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Development of a Novel Method to Express and Purify Vaccinia Virus Early Transcription Factors A7 and D6 using Bacteria

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By

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Seton Hall University

Department of Biological Sciences

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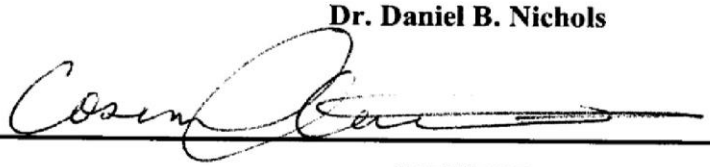

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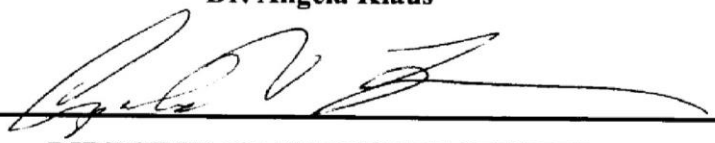

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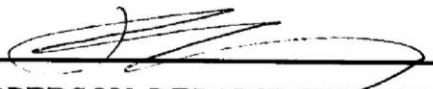

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Abstract:

The eradication of Smallpox is one of the greatest human achievements. However other poxviruses still exist infecting humans (Molluscum Contagiosum, monkeypox, and Vaccinia) or livestock (cowpox, sheeppox). Though vaccines and anti-virals exist, the side effects are serious, and viral resistance has been detected. The genome amongst poxviruses is highly conserved, early gene promoters maintain a specific critical consensus region that can be targeted for development of novel broad spectrum therapeutics. Using Vaccinia early transcription factors (VETFs) a novel method of expressing and purifying VETF A7 and D6 was developed. A7 and D6 are required for transcription of early genes recruiting the RNA polymerase to the site of transcription. Cloning of A7L and D6R into the bacterial expression vector pET302/303 N-terminus/C-terminus His-Tag was done in order to express A7 and D6 in the *Escherichia coli* (*E. coli*) DH5α and BL21 DE3 strains. Under Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, BL21s can be controlled to express proteins under time courses and different concentrations of the inducer. Optimizing provided a method where concentration of VETFs A7 and D6 expression was controlled but abundant. After, different methods of extraction were used to solubilize A7 in order for purification to be done. Nonionic buffers were not strong enough to solubilize the majority of A7 from samples, however anionic buffers are able to extract the majority of the VETF. Purification of A7 was successful but further optimization is required, and although D6 was successfully solubilized purification still has to be done. This novel system of expression and purification using bacteria is the first known attempt to purify VETFs A7 and D6, and is significantly cheaper and safer than known methods. Thus, this system opens the door for a cost effective and safer method for future studies.

Introduction:

Despite the eradication of Smallpox, poxviruses remain a concern to the public health. Poxvirus infections result in numerous human and veterinary diseases. For example, the Molluscum Contagiosum Virus is the third most prevalent virus infection of the skin. Further, in recent years, there have been increased reports of emerging infectious diseases caused by poxviruses such as Monkey pox and Vaccinia Virus outbreaks. As the general population is no longer vaccinated, accidental or intentional exposure to Smallpox remains a concern. While the majority of Smallpox stocks are known to be contained or destroyed, undocumented vials of Smallpox were recently found in an FDA facility. More troubling was the fact that the virus was found to still be infectious. In light of recent events, continued research on poxviruses and the identification of novel poxvirus inhibitors should remain a research priority.

Poxviruses are large double-stranded (ds) DNA, enveloped viruses. With a genome of 200kb consisting of approximately 200 genes, the *Poxviridae* family is amongst the largest of the DNA viruses. Poxviruses differ from most other DNA viruses as they reside in the host cell cytoplasm rather than traveling to the nucleus during infection. The virus must therefore bring its own transcription machinery when it infects a cell, as it cannot hijack the host DNA or RNA polymerase. Upon entry the virus fuses its envelope to the host cell allowing the viral capsid to enter into the cytoplasm (Fig. 1). Transcription occurs initially inside the capsid, which acts as a barrier from the host cell. As the virus matures two virions are formed, intracellular mature virions (IMV) or external enveloped virions (EEV).

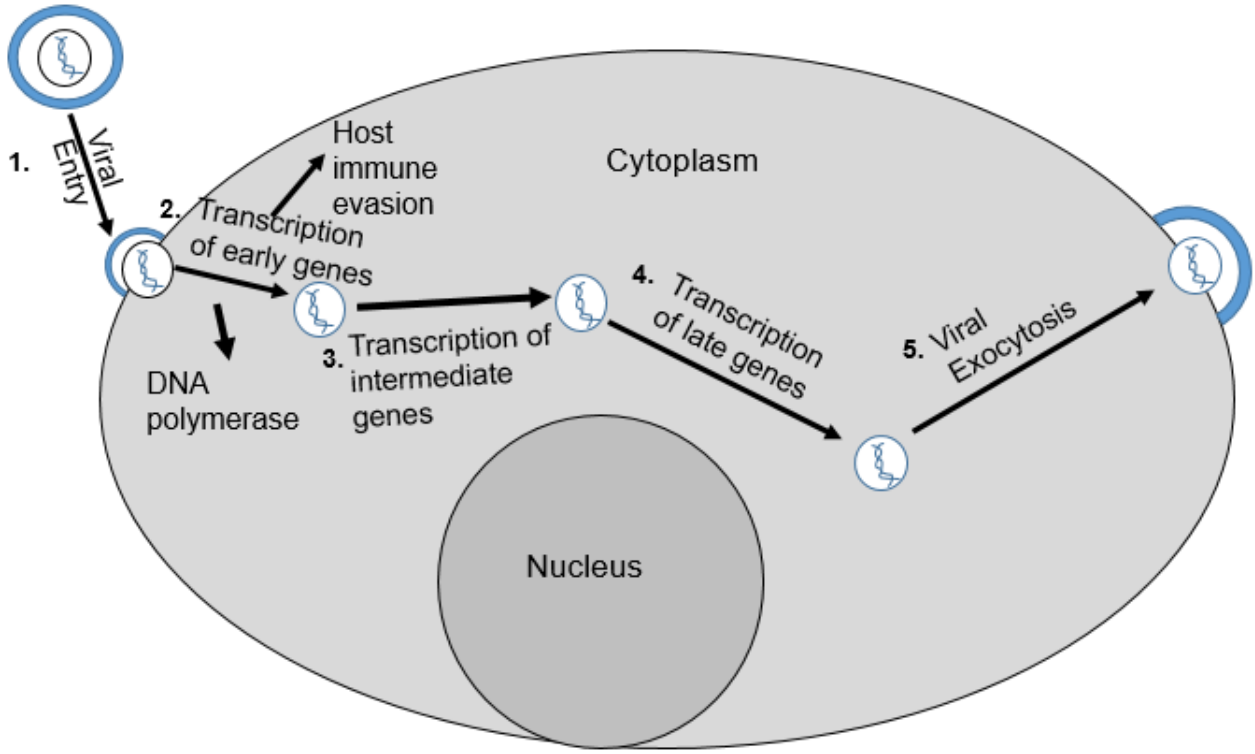


Figure 1: Life cycle of a poxvirus from entry to exocytosis: Poxvirus replication occurs within the cytosol and gene transcription occurs in a temporal manner

Poxvirus Diseases:

Smallpox caused by the Variola Virus (VARV), plagued humanity for 3,000 years up until its eradication in the late 1970's. VARV, a member of the *orthopoxviridae* subfamily caused the known deaths of 300 million to 500 million people over this 3,000 year plague (Ristanovic et al., 2016). Smallpox manifests as skin lesions upon the surface of the skin leading to severe scarring, deformities, and possibly to death resulting from host systemic infection (Kennedy et al., 2016). During the 20th century, the Smallpox was cited to have resulted in the morbidity of 48,000 people annually (The Centers for Disease Control and Prevention [CDC], 1999). Vaccination was first attempted by the Edward Jenner, using cowpox virus as a means to evoke immune response and protection. The modern vaccine, developed during the 20th century came from another *orthopoxviridae*, Vaccinia Virus (VACV). Vaccination was continued until the early 1980's until the virus was declared eradicated. However, the threat of Smallpox still exists due to its potential use as a bioweapon. Epidemiological, antigenic, and genetic characters make Variola classified as a potential biological weapon (Ristanovic et al., 2016). Those born after the last released vaccination have no acquired immunity against Smallpox. VACV and VARV contain similar structural genes, and similar promoters for many of their genes (Weltzin et al., 2003). Therefore, VACV could easily be engineered to become more virulent.

Monkeypox (MPV), another orthopoxvirus, is a zoonotic virus that was first seen in Western and Central Africa (Abrahão et al., 2015). In 2003, Monkeypox virus spread to the United States, causing outbreaks localized with major incidents in Illinois, Indiana, Wisconsin, amongst other states (Guarner et al., 2004; Croft et al., 2007; The Center for Disease Control and Prevention, 2003). Containment and control of outbreaks was quick due to rapid detection and coordination between federal, state and local public health efforts in the initial cases (Damon,

2011). By quarantining those who had developed the disease, and euthanization of the ill prairie dogs that transmitted the disease, containment and prevention of further cases was successful. Although first detected in primates, the virus primarily infects rodents, and is able to be transmitted to humans from rodents such as prairie dogs, during the outbreak, or from squirrels and monkeys as seen in cases in Africa (Guarner et al., 2004; Damon, 2011; Croft et al., 2007). It is no surprise that like other poxvirus, MPV manifests as skin lesions and causes tissue disfigurement and scarring and in some cases causes breathing difficulties or encephalitis (Damon, 2011). Though MPV does not primarily infect humans, it is still a cause for concern because it is unknown if the virus is transmittable between humans. The genome between orthopoxviruses are highly conserved in essential regions. If a mutation does arise, the virus could possibly become more virulent and be transmittable between humans.

Vaccinia virus (VACV) is an orthopoxvirus, genetically similar to VARV, yet believed to have originated from the cowpox virus (Olson et al., 2014; Gilsdorf & Zilinskas, 2005). Through its use as the vaccine against Smallpox (VARV), VACV became the model poxvirus used to characterize numerous aspects regarding the biology of poxvirus infections (Broyles, 2003). VACV is nearly identical to VARV in terms of conserved genes involved in viral replication and transcription processes, but relative to VARV, VACV is significantly less virulent. However, the varying strains of VACV are known to be virulent and cause disease. In fact, recent outbreaks have occurred, such as in Brazil in 2010: where cattle and workers became infected with a strain of VACV (Abrahão et al., 2015). The cattle and workers develop scarring deformities, and provide a cause for concern about handling and controlling the viral infection. One concern is the ability to modify VACV into a recombinant VARV (Gilsdorf & Zilinskas, 2005), as the two viruses are similar enough that VACV could be modified to increase its virulence. It should be

noted that a variant termed Modified Vaccinia Ankara (MVA), is a highly attenuated VACV strain (Kremer et al., 2012). MVA lacks the majority of the immune evasion genes found in the parental VACV and thus can safely be used in a BSL-1 setting (Sutter et al., 1992; Tuppurainen et al., 2015). MVA as a model, provides researchers a means to study a virus that maintains the essential genes needed for all poxviruses, with a lower risk of being infected or causing an outbreak.

Molluscum Contagiosum Virus (MCV), is a member of the genus *molluscopoxvirus*, differing from the *orthopoxviridaes*. With the eradication of Smallpox, MCV remains the only virus that exclusively infects humans, having no animal host (Nichols & Schisler, 2009). MCV infection manifests as benign skin lesions on the appendages or trunk of a host, causing itchiness and mild discomfort. Unlike VACV, MCV cannot be grown in tissue culture (Postlethwaite, 1970). Therefore, MCV is difficult to study as there is no way to easily maintain a live virus culture for extended periods of time. Due to the lack of a tissue culture model, the ability to discover anti-MCV therapeutics has been severely impacted.

Poxviruses also causes numerous veterinary diseases. Recent history shows the relevance in the form of livestock belonging to Syrian refuges, who have sought asylum in neighboring countries due to war and strife. An example is Lebanon, where 1.2 million refugees have relocated and consequently increased the number of livestock by the Syrian border to 60% (Tuppurainen et al., 2015). Refugees traveling with their domesticated animals: goats, sheep, and cows, may inadvertently bring poxviral diseases with them such as goatpox, sheep pox, or lumpy skin disease. The poxviruses causing these diseases are members of the *Capripoxvirus* (CaPV) genus and are capable of being transmitted to healthy livestock residing in these asylum countries. The effect of transmittance of these diseases is devastating agriculturally and

economically. Lesions formed by the virus leave permanent scars decreasing the value of skins and hides, thus devastating the leather industry (Tuppurainen et al., 2015). Incidents similar to the spread of cowpox and VACV outbreaks may occur, thereby allowing the virus and disease to propagate amongst livestock. One concern is the lack of consistent vaccination along with animal movement control acting as the cause for uncontrollable spread of CaPV (Tuppurainen et al., 2015). Therefore continued research should be applied for veterinary poxviruses in order to prevent damage to necessary industries and control transmittance of veterinary poxviruses.

Viral Life Cycle:

The life cycle of a poxvirus differs greatly from most double-stranded DNA viruses. Once a cell has become infected, most dsDNA viruses travel to the nucleus requiring the host transcriptional machinery. However, poxviruses reside in the cytoplasm of the host (Condit et al., 2006). Since the host's DNA polymerase and RNA polymerase reside within the nucleus, the virus must provide its own transcriptional machinery. Transcription of poxvirus genes occur in a temporal manner (Fig. 1) (Broyles et al., 1991; Cassetti et al., 1996; Broyles, 2003). Three sets of promoters, early, intermediate, and late control the transcription of poxvirus genes. Early genes are transcribed shortly after the virus enters the cell (Fig. 1). Products of the early genes include proteins involved in genome replication, the viral DNA polymerase, host interaction/evasion proteins, and intermediate gene transcription factors (ITFs) for intermediate genes. Intermediate genes transcribe transcription factors for late genes, and late genes transcribe structural proteins as well as early transcription factors for the progeny virions (Fig. 1) (Broyles, 2003). The replication of the genome before producing structural genes ensures that progeny virions contain a copy of the genome. Poxvirus transcription of proteins is dictated through transcription factors (Broyles et al., 1991; Li & Broyles, 1992), which bind to or near the promoter region of a

specific target gene, thus recruiting the RNA polymerase to bind to the region and initiate transcription of mRNA (Broyles et al., 1988). The viral DNA polymerase promoter contains a highly conserved structure between *orthopoxviridae* and similarity amongst other poxviridae (Fig. 4). Davison identified specific regions of early gene promoters: the critical region, spacer region, and initiation region, which are conserved between poxviral genes (Davison & Moss, 1989). Davison and Moss identified the critical regions of the early gene promoters by introducing single point mutations as well as complex mutations (Davison & Moss, 1989). To study the effects of different mutations on the promoter, Davison developed recombinant VACV by designing synthetic VACV promoters to control transcription of the *lacZ* gene (Davison & Moss, 1989). Mutations of key nucleotides in the Critical region reduced expression while other mutations in the same region seemed to increase expression, thus establishing the consensus sequence for the critical region to be AAAAgTaGAAAataTA (Davison & Moss, 1989). The critical region is highly conserved with common nucleotide sequences especially for the same gene amongst different poxviruses shown in figure 2. Further the critical region is essential for the initiation of transcription similar to the TATA box in mammalian genes (Davisons & Moss, 1989). Poxvirus early promoters are recognized by viral early transcription factors (ETFs) A7 and D6 (Cassetti et al., 1996). The two transcription factors recognize and bind to the critical region to recruit the viral RNA polymerase to initiate transcription of early genes (Li & Broyles, 1993). Transcription of early genes is quick, implicating that these transcription factors bind to the early gene promoters upon entry into a host cell within the viral capsid. Thus, by prepackaging A7 and D6, the virus is able to swiftly initiate protein transcription and further initiating DNA replication and evasion of the host innate and adaptive immunity.

Poxvirus Inhibitors:

To control and prevent outbreaks of poxvirus diseases, the development and study of anti-poxviral therapeutics is necessary. The Smallpox vaccine does exist if the re-emergence of Smallpox occurs. Only two known locations, the CDC and a laboratory in Moscow, Russia, maintain the vaccine and impact the efficiency of dispersing the vaccine rapidly. However, vials labeled Variola were recently found in an FDA storage unit, and were confirmed to have contained VARV (The Centers for Disease Control and Prevention, 2014). Cases such as this are rare, yet provide evidence that VARV may not just be located in two locations and in fact might be found in even more areas. The vaccine also contains weakened yet active viruses, meaning those vaccinated are at risk for disease due to the virus reverting, such as those with eczema, dermatitis or other skin diseases (The Centers for Disease Control and Prevention, 2017; Stittelaar et al., 2006). Still, those who are vaccinated may inadvertently spread the virus to other people due to carrying the live virus within their body (Moss, 2005). Vaccines also may not be capable of being used on patients with a weakened immune response, such as patients on immunotherapies, human immune-deficiency virus (HIV) patients, or people that lack a healthy immune response due to other immune deficiencies (Edghill-Smith et al., 2003). These patients are at greater risk of developing disease, as their immune system is not capable of properly mounting an immune response. Therefore alternative means of therapy are necessary for those lacking a functional immune system.

Aside from vaccination, pharmaceutical compounds exist as a means to control and combat poxvirus infections too. Cidofovir, a drug developed to be used against cytomegalovirus, was used to combat the monkeypox virus outbreaks in Illinois (Stittelaar et al., 2006). The drug acts by inhibiting DNA synthesis through selectively inhibiting the viral DNA polymerase from

functioning and replicating the genome (Clercq, 2001). By stopping replication, progeny virus are able to infect cells, but may not have a fully replicated genome preventing the virus from producing future progeny. Recently, brincidofovir was developed in order to be more specific towards poxviruses (Kern et al., 2008; Olson et al., 2014). While the drug is capable of treating patients it is very taxing with many side effects. Minor side effects include: nausea, vomiting, hairloss, diarrhea, and weakness, with major side effects consisting of neutropenia, kidney failure, and cancer (Florescu et al., 2012). The drug is also capable of interacting with the host DNA polymerase, and can shut down cellular replication which will stop the virus but unintentionally kill the host cell. Therefore cidofovir and its analogs provide therapy for poxvirus infection, but the side effects on the patient are significant. Thus, less taxing drugs are necessary for treatment of infections.

Other drugs have entered testing and show promise for poxviral treatment, such as the drug ST-246 commonly known as tecovirimat (Mucker et al., 2013). Tecovirimat provides treatment by inhibiting the viral phospholipase (F13L). F13L is necessary for the formation of infectious virions to be released from the host to spread infection (Berhanu et al., 2015). Tecovirimat targets the F13L proteins by preventing the formation of extracellular enveloped virions, thus preventing the formation of infectious virions.

Limitations:

A primary concern with ST-246, as well as other anti-viral therapies, is that viral resistance has been identified (Kern et al., 2008). Due to singular targeting by anti-viral compounds, a virus is still capable of adapting through genetic mutations. These genetic mutations can produce poxviral strains that have developed drug resistance. A method to prevent this is to use multi-targeted anti-viral therapies, because the virus would have to adapt and

potentially change multiple genes. The drastic adaptation that VACV, for example, would have to go through has a high chance of causing the virus to lose functional and necessary genes, thus causing the virus to weaken and lose its virulence (Broyles, 2003; Li et al., 1993).

The development of anti-poxvirus therapeutics is highly challenging due to the cost of equipment, culturing methods and viral propagation. Most strains of poxvirus require a minimum BSL-2 training, and equipment, which may include a medical surveillance unit, proper immunizations, amongst cost of equipment for disposal and decontamination. Another limitation is that some poxviruses cannot be grown in the lab. For example, MCV has limited anti-viral drugs to effectively treat it. MCVs inability to be cultured *in vitro* prevents the capability to properly test compounds for therapeutic effect (Senkevich et al., 1997). Culturing the virus requires proper cell lines, media, and time in order to reach optimal viral titers for testing particular drugs. Viruses, such as Molluscum Contagiosum, make even propagation difficult because culturing and maintaining the virus *in vitro* is difficult (Senkevich et al., 1997) and MCV's lack of an infectious animal model prevents *in vivo* drug testing. Therefore, developing a novel virus free method of testing compounds without an initial live virus test would facilitate the capability of developing novel anti-poxvirus therapeutics. Further, this type of test would allow smaller institutions to participate in basic science by allowing research to be conducted in BSL-1 level facilities. The goal of my thesis is to develop a virus-free system in which to screen poxvirus inhibitors.

Designing a Novel Approach to Inhibit Poxvirus Replication:

Inhibition of VETFs binding to early gene promotors makes an attractive target in the development of novel anti-poxviral strategies. The early gene promoters are highly conserved. By identifying small molecules that bind these conserved regions of the poxvirus genome (Fig.

2), one can prevent or reduce the virus's ability to transcribe gene products, such as the DNA polymerase or immune evasion molecules. The inevitable outcomes would include stalling the viral replication cycle and allowing the host immune system to rapidly clear the virus.

Theoretically drugs that target poxvirus early promoters would have a high barrier to resistance. To become resistant to drugs targeting poxvirus promoters, the virus would have to drastically change its promoter and alter transcription factors, thereby losing the conserved critical region which is needed for promoter recognition. It is likely that these alterations would result in decreased virus-fitness. Furthermore, given the degree of conservation in a poxvirus promoter, a drug capable of binding the promoter for one viral strain could potentially have broad spectrum anti-poxvirus activity such as with brincidofovir (Olson et al., 2015).

VACV provides a strong model to test compounds for promoter binding because it has been very well studied. The study of vaccinia replication is widely accepted as being the same for other poxviruses, making it the perfect model for poxviruses that are dangerous or difficult to culture. Although the virus is a BSL-2 strain, further attenuation has developed vaccinia virus Ankara (MVA) which is very safe and easily handled in a BSL-1 facility.

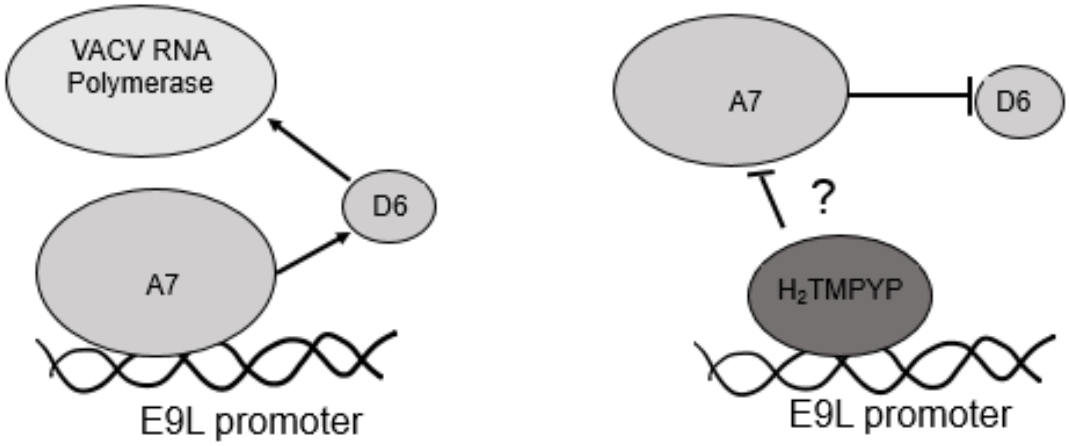


Figure 2: Recruitment of the RNA polymerase to the E9L, VACV DNA polymerase promoter. Normal recruitment of the viral RNA polymerase (left), predicted effects of compound H_2TMPYP interaction with the promoter (right).

The goal of this thesis work was to develop a novel system in which poxvirus early transcription factors could be expressed using bacteria. To this end, VACV early transcription factors (ETFs), A7 and D6, were cloned into a bacterial vector for protein expression. BL21 DE3 were then transformed with the cloned A7 and D6 for and nicely expressed *in vitro* without the need to culture VACV in tissue culture. Research described, herein, describes progress towards a method that is both cheaper and safer than traditional means to express these proteins and conduct initial drug screening.

Methods

Poxvirus Early Gene Promoter Alignments:

Using the previous literature which describes the conserved properties of the poxviral early gene promoter, different poxviral early gene promoter sequences for the viral DNA polymerase were aligned through Clone Manager 9 (Sci – Ed software). Sequences were taken +1 nucleotides downstream of the promoter, at the first nucleotide to be transcribed, and upstream at the -35 base pair.

Cloning of D6R and A7L into Bacterial Expression System:

Vaccinia early transcription factor D6R and A7L were cloned into the pET303 C-terminal His (pET CT) vector through polymerase chain reaction (PCR), restriction digest, and ligation (Fig. 3). Step 1, involved PCR, restriction digest, and gel extraction of linearized VETFs. Step 2 involved restriction digest and gel extraction of linearized pET CT. Step 3 ligates the VETFs into the pET CT vector, and step 4 involves transforming bacteria for plasmid maintenance and bacterial expression. Primer design integrated sites for restriction endonucleases XbaI (forward) and XhoI (reverse) (Table 1). The D6R forward primer was 5'-TTC TCT AGA ATG AAT ACT GGC ATT ATC GAC-3' and reverse primer as 5'-AGA CTC GAG AGG GCT GCT G-3' (underlined nucleotides indicate restriction sites). A7L forward primer was 5'-TTC TCT AGA ATG AGG TAT ATC GTG-3' and reverse primer 5'-AGA CTC GAG ATT GAT CTG TG-3'. Underlined nucleotides denote restriction enzyme cleavage sites. PCR was accomplished with plasmid D6R_pcDNA 3.1 and A7L_pcDNA 3.1 as the template (Table 1) and completed using the Phusion® High-Fidelity PCR Kit (New England BioLabs® *Inc.*) D6R amplification was optimized for the annealing temperature of 57^{o}C for the Multi Gene II thermocycler, while A7L

required an annealing temperature of 54ºC. After PCR, 5µl of the sample was loaded into a 0.7% agarose gel set to 100 volts for 40 minutes to detect an amplicon of the right size 1963bp for D6R amplicons or 2172bp for A7L. Amplicons were visualized with the 2UV Transilluminator GelDoc-IT System (UVP) and imaged with VisionWorksLS software. Samples were purified following the Wizard® SV Gel and PCR Clean-Up System protocol (Promega).

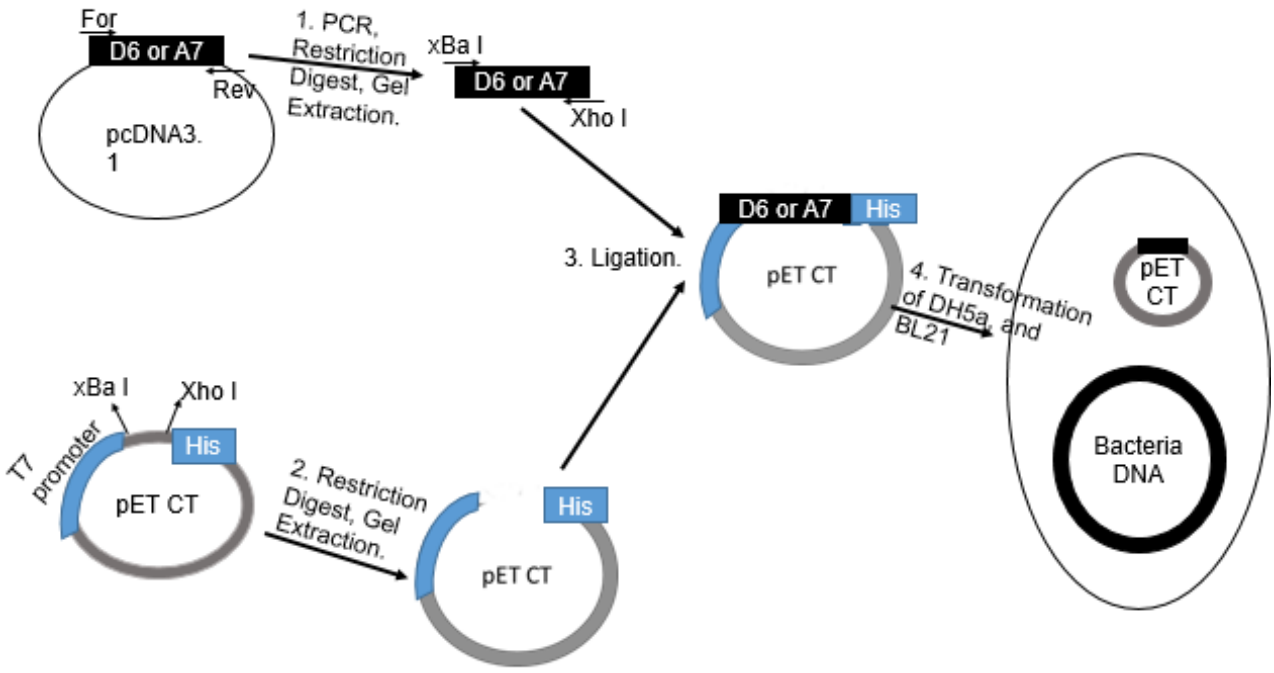


Figure 3: Cloning of VETFs into bacterial expression vector pET CT. PCR using primers (Table 1) was done to clone VETFs D6 and A7 into the pET CT bacterial expression vector.

Restriction digests, of the aforementioned restriction sites were performed for 1μg of DNA of D6r, A7L and pET CT following New England BioLabs® *Inc.* protocol in 50μl reactions in a 37^{o}C water bath overnight. The linearized products were loaded on a 0.6% agarose gel set to 100 volts for 30 minutes and bands for D6R, A7L and pET CT. After, the gel was visualized with MaestroGen illuminator, before bands were excised for purification by following the Wizard® SV Gel and PCR Clean-Up System protocol (Promega).

D6R and A7L inserts were ligated into pET CT containing the *amp* resistance gene using the T4 DNA ligase protocol (New England BioLabs® *Inc.*) in twenty microliter reactions at room temperature for 25 minutes. The resulting ligated plasmid was transformed into *Escherichia coli* (DH5α) following the MAX Efficiency® DH5α^{TM} Competent Cells protocol (Fig. 3). The transformed DH5α cells were plated onto Luria-Bertani (LB) agar plates in the presence of ampicillin (100μg/mL) and grown overnight to produce colonies. The *amp* positive colonies were picked and grown overnight in 3.5ml of LB broth containing ampicillin (100μg/mL) which was incubated at 37^{o}C with shaking at 225rpm to produce glycerol stocks for plasmid production and DNA extraction. Samples were submitted to Genscript for sequence verification.

Primer	Primer Name	Sequence	Tm	GC Content	Restriction enzyme	Template
P1	D6CT For	5’-TTC TCT AGA ATG AAT ACT GGC ATT ATC GAC-3’	56.3ºC	36.7%	Xba I	D6_pcDNA 3.1
P2	D6CT Rev	5’-AGA CTC GAG AGG GCT GCT G-3’	59.0ºC	63.2%	Xho I	D6_pcDNA 3.1
P3	A7CT For	5’-TTC TCT AGA ATG AGG TAT ATC GTG-3’	51.2ºC	37.5%	Xba I	A7_pcDNA 3.1
P4	A7CT Rev	5’-AGA CTC GAG ATT GAT CTG TG-3’	51.3ºC	45.0%	Xho I	A7_pcDNA 3.1
P5	D6NT For	5’-GGC TCG AGA ATA CTG GCA TTA TCG-3’	57.4ºC	50.0%	Xho I	D6_pcDNA 3.1
P6	D6NT Rev	5’-ACA GGA TCC CTA AGG GCT G-3’	56.3ºC	57.9%	BamH I	D6_pcDNA 3.1
P7	D6NT Rev 2	5’-ATA GGA TCC CTA AGG GCT GCT GAC-3’	60.0ºC	54.2%	BamH I	D6_pcDNA 3.1
P8	T7 Promoter For	5’-TAA TAC GAC TCA CTA TAG GG-3’	47.5ºC	40.0%	-	D6_pET303CT
P9	T7 Promoter Rev	5’-TAG TTA TTG CTC AGC GGT GG-3’	54.8ºC	50.0%	-	D6_pET303CT
P10	D6_internal	5'-GAG CGA GAT TAC ATG ATC AC-3'	50.5ºC	45.0%	-	D6_pET303CT
P11	A7_internal	5'-TTA TCC TGA ACG ACG AGC -3'	51.7ºC	50.0%	-	A7_pET303CT
P12	D6NT_internal	5’-AAG CTG CAG GAG CGA GAT TA-3’	56.3ºC	50.0%	-	D6_pET302NT

Table 1: List of primers used for cloning and sequencing, containing: melting point temperatures.

GC nucleotide Content, restriction enzyme sites, and templates.

Plasmid DNA Extraction:

DNA plasmids were extracted using the PureYield™ Plasmid Miniprep System protocol (Promega). DH5α cells harboring plasmid D6R or A7L were grown for 16-20 hours in LB broth supplemented with ampicillin (100μg/mL). Bacteria were centrifuged at 14,000 rpm for 30 seconds, then resuspended in 600μL of nuclease-free water. The Promega protocol was followed and finally plasmid A7 and D6 were eluted in nuclease-free water. The concentration and purity of each sample was evaluated using the NanoVue™ Plus Spectrophotometer (GE Healthcare).

Protein Expression:

DH5α stocks containing D6R or A7L were grown overnight and harvested using the aforementioned PureYield™ Plasmid Miniprep System (Promega). Plasmids with A_{260}/A_{280} reading 1.8-2.0 were considered satisfactory for transformation into *Escherichia coli* strain BL21 for protein expression. BL21s were transformed following the MAX Efficiency® DH5α™ Competent Cells protocol. Colonies were selected and grown in 2ml of LB broth with ampicillin (100μg/mL) in 10mL Falcon polypropylene centrifuge tubes (Falcon) overnight before being transferred to new Falcon tubes containing 5mL LB broth and incubated at 37°C with shaking at 225rpm for 2 hours or reaching OD_{600}=0.8. D6 and A7 expression was induced through Isopropyl β-D-1-thiogalactopyranosid (IPTG) stimulation at 1mM concentration for 4 hours. BL21 cultures were centrifuged at 5000x gravity for 10 minutes and LB media was discarded. A7 was verified by SDS-PAGE and proteins were visualized with Coomassie staining and immunoblotting. Samples were boiled for 3 minutes with 5% 2-mercaptoethanol in SDS sample buffer (62.5mM Tris pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and chilled on ice until resolved through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) to confirm expression. Polyacrylamide gels were stained with Coomassie blue (40% distilled water, 50% methanol, 10% glacial acetic acid, 0.1g Brilliant Blue) or transferred to a Polyvinylidene fluoride (PVDF) membrane (VWR) for antibody probing. Membranes were blocked overnight in 5% milk (Carnation non-fat dry milk) 1x TBST (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% Tween-20), then probed with monoclonal Mouse anti-His (Thermofisher) diluted (1:2,500) in 0.5% milk TBST for 1 hour. Membranes were washed 3 times with 0.5% milk TBST for 10 minutes, then incubated with the secondary antibody Goat α-mouse-HRP diluted (1:5,000) in 0.5% milk TBST for 1 hour. Finally Membranes were again washed with 0.5% milk TBST and visualized using SuperSignal™ West Pico or SuperSignal™ West Femto (Thermo Scientific) chemiluminescent reagent. Membranes and Coomassie stained gels were visualized using a FluorChem E imaging system (Protein Simple).

Cell Viability Screening of H2TMPyP:

Human embryonic kidney cells (HEK 293 cells) and HeLa cells were seeded into clear bottom 96-well plates and incubated overnight at 37ºC in a 5% CO_2 humidified incubator for 24 hours until the plates reached 50% confluence. Serial dilutions using Opti-MEM (Gibco/Life Technologies) and 5, 10, 15, 20 tetrakis(1-methyl-4-pyridyl) 21H, 23H-porphine (H_2TMPyP) was added to the media at 50μM, 25μM, 12.5μM, 6.25μM and 0μM (control), and plates were incubated at 37ºC for 24 hours. Cell viability was determined following the Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay) (Promega 2012). In the procedure, 20μL of reagent was pipetted into each well and incubated for 4 hours in the 37ºC, 5% CO_2 humidified incubator. The plates were placed in the Spectramax M5 spectrophotometer to obtain absorbance readings at 490nm. The SoftMaxPro software was used to interpret the resulting absorbance readings, and significance was determined using Student's t-test.

Protein Extraction:

BL21s induced with IPTG at 0.125mM were centrifuged at 14,000rpm for 30 seconds to discard the supernatant. IPTG concentration was reduced to prevent the formation of insoluble bodies. To maintain similar buffer conditions for purification, lysis was done using Buffer 1 (Table 2; list of buffers and components used for lysis and extration) along with 10mM Dithiothreitol and 1mM phenylmethylsulfonyl fluoride (PMSF). Without lysozyme this buffer was ineffective, pellets were lysed with the Equilibration buffer along with 1mg/mL lysozyme at 37^{O}C for 1 hour before being centrifuged at 14,000rpm. The supernatant was separated from the debris and both stored for western blot detection.

Bfr #	Buffer Name	Components
1	Equilibration Buffer	50mM NaPO4, pH 7.4, 300mM NaCl
2	STET Buffer 8.0	10mM Tris-HCl, pH 8.0, 0.1M NaCl, 1mM EDTA, 1% Triton
3	STET Buffer 6.2	10mM Tris-HCl, pH 6.2, 0.1M NaCl, 1mM EDTA, 1% Triton
4	Denaturing Buffer	50mM NaPO4, pH 8.0, 300mM NaCl, 8M Urea
5	10% SDS Buffer	50mM Tris-HCl, pH 8.0, 8M Urea, 10% SDS10mM DTT
6	1% SDS Buffer	50mM Tris-HCl, pH 8.0, 8M Urea, 1.0% SDS
7	0.5% SDS Buffer	50mM NaPO4, pH 8.0, 300mM NaCl, 8M Urea, 0.5% SDS
8	Equilib: 1% Tween	50mM NaPO4, pH 7.4, 300mM NaCl, 20mM imidazole, 1% Tween-20
9	Equilib: 8M Urea	50mM NaPO4, pH 8.0, 300mM NaCl, 8M Urea, 20mM Imidazole
10	Equilib: Urea/Tween	50mM NaPO4, pH 7.4, 300mM NaCl, 8M Urea, 20mM imidazole, 1% Tween-20
11	Equilib: 1% Triton	50mM NaPO4, pH 7.4, 300mM NaCl, 20mM imidazole, 1% Triton X-100
12	Equilib: Urea/Triton	50mM NaPO4, pH 7.4, 300mM NaCl, 8M Urea, 20mM imidazole, 1% Triton X-100

Table 2: List of buffers used for lysis and denaturation for reference.

Buffer compatibility and lysozyme storage may have been an issue, thereby cells were lysed in Buffer 2 or 3 (Table 2), lysozyme (1mg/mL), 2μL of DNAse I per 100μL, and supplemented with 1mM PMSF. Samples were centrifuged at 10,000rpm at 4^{O}C to separate lysates from cell debris. Cell debris was resuspended in Buffer 4 (Table 2) and incubated for 1 hour at 37^{O}C to determine how effective denaturation conditions might be.

Lysates showed minimal extraction of the protein, A7. Pellets were lysed with Buffer 2(Table 2) and the cell debris was further denatured upon resuspension in Buffer 5 (50mM Tris-HCl, pH 8.0, 8M Urea, 10% SDS 10mM DTT) supplemented with 1mM PMSF and incubated for 30 minutes in a 37^{o}C water bath. Samples were centrifuged at 8,000rpm at room temperature and the supernatant was separated from the pellet for western blotting and Coomassie staining. The amount of SDS was too high for purification purposes, hence Buffer 6 (Table 2) was designed with 1% SDS for denaturation. The final concentration of SDS in the new Buffer 7 (Table 2) was 0.5% (50mM Sodium Phosphate, pH 8.0, 8M Urea, 300mM Sodium Chloride, 0.5% sodium dodecyl sulfate (SDS)) supplemented with 1mM PMSF for 1 hour in a 37^{o}C water bath. Samples were centrifuged at 8,000rpm and the supernatant was separated from the debris.

To test nonionic detergent conditions relative to SDS conditions, Buffers 4, and 8-12 were used to denature debris after lysis with Buffer 2 (Table 2). Denaturation followed for 1 hour in a 37^{o}C water bath, samples were then centrifuged at 10,000rpm at 4^{o}C and the supernatant and debris were separated for western blotting.

A7 Purification:

Protein purification was established through Capturem^{TM} His-Tagged Purification Miniprep Kit (Clontech). Columns were equilibrated at room temperature with 400µl of denaturing buffer and centrifuged at 11,000rcf. Three hundred microliters of clarified lysate was added to the column and centrifuged for 3 minutes at 6,500rcf. The resin in the column was then washed with 300µl wash buffer (50mM Sodium Phosphate, 300mM Sodium Chloride, 10mM Imidazole, 8M Urea, .5% SDS) at 11,000rcf. A7 was eluted with 300µl of non-denaturing elution buffer first (50mM Sodium Phosphate, 300mM Sodium Chloride, 500mM Imidazole) at 6,500 rcf. A second elution was done with 300µl of a second elution buffer (50mM Sodium Phosphate, 300mM Sodium Chloride, 10mM Imidazole, 8M Urea, .5% SDS). Twenty-five microliters of the flowthrough from each step after equilibration of the column was separated to be run through SDS-PAGE. SDS-PAGE gels were stained with coomassie blue or transferred to PVDF membranes to be His-probed and immunoblotted. Eluted samples were stored in 20% glycerol at -20^{o}C for further analysis and use.

Extraction of D6:

Lysis of D6 was started with 100µL Buffer 2 supplemented with 1mg/mL lysozyme, 1mM PMSF, and DNAse I at 37^{O}C for 1 hour. Samples were centrifuged at 10,000rpm for 10 minutes to separate the supernatant from the debris. Cellular debris was resuspended in the aforementioned denaturing buffer 7 and denaturation was done for 1 hour at 37^{O}C. Extraction of D6 was visualized through Coomassie staining and antibody probing.

Results:

Early Gene Alignments:

Compounds binding to the VACV DNA polymerase promoter (Fig. 4) may be capable of binding to other poxviral DNA polymerase promoters. Thus, sequences from different poxvirus promoters for the viral DNA polