

AN ABSTRACT OF THE THESIS OF

Jessica M. Page for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on May 25, 2007. Title: Investigation of the Roles of Viral Proteinases in the Vaccinia Virus Life Cycle.

Abstract approved: _____
Dennis Hruby

An inducible, mutant virus, designated vvtetO:I7L/G1L, was used to study the morphogenic proteolysis step of the vaccinia virus life cycle. The vvtetO:I7L/G1L controlled the expression of two genes, I7L, a cysteine proteinase, and G1L, a putative metalloproteinase. These proteins are involved in the maturation of viral core proteins, p4a, p4b, and p25K, to form infectious virions. DNA extraction and genomic sequencing verified the correct insertion of the tetracycline operators. The multiplicity of infection (MOI) was optimized, and a MOI of 0.5 was best, with a 99.25% reduction in viral plaque formation compared to the wild type vaccinia virus. A growth curve over 12 hours was done and the vvtetO:I7L/G1L in the “on” state closely followed the growth kinetics of the wild type vaccinia virus and the vvtetO:I7L/G1L in the “off” state had significantly lower viral titers throughout the last 6 hours of the cycle. Viral core protein processing in the “on” and “off” states, and in rescue experiments with transfected plasmids containing the I7L or G1L genes, was examined by immunoblot assay. Protein processing was difficult to effectively visualize, with the best processing occurring at a MOI of 0.2, using Fugene as a transfection reagent.

Key Words: I7L, G1L, proteolysis, vaccinia, tetracycline

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Investigation of the Roles of Viral Proteinases
in the Vaccinia Virus Life Cycle

By

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I understand that my project will become a part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Investigation of the Roles of Viral Proteinases in the Vaccinia Virus Life Cycle

Introduction

Smallpox, caused by variola virus, has become a recognized threat in bioterrorism, and due to the nature of its life cycle, a post-exposure drug is needed in addition to the current vaccine to prevent and treat disease. Variola virus has a 14 day incubation period during which it is replicating but symptoms are not visible. After two weeks have passed, flu-like symptoms occur with pock formation that initially could be mistaken for chicken pox or allergic dermatitis. The patient is highly contagious at this point. Thus, it may be nearly three weeks before a smallpox diagnosis is made, allowing the virus to spread and become a major problem. Vaccinations would be effective in the unexposed population, but the individuals previously exposed or unable to be vaccinated would need an antiviral drug to prevent disease [1]. Vaccinia virus belongs to the orthopoxvirus family and is closely related to variola virus, the causative agent of smallpox. Vaccinia virus is being used to study replication mechanisms that are conserved in variola virus. These studies should enable the identification of drug targets that can be used to create antiviral drugs to prevent or treat smallpox.

The life cycle of vaccinia virus begins with the attachment and entry of the EEV, extracellular enveloped virus, into a host cell. In the cytoplasm, the membranes are removed and the viral core is activated to initiate early gene expression [2]. DNA

replication begins concurrently with intermediate gene expression resulting in the formation of the virosome. This signals the beginning of late gene expression and virion assembly into the IV, intracellular virus [3]. The membranes are acquired to envelope IV. The origin of these membranes is unknown. They may be formed *de novo* or derived from budding through the intermediate compartment, between the endoplasmic reticulum and Golgi network [4]. The core proteins within the IV are cleaved via morphogenic proteolysis to form the characteristic bi-concave core producing the infectious IMV, intracellular mature virus. The IMV then acquires membranes from trans-Golgi network and becomes the IEV, intracellular enveloped virus [4]. The IEV fuses with the plasma membrane, resulting in either the CEV, cell-associated enveloped virus, or the EEV, extracellular enveloped virus [5].

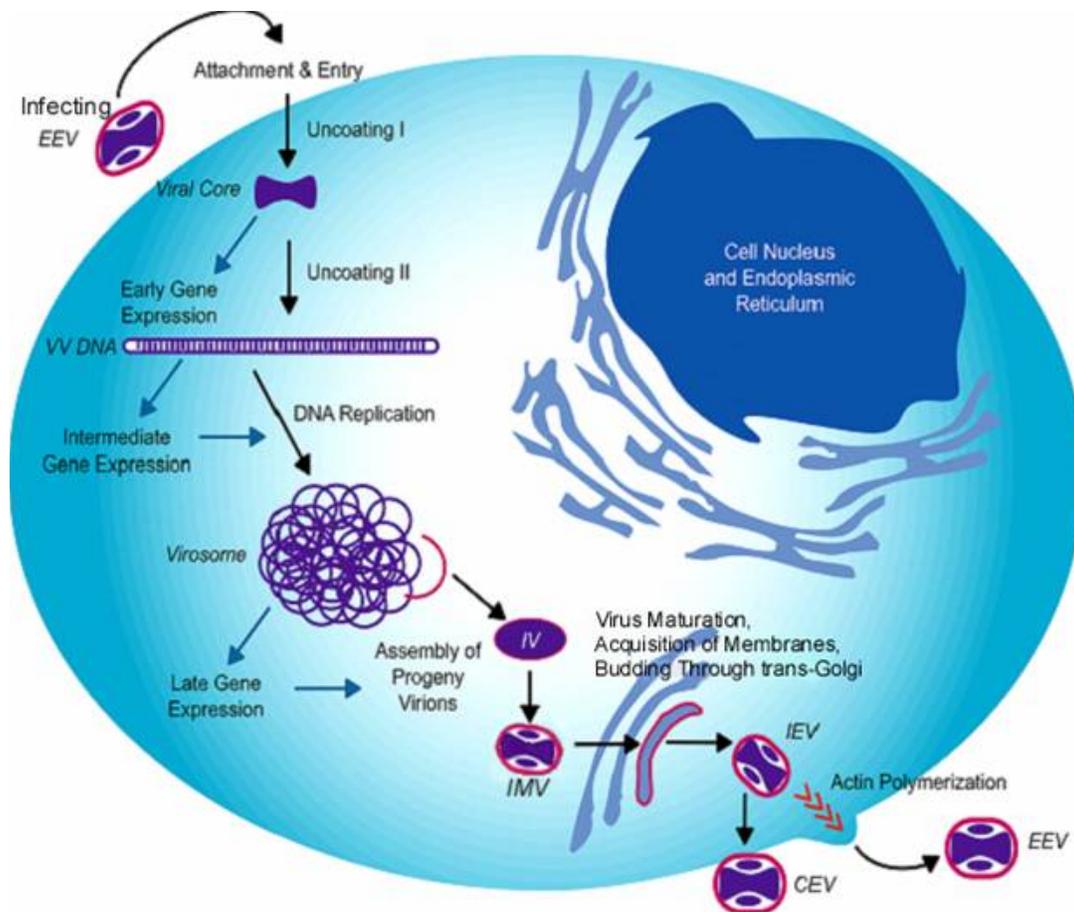


Figure 1. Vaccinia virus life cycle. The vaccinia virus life cycle, beginning with entry and attachment and ending with mature virion exit is shown, with all viral stages indicated [adapted from Hruby *et al*, unpublished data].

A major step used during the vaccinia virus life cycle is morphogenic proteolysis that allows the virion to acquire infectivity through cleavage of the core proteins to produce the IMV [6]. Proteolysis is a process by which large polyprotein precursors are selectively cleaved to produce functionally active smaller proteins. There are two types of proteases, peptidases and proteinases [7]. Peptidases hydrolyse single amino acids from the N' or C' termini while proteinases cleave between specific amino acids from within a protein [7]. There are four subtypes of proteinases, serine, cysteine, aspartic, and metalloproteinases [8]. In general, proteinases have a catalytic site and substrate binding

pocket [9]. The substrate binding pocket is usually formed by two globular domains which create unique conformations that dictate proteinase specificity. In order for a protein to be cleaved, it must contain amino acids with specific side chains that define the susceptible bond [7]. A protein must also have the susceptible bond located adjacent to a flexible region that is accessible to the proteinase and fits the active site binding pocket [10]. Morphogenic proteolysis is a process in which viral structural proteins are cleaved to produce proteins that will produce a functional structure [11]. This is often required to achieve infectivity, as is the case with vaccinia virus [11,12] Two proteins involved in the morphogenic proteolysis step of the vaccinia virus life cycle have been identified, a putative metalloproteinase, G1L, and a cysteine proteinase, I7L. Byrd and Hedengren-Olcott have demonstrated that these proteins must be expressed for successful production of progeny virions. [13, 14] A tetracycline operator/repressor system was used to investigate the activity of the I7L proteinase which was found to be a late gene involved in viral core formation. When I7L was inhibited, virion core maturation did not occur, preventing the formation of infectious IMV particles. Electron microscopy revealed crescent shaped cores, indicating that I7L is involved in the maturation of the viral core proteins to the infectious, biconcave phenotype [13]. A tetracycline operator/repressor system was also used to investigate the activity of the putative G1L metalloproteinase. It was found that this proteinase is involved in the late stages of viral development, apparently after I7L has acted [15]. Electron microscopy showed an accumulation of immature oval shaped viral particles without progression to IMV. However, G1L has not been biochemically confirmed as a metalloproteinase, but has been identified as necessary for viral maturation. [14] It is believed that G1L and I7L act in tandem in a

proteolytic cascade to form the bi-concave, mature viral core particles. However, the regulation and biochemistry of this process has not yet been elucidated [15].

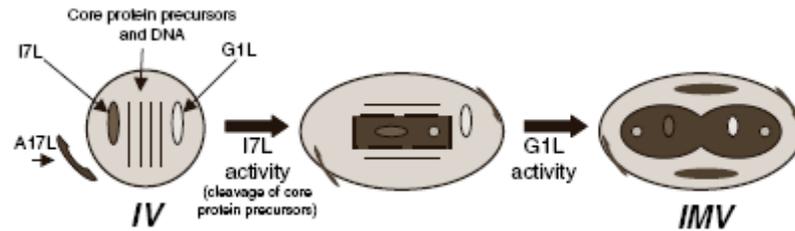


Figure 2. Model of proteolysis in vaccinia virus morphogenesis. During the transition from an IV to an IMV particle there are a series of proteolytic cleavage events including the cleavage of the major core protein precursors by I7L and followed by the activity of G1L to lead to infectious virus particles [15].

The major core proteins involved in this morphogenic proteolysis step are the p4a, p4b, and p25K proteins, derived from the A10L [16], A3L [17], and L4R [18] open reading frames (ORF) respectively. They are cleaved as shown in figure 3.

