), although it was still severely reduced compared to viD6R grown in the presence of doxycycline. These results are similar to those generated using the *lac* operon system to control the *A7L* gene. In the absence of inducer (IPTG) no plaques formed but the virus titer did increase slightly (Hu, et al., 1996).

Tetracyclines are a class of broad spectrum antibiotics. This class includes naturally occurring antibiotics such as tetracycline, and synthetic tetracyclines such as doxycyclines. Three different tetracyclines (tetracycline, doxycycline, and anhydrotetracycline) were tested for their effect on viral replication of the recombinants. Typically tetracycline or doxycycline are used as inducers of tet operon systems. Anhydrotetracycline is known to bind TetR with 35-fold higher affinity than tetracycline (Degenkolb, et al., 1991). Viruses treated with different tetracyclines (doxycycline, tetracycline, and anhydrotetracycline) at a concentration of 1 µg/ml all produced plaques of similar size. While this is strongly indicative that the different tetracyclines do not have an effect on viral replication, this cannot be definitively concluded without the testing of viral titers.

The observation that anhydrotetracycline does not increase the plaque size of the recombinant viruses in comparison to equal concentrations of doxycycline suggests that 1 µg/ml of doxycycline is sufficient to induce maximum expression levels of the essential gene products and that the apparent attenuation of viF17R is not due to insufficient levels of inducer.

The goal of this research was to develop recombinant VACVs in which viral replication can be controlled through the addition/removal of tetracyclines. By placing the *A6L*, *A7L*, *D6R*, and *F17R* genes under the control of the *tet* operon, inducible viruses were generated. Each recombinant responds uniquely to doxycycline. Based on the current analysis of the recombinants the viA6L virus appears to have the most desirable growth characteristics for VACV vectors. The ability of viA6L to replicate at relatively low doxycycline concentrations (10 ng/ml) at a rate similar to wild type would allow the antibiotic dose given with the vaccine to be kept at a low and safe level for the vaccine recipient. The abrupt cease of viral replication between 10 ng/mL and 1 ng/mL that viA6L displays is also a desirable trait in a doxycycline dependent vaccine. If the vaccine can be induced with a low dose of doxycycline, when treatment is stopped the doxycyline concentration within the body should fall to the critical concentration quickly, rapidly stopping virus replication. Similarly, viA7L is also a good vaccine candidate; however, this virus did replicate at reduced levels at 1 ng/ml doxycycline. For replication to be induced at wild-type levels, 10 ng/ml doxycycline had to be used. This suggests that for a good take to occur upon vaccination, viA7L would require a dose of doxycycline similar to A6L. However, viral replication could be more difficult to stop, as 1 ng/ml doxycycline would be sufficient to induce the replication of viA7L. If an adverse event occurs after vaccination once doxycycline treatment is stopped, the level within the body would have to decrease to below 1 ng/ml (rather than to below 10 ng/ml for viA6L) to stop viral replication and the adverse event.

The recombinant viD6R appears to be a good vaccine candidate based on the plaque size assays, which show no evidence of viral replication in the absence of

doxycycline. However, the viral titers of viD6R do increase even in the absence of doxycycline, raising the question of whether this gene would be ideal for vaccine and therapy vectors.

While viF17R was inducible by tetracyclines, it appears to be attenuated which is not ideal for smallpox vaccination. This recombinant was unable to replicate at levels similar to wild type even in the presence of high levels of inducer. Recombinant viA3L and viE8R were not dependent on tetracyclines for replication, and in their current state are not good vaccine vector candidates. However, this does not indicate that these genes may not be useful for controlling VACV replication. There is strong evidence suggesting that both viA3L and viE8R are essential to VACV replication. As mentioned before, the *tetO* sequences may have been inserted in a non-ideal location, allowing leaky or full expression of the gene. It would be interesting to test whether viral replication would be controlled if the P_{i11} promoter was used in place of the natural promoters.

This work also suggests that more knowledge is needed about VACV promoters to be able to utilize natural VACV promoters in the control of gene expression. In this research three natural promoters and three P_{i11} promoters were used to control gene expression. All of the viruses that utilized the P_{i11} promoters were successful in producing tetracycline dependent recombinants (viA6L, viA7L, viF17R), whereas only one of the viruses that utilized a natural promoter with a *tetO* sequence was inducible (viD6R). This may suggest that incorporation of the *tetO* sequence after the TAAATA initiator may not be ideal for every late VACV promoter or that unrecognized sequences within the promoters are acting as initiator sequences.

DISCUSSION AND FUTURE DIRECTIONS

Four successful inducible recombinant VACVs were generated (viA6L, viA7L, viD6R, and viF17R) that have many potential uses. The same strategy could be used for the development of safer, new generation smallpox vaccines, replacing ACAM2000. They could also be used for the development of new generation viral vectors for both human and animal vaccines, and for oncolytic vectors. They would provide a safety mechanism that would benefit not only the recipients of the vaccine or therapy, but also personnel administering the vectors (e.g., vaccinators) and contacts of the vaccine/therapy recipients.

Another important potential use of the inducible VACVs would be to replace the *TetR* with a *reverse TetR* gene, thus creating a repressible VACV. Mutagenesis studies have shown that the response of the TetR repressor can be reversed, causing TetR to act as an inducible repressor. A variety of single and multiple mutations in the *tetR* gene are able to produce this phenotype. The reverse form of the protein, revTetR, is only able to bind to the operator sequence and block transcription in the presence of tetracyclines (Gossen, et al., 1995, Resch, et al., 2008, Scholz, et al., 2004). While different tetracyclines can be used with the TetR repressor, the effect of tetracyclines on revTetR varies greatly (Gossen, et al., 1995). The revTetR repressible system is currently being adapted to the VACV system (Titong and Verardi, unpublished data).

The repressible VACVs would have similar applications as the inducible VACVs, but instead of requiring tetracyclines for viral growth, tetracyclines would stop viral replication. A repressible system would be especially beneficial in animal vaccines, such as the oral rabies vaccine. The oral rabies vaccine, which is composed of a VACV vector, is used across the United States to vaccinate wildlife against rabies. There are

currently 16 states distributing the vaccines in baits for wildlife (Slate, et al., 2009). The baits are composed of a plastic packet containing the vaccine and coated in fishmeal to attract animals. Once an animal bites the bait the packet of vaccine is broken and the vaccine leaks into the mouth, resulting in viral replication and immunization of the animal (Slate, et al., 2005). Although oral rabies vaccination programs are careful of where baits are dropped, inevitably people come in contact with the baits and the VACV vaccine vector they carry. One such incident occurred in Pennsylvania in 2009 when a dog brought its owner a ruptured vaccine bait. The owner had cuts on her hands and developed a VACV infection from handling the bait. As the owner was on immunosuppressive medication for inflammatory bowel disease, treatment with VIGIV (Vaccinia Immune Globulin Intravenous) and investigational antiviral agents were required to clear the VACV infection. However, she was not able to remain off her immunosuppressive medication for an extended period, making her treatment difficult (Centers for Disease Control and Prevention, 2009). If a repressible VACV had been used in the rabies vaccine the woman could have been treated with tetracyclines to stop the VACV infection and may not have needed to be removed from her medication.

The inducible VACV generated have many practical uses and will hopefully allow VACV to be utilized more frequently as a vector to develop life-saving vaccines and therapeutics.

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