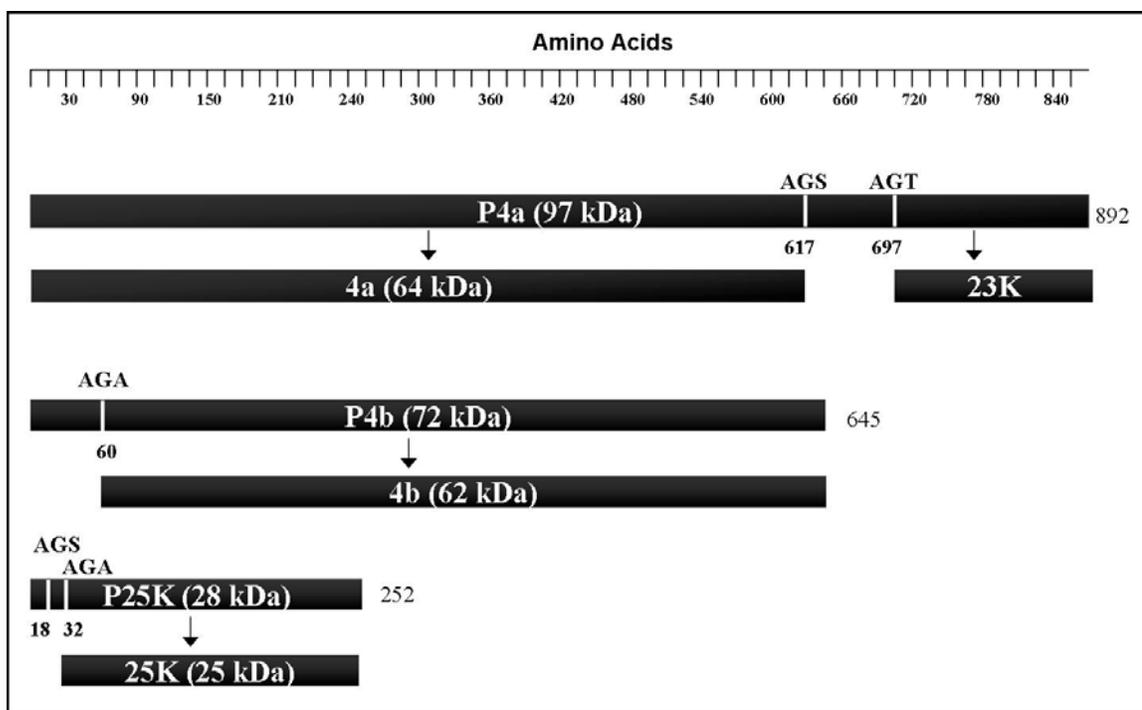


Figure 3. Viral core protein cleavage sites. The core vaccinia viral proteins are shown with cleavage sites indicated and resulting cleavage products. The amino acid locations of the AGX cleavage sites are shown according to the amino acid map above [19].

These proteins are cleaved at AGX cleavage sites, a conserved sequence throughout the major open reading frames of core protein precursors in the vaccinia virus genome. The viral core is produced through contextual processing, a procedure in which the viral proteins are cleaved as they are assembled [15].

Since the morphogenic proteolysis step is crucial for viral development, investigation of its mechanisms could lead to identification of a potential drug target. By characterizing and learning more about the I7L and G1L proteins the vaccinia virus life cycle, and thus the variola virus life cycle will be better understood.

Methods and Materials

Construction of vvtetO:I7L/G1L

An inducible, mutant strain of Western Reserve vaccinia virus with tetracycline operators inserted directly before the I7L and G1L ORFs was obtained and is designated vvtetO:I7L/G1L. The vvtetO:I7L/G1L was made by performing a double infection with the single mutant viruses, vvtetO:I7L and vvtetO:G1L. The single mutants were each made by constructing a plasmid containing the tetracycline operator just upstream of either the I7L or G1L ORF as previously reported by Byrd and Hedengren-Olcott [13, 14]. Briefly, plasmids containing the I7L or G1L ORF with their respective native promoters were created with tetracycline operators inserted directly before each of the reading frames and after the promoter sequences. These plasmids were used to create the single inducible, mutant viruses. After co-infection at a multiplicity of infection (MOI) of 5 for each virus, the cells were harvested 24 hours post infection (HPI) and plaque purified. A genomic preparation was done on individual amplified plaques and double mutants were identified using PCR. One plaque was confirmed to contain the vvtetO:I7L/G1L and it was purified, amplified and sequenced [Byrd *et. al*, unpublished data].

Cells and virus

The tetracycline operator/repressor system allows viral replication to essentially be turned “on” or “off”. In order to use this system, cells that constitutively express the tetracycline repressor must be used. T-REx 293 cells (Invitrogen) were used in these experiments and

are adherent human embryonic kidney cells (Graham *et al.*, 1977). T-REx 293 cells achieve constitutive expression of the tetracycline repressor by expressing it from the pcDNA6/TR plasmid. The T-REx 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with glutamax. 10% tetracycline-free fetal bovine serum (FBS) and 1% penicillin-streptomycin were added to the media and 5µg/ml blasticidin was added directly to the plates during cell passaging. The penicillin-streptomycin aided in preventing infection with foreign bacteria and the blasticidin was the selection agent for the T-REx 293 cells containing the tet repressor expressing plasmid.

For virus titers and various steps in experimentation, BSC40 cells were used. BSC40 cells are African green monkey kidney cells and were cultured in minimum essential media (MEM) with 10% FBS, 1% L-glutamine, and 0.25% Gentamicin.

Genomic DNA extraction

A genomic DNA extraction was done in preparation for genomic sequencing. BSC40 cells (at 90% confluence) were infected at a MOI of 10 with vvtetO:I7L/G1L. For infection, the media used was modified to contain 5% FBS as opposed to the 10% used for culture. The cells were harvested 24 HPI, suspended in 1x PBS and centrifuged at 2,000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 900 µl 1x PBS. The cells were lysed via freeze-thawing and the crude virus was treated with 0.5M EDTA, 0.5M β-ME, and 10% Triton X-100, and centrifuged at 3,000 rpm for 2.5 minutes at 25°C to remove cellular fragments. Then the supernatant was transferred to a new tube and spun again at 15,000 rpm for 10 minutes to pellet the

viral DNA. The pellet was then resuspended in 100 μ l of master mix containing, 1mM Tris-HCL, 0.5M EDTA, 0.5M β -ME, 3M NaCl, 10% SDS, and Proteinase-K (7.5 μ l of 20 μ l/ml). This solution was incubated at 50°C for 40 minutes to promote solvation of the pellet. Once solvation had been achieved, impurities were precipitated using phenol-chloroform-isoamyl alcohol. The solution was centrifuged at 15,000 rpm for 10 minutes at 25°C and the top layer was removed and placed in a new tube. The viral DNA was precipitated from this layer using sodium acetate, glycogen (3 μ l of 20 μ l/ml), and 100% ethanol and cooling at -20°C. The mixture was spun at 14,000 rpm at 4°C for 10 minutes and the supernatant was removed and the pellet was air dried for approximately 45 minutes. The pellet was resuspended in 25 μ l of TE buffer (1mM EDTA, 10mM Tris, pH 7.5). The viral DNA was quantified using spectrophotometry and the concentration was determined to be 2518.3 μ g/ml.

Viral DNA amplification and sequencing

In order to sequence the G1L and I7L reading frames effectively, they were each amplified via PCR. For I7L the CB26 (5'-GAG CTC GTT TTC CTA GTG ATG GAG GAG-3') and CB90 (5'-CCT TTG ATT ATC ATC TTC TCG TAG GCG-3') external primers were used and for G1L the CB29 (5'-CCG TCG CGA GTG CGC AAA TAC ACC-3') and CB19 (5'-AAG CTT TCA AAC TCT AAT GAC C-3') external and CB79 (5'-GAA GTA TTC CAT TGT ATG GAT ATA CTA ACG-3') internal primers were used. 100 ng of viral DNA was used and the following cycle was used, 94°C for 2 minutes 1x, 94°C for 30 seconds and 55°C for 30 seconds and then 68°C for 2 minutes 35x, and 72°C for 7 minutes and was held at 40°C. The PCR product was purified using a

Qiagen purification kit and the DNA was quantified with spectrophotometry. Two samples for each open reading frame were made and analyzed and the G1L concentrations were 32.5 $\mu\text{g/ml}$ and 32.4 $\mu\text{g/ml}$, and the I7L concentrations were 34.2 $\mu\text{g/ml}$ and 44.2 $\mu\text{g/ml}$. These samples, with the primers used in PCR, were submitted to The Center for Gene Research and Biotechnology for sequencing. The sequence was obtained and the I7L and G1L open reading frames were compared with those in the Western Reserve vaccinia virus (sequence obtained from National Center for Biotechnology Information website). The open reading frames matched with the tetracycline operators inserted just before the methionine start sequence of each gene.

Virus preparation

The virus prep was begun by infecting 20 150mm plates of BSC40 cells at approximately 80% confluence with vvtetO:I7L/G1L at a MOI of 0.01. The infected cells were harvested 48 hours post infection (HPI) by scraping with a bent pipette tip and were transferred into 50ml conical tubes. Eight tubes were used, with 50ml of cells suspension per tube. The tubes were centrifuged at 4°C for 10 minutes at 2,350 rpm. The supernatant media was aspirated and the cell pellets in two of the tubes were resuspended in 4 ml of 10mM Tris-HCL (pH 8.0). The cell solutions were then transferred to the third and fourth tubes and those pellets were resuspended using the cell solutions to increase concentration. The cells were then homogenized in the Dounce Homogenizer and centrifuged for 10 minutes at 2,350 rpm. The homogenization allows cell lysis and release of the virus. The supernatant was layered on a 36% sucrose cushion and stored in a refrigerator while the resuspension and homogenization was repeated on the pellet. For

the second homogenization, the pellet was resuspended in 2 ml of 10mM Tris-HCL (pH 8.0). After centrifugation, the supernatant was added to the 4 ml previously layered on the 36% sucrose cushion. Next, the samples were centrifuged at 18,000 rpm for 80 minutes at 4°C. The supernatant was removed and the pellets were resuspended in 0.5 ml of 1mM Tris-HCL (pH 8.0). The suspensions were homogenized in a Duall Homogenizer and were layered onto a 25-40% sucrose gradient. These were centrifuged at 13,500 rpm for 40 minutes. This centrifugation results in a cloudy band near the middle of the sucrose gradient which contains the desired vvtetO:I7L/G1L. To extract the virus, a syringe is used to puncture the side of the tube, just below the cloudy level, and the virus is pulled out using the syringe. The virus was transferred to a new centrifuge tube and filled with 1mM Tris-HCL (pH 8.0) and spun at 13,500 rpm for 40 minutes. The pellet left in the bottom of the sucrose gradient was retained as partially purified virus and stored at -80°C. The supernatant was removed and the pure virus pellet was resuspended in 0.5 ml of 1mM Tris-HCL (pH 8.0) in an eppendorf tube and stored at -80°C.

Titration experiments

The purified vvtetO:I7L/G1L was titered to determine viral concentration. BSC40 cells were grown in two 6-well plates to approximately 80% confluence and infected with vvtetO:I7L/G1L in serial dilutions from 10^{-3} to 10^{-11} . These titer dilutions were used for all subsequent titers described below. The cells were stained and counted 24 HPI using 0.1% crystal violet, and the viral titer was determined to be 6×10^9 PFU/ml. This virus stock was used for all of the following experimentation involving vvtetO:I7L/G1L.

Plasmid preparation

A plasmid preparation of pRB21 and pRB21:I7L was performed to amplify the plasmid stock for transfection experiments. These plasmids were obtained from Moerdyk and pRB:I7L and pRB:G1L were originally constructed by Byrd [20]. A Qiagen MaxiPrep kit was used and the final concentrations for each plasmid were determined by spectrophotometry and were 1897.25 µg/ml pRB21 and 520.35 µg/ml pRB21:I7L. A stock solution of pRB21:G1L was obtained pre-purified from Moerdyk.

MOI optimization

Next, the MOI was optimized. This optimization identifies the amount of virus needed to establish effective infection without overwhelming the tetracycline operator/repressor system. When this system is saturated, or “leaky”, virion production is seen in the “off” state or the in the cells without tetracycline. TReX-293 cells were grown to ~80% confluence in 6-well plates and were infected at three different MOIs, 0.1, 0.5, and 1 PFU/cell (plaque forming units/cell). The infection media was modified from the culture media to contain 5% tetracycline-free FBS, and 2.5µg/ml blasticidin. The cells were harvested, freeze-thawed, and titered on BSC40 cells, using 0.1% crystal violet for plaque visualization.

Growth curve

A growth curve was used to further test the conditional lethality of the tetracycline operator/repressor system. Plates were grown to ~85% confluence with TReX-293 cells and infected with vvtetO:I7L/G1L with and without tetracycline (1µg/ml). A VV

infection was also done as a positive control to show the viability of the vvtetO:I7L/G1L in the presence of tetracycline. The vaccinia virus replication cycle takes ~12 hours to complete and the cells were harvested every two hours and titered and stained with 0.1% crystal violet on BSC40 cells to examine the viral progression.

Immunoblot experiments

Next, immunoblots were used to investigate viral core protein cleavage. First, p4b processing was examined by infecting T-REx 293 cells at various MOIs with vvtetO:I7L/G1L in the presence (1 μ g/ml) and absence of tetracycline and with Western Reserve vaccinia virus as a positive control. A transfection was also done using T-REx 293 cells infected with vvtetO:I7L/G1L without tetracycline. 2 μ g/ml of plasmid DNA was added, and both pRB21:I7L and pRB21:G1L were added. For the transfection, DMRIE-C (Invitrogen) reagent was used (6 μ l/ μ gDNA). The DMRIE-C and plasmid DNA were added to 1ml of T-REx 293 infection media and let sit for 20 minutes to allow micelle formation. Next, the virus was added and the mixture was applied to T-REx 293 cells at approximately 70% confluence. The infected cells were harvested 24 HPI and centrifuged at 14,000 rpm at 4°C for 5 minutes and the pellet was resuspended in 100 μ l or 50 μ l 1x PBS, varying by experiment. The cell solution was freeze-thawed to release the virus and was briefly centrifuged again to separate the viral supernatant and cell debris in the pellet. The viral samples were heated at 70°C for 10 minutes and loaded into a pre-poured 4-12% SDS-PAGE gel (Invitrogen). The gel was run at 200V and when finished, Western blotted onto a PVDF membrane. The Western blot was run at 30V for 1 hour 15 minutes and washed with 1x TBS. The membrane was blocked with 3% gelatin

for 2 hours and then washed 3x10 minutes with 1x TTBS (TBS with Tween 20). Next, the membrane was incubated in 10ml 1% gelatin with 10 μ l polyclonal rabbit anti-p4b antisera [20] overnight. The membrane was then washed 4x10 minutes with 1x TTBS and 5ml of the secondary goat anti-rabbit IgG antisera (Zymed) was applied in 10ml of 1% gelatin for 1 hour. The membrane was washed 3x10 minutes with 1xTTBS, followed by 2x5 minutes with 1xTBS. Alkaline phosphatase was used to visualize the protein bands, a BCIP/NBT developing tablet (Rad-Free) was dissolved in 30ml of ddH₂O and the membrane was placed in 10ml of the developing solution until bands were visible. The membrane was washed three times with ddH₂O and dried on paper toweling.

In some immunoblots, a gentle transfection reagent was used, Fugene 6 (Invitrogen), at 1.5 μ l/ μ gDNA. For these transfections, 3 μ l Fugene and 2 μ g of each plasmid DNA was added to 100 μ l Opti-Mem media (Invitrogen) and allowed to sit for 30 minutes. The transfection solutions were added to the plates containing 1ml T-REx 293 infection media, virus, and tetracycline (1 μ g/ml). These cells were infected at 0.2 MOI with vvtetO:I7L/G1L to further increase protein visibility and after harvesting, the cell pellet was resuspended in 50 μ l 1x PBS to increase protein concentration. The rest of the experiment was performed according to the protocol described above.

Results and Discussion

Investigation of the morphogenic proteolysis step in the vaccinia virus life cycle was performed using an inducible, mutant virus, vvtetO:I7L/G1L, in which tetracycline operators had been inserted before the ORF for the I7L and G1L proteins. The cell system used to achieve the conditional-lethal character of the virus was the T-REx 293 (tetracycline repressor expressing) cell line from Invitrogen. This system allows viral expression to essentially be turned “on” or “off”. When the system is in the “off” state, the tetracycline repressor is bound to the tetracycline operator, thereby inhibiting expression of the G1L and I7L proteins and viral replication. When the system is in the “on” position, tetracycline is added to the system and binds the tetracycline repressor, removing it from the operator and allowing expression of the proteins and subsequent viral replication. The viral DNA was purified and sequenced prior to experimentation to ensure that the tetracycline operators had been inserted correctly and that the I7L and G1L ORF were intact.

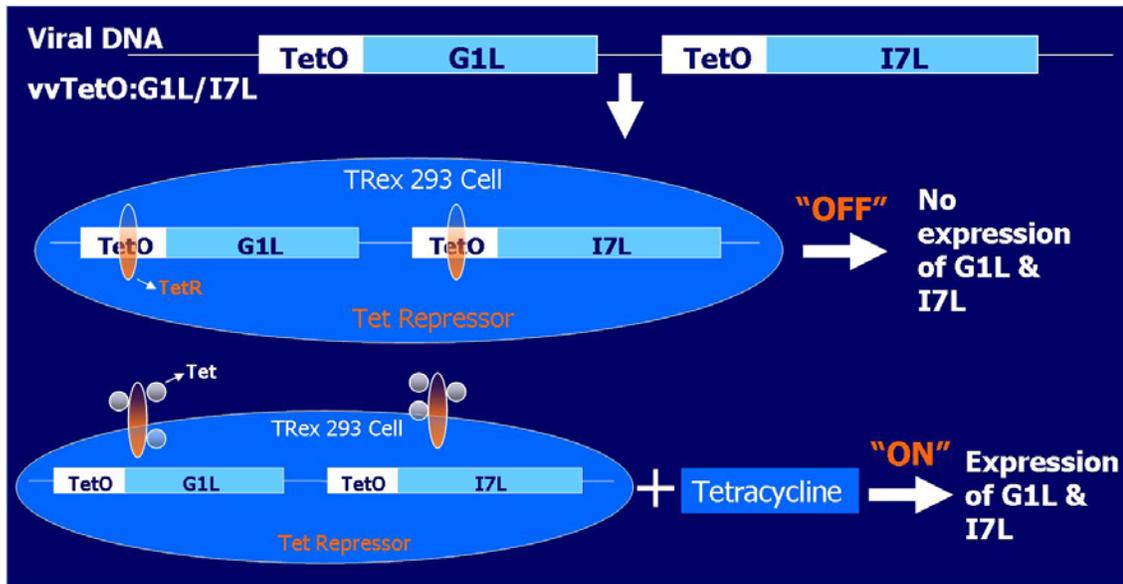


Figure 4. Schematic of inducible expression system. The double mutant vaccinia virus with tetracycline operators inserted before the I7L and G1L ORFs, and the cellular model of the system in the “on” and the “off” states.

The genomic DNA extraction and purification resulted in a viral DNA concentration of 2518.3 $\mu\text{g/ml}$. The DNA sequencing confirmed that the tetracycline operators were inserted correctly as shown in figure 5.

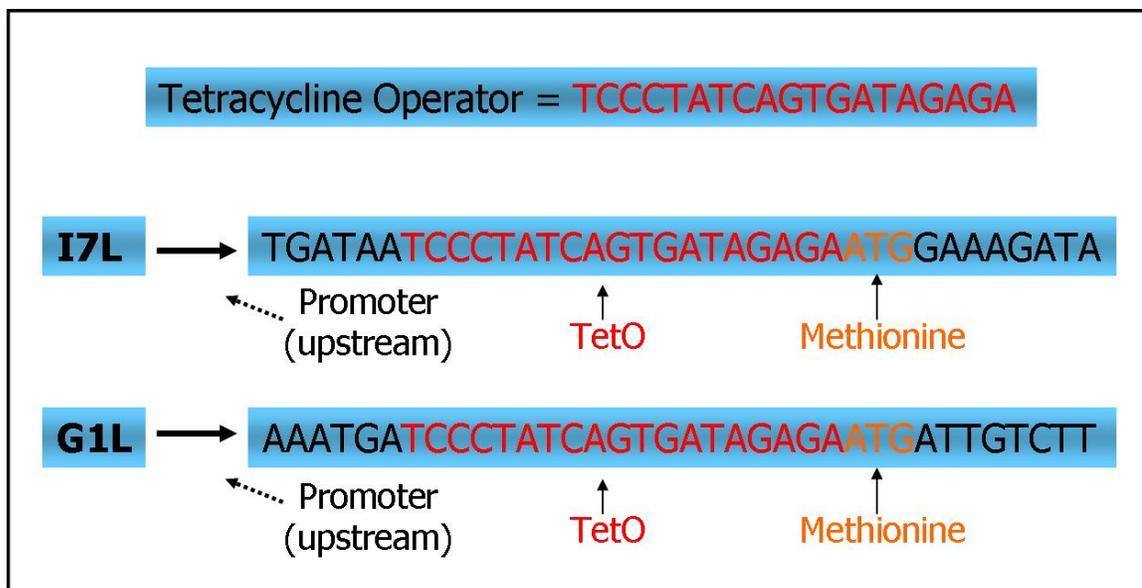
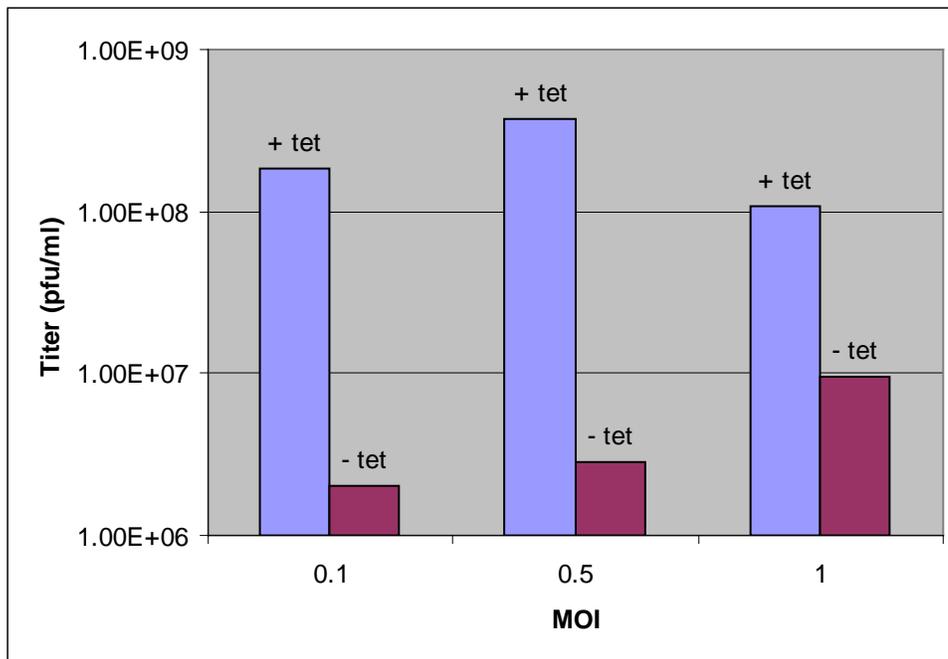


Figure 5. Verification of tetracycline operator sequence location and quality. The vvtetO:I7L/G1L viral DNA was sequenced to verify that the tetracycline operator was intact and located before the I7L and G1L ORFs and after the native promoters.

The virus preparation effectively purified and concentrated the vvtetO:I7L/G1L to a final concentration of 6×10^9 PFU/ml. This virus stock was used for all subsequent experimentation.

Next, a MOI optimization was performed to determine the best amount of virus to infect the T-REx 293 cells at without saturating the conditional-lethal system and thus causing “leakiness”. “Leakiness” refers to core protein processing and thus viral growth in the cells that are in the “off” state, or without tetracycline. The results for each MOI with and without tetracycline are shown in figure 6.



MOI	Tet	Pfu/ml	%	% reduction
0.1	+	1.85E+08	100	
	-	2.00E+06	1.1	98.9
0.5	+	3.71E+08	100	
	-	2.80E+06	0.75	99.25
1	+	1.08E+08	100	
	-	9.50E+06	8.8	91.2

Figure 6. MOI Optimization Data. The effect of tetracycline on viral replication was determined by infecting TReX-293 cells at varying MOIs with and without tetracycline (1 μ g/ml). The cells were harvested and freeze-thawed 24 HPI. They were titered on BSC40 cells and plaques were visualized by staining with 0.1% crystal violet.

The MOI optimization data was analyzed to determine which situation produced the highest viral titer in the “on” state and the lowest viral titer in the “off” state. The 0.5 MOI was best, with a 99.25% reduction in viral titer, or PFU, from the “on” state to the “off” state.

Next, a single step growth curve was done to demonstrate the fitness and conditional-lethal characteristic of the vvtetO:I7L/G1L. The data is shown in figure 7.

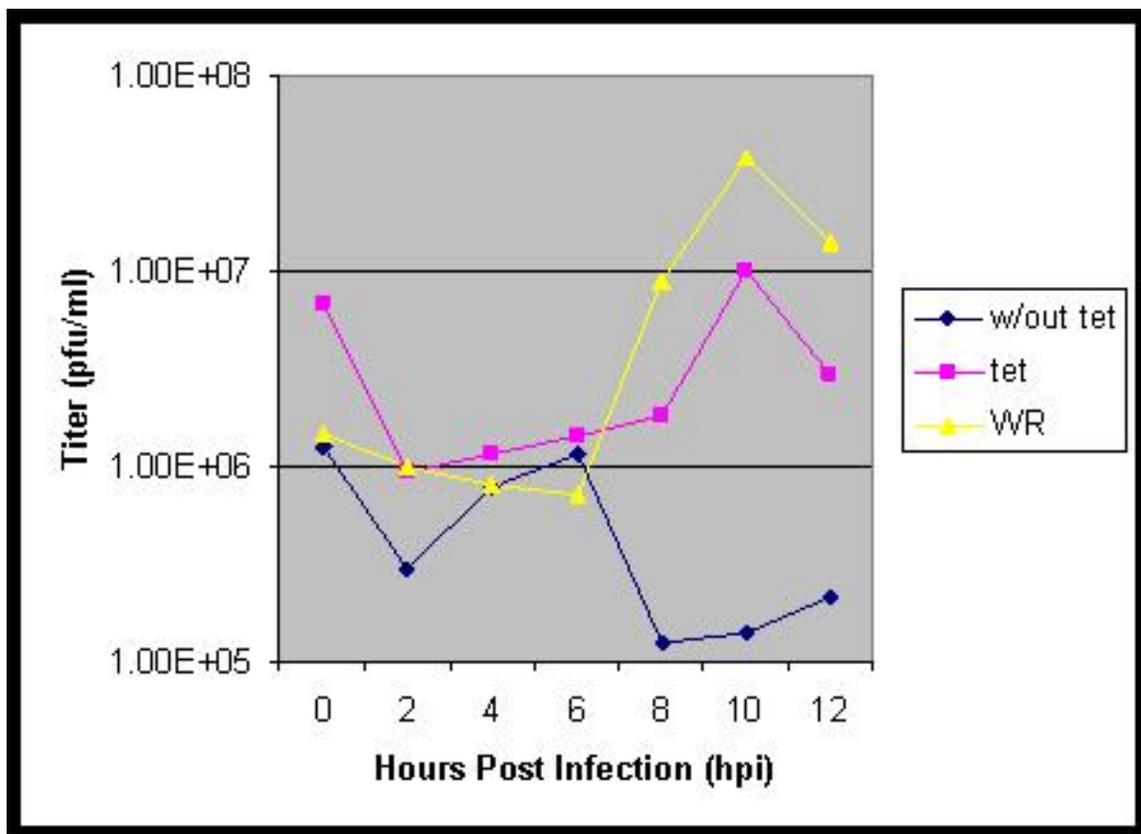


Figure 7. Demonstration of conditional lethality and viral fitness of vvtetO:I7L/G1L. TREx-293 cells were infected with vvtetO:I7L/G1L in the presence and absence of tetracycline (1 μ g/ml), and a vv Western Reserve infection was used as a control. The cells were infected at 0.5 MOI and harvested every 2 hours for 12 HPI. They were titered on BSC40 cells and plaques were visualized by staining with 0.1% crystal violet.

The sample in the “off” position progressed with lower viral titers consistently through the first six hours with significant decrease in the second half of the cycle. The sample in the “on” position grew similarly to the VV-WR sample, with a slightly lower viral titer at the end of the 12 hour cycle, demonstrating strong conditional lethality and viral fitness.

With confirmation of viral fitness and conditional-lethality, immunoblots were performed to examine core protein processing under the tetracycline operator system. However, the

vvtetO:I7L/G1L core protein cleavage under the desired experimental conditions (+tet, -tet, transfections) could not be effectively visualized via immunoblot procedure. Various MOIs and protein concentrations were used and the vvtetO:I7L/G1L was reititered during experimentation to ensure its viability and activity. Detection for p4b and 4b proteins was moderately successful in the +tetracycline and -tetracycline samples as shown in figure 8.

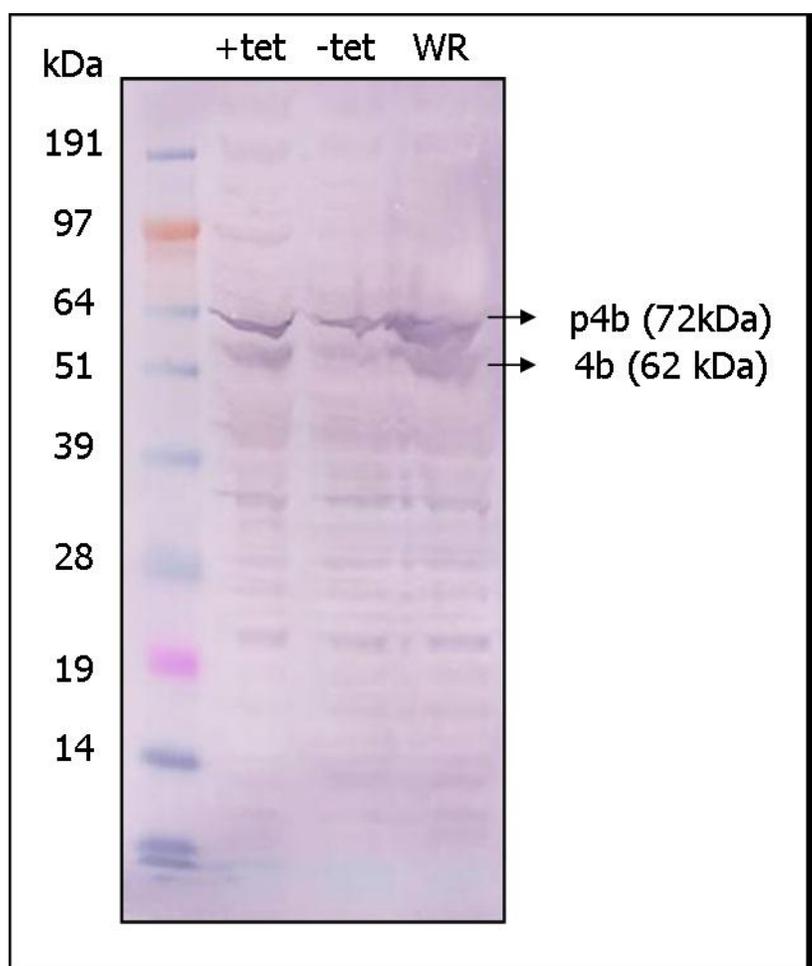


Figure 8. Immunoblot detection of proteolytic processing of the p4b core protein. T-REx 293 cells were infected at a 0.05 MOI, harvested, and freeze-thawed at 24 HPI. Proteins were separated on a 4-12% SDS-PAGE gel, Western blotted, and p4b proteins were visualized with polyclonal rabbit anti-p4b antisera [20].

The 0.2 MOI with increased protein concentration (resuspension in 50 μ l 1x PBS during harvesting) did improve viral protein cleavage visualization in the +tetracycline sample and kept the -tetracycline sample at a low level of “leakiness” with little cleavage product visible. Using Fugene 6 (Invitrogen) as a transfection reagent improved viral and cell viability but did not aid in revealing successful rescue of viral protein cleavage. The results are shown in the figure 9.

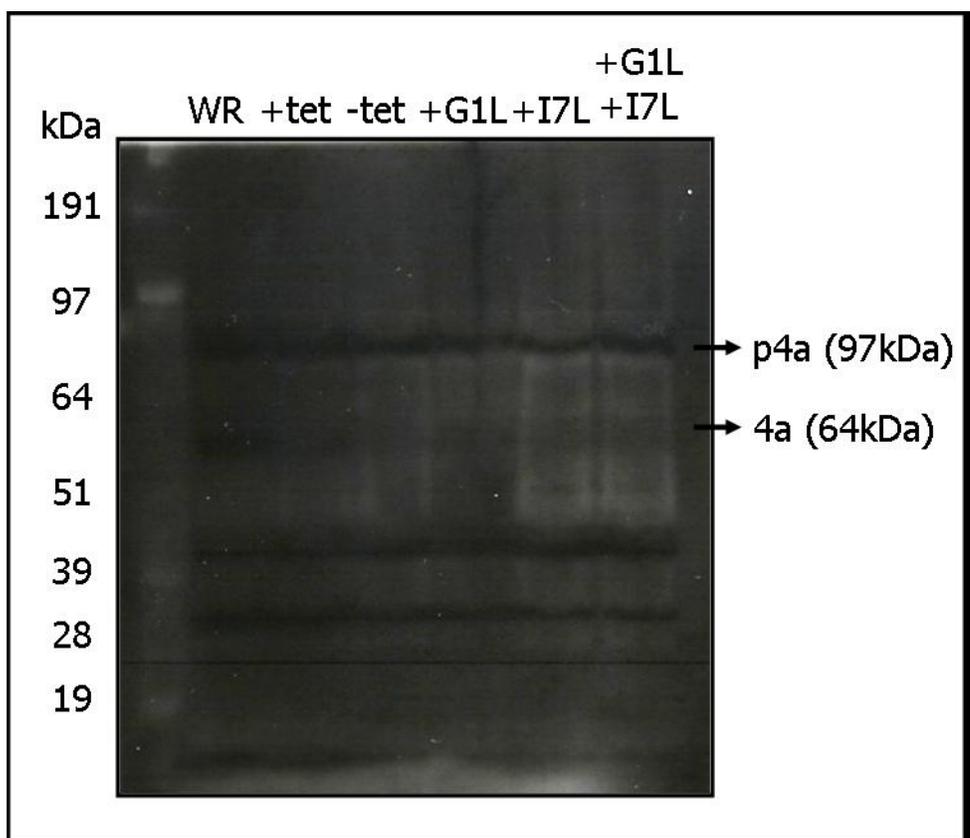


Figure 9. Immunoblot detection of proteolytic processing of the p4a core protein. T-REx 293 cells were infected at a 0.2 MOI, harvested, and freeze-thawed at 24 HPI. The proteins were separated on a 4-12% SDS-PAGE gel, Western blotted, and p4a proteins were detected with polyclonal rabbit anti-p4a antisera [20].

An increased MOI could have improved the visibility of the processed core proteins, but would have saturated the conditional-lethal system, compromising the inducible nature of

the virus. The difficulty in achieving successful protein visualization in the transfections rendered significant viral replication study unfeasible.

Insertion of the tetracycline operators in front of both ORFs may have compromised effective protein detection in the conditional-lethal system due to excessive leakiness. The single mutants, vvtetO:I7L and the vvtetO:G1L, both achieved greater success in transfection experiments, but still experienced problems with leakiness. The vvtetO:I7L virus behaved similarly in the MOI optimization with an average reduction of 99.0% in the 0.5 MOI sample, but its yield and growth kinetics more closely resembled the wild type virus in the growth curve than did the vvtetO:I7L/G1L. Successful visualization of core protein cleavage, as well as I7L proteinase localization was achieved with immunoblots using the vvtetO:I7L, indicative of a more stable virus, thus allowing further and more conclusive experimentation [13]. The vvtetO:G1L also produced more conclusive results, with MOI having little effect on the ratio of viral expression of the +tet and -tet samples, and protein expression and virion assembly were visualized successfully [14]. For future experimentation a double infection with these single mutant viruses could be used and may be more effective than the double mutant virus.

Another explanation for poor protein detection could be that the antisera weren't working optimally. It may not have been sensitive enough to specifically recognize the vvtetO:I7L/G1L, resulting in the strong bands visible in the Western Reserve wild type samples and the faint ones in the mutant samples. These experiments may be more successful with production of new antisera.

For more successful investigation of vaccinia virus replicative mechanisms in a double mutant, inducible virus another operator/repressor system could be used. An inducible system that has been successful in vaccinia in previous experimentation is the lacO operator/repressor system. Moss and Ansarah-Sobrinho effectively used this system to regulate I7L expression with IPTG as the inducer [21].

Here I have shown that the vvtetO:I7L/G1L was constructed successfully through DNA extraction and genomic sequencing. The vvtetO:I7L/G1L inducible expression system effectively achieved reduced viral titers in the “off” state in the MOI optimization and growth curve experiments. However, it was difficult to effectively visualize core protein processing the “on” and “off” state and in the transfection experiments. It was determined that this virus was not suitable for conclusive core protein processing examination and that another inducible, mutant system, such as the lac operon system, may be more successful.

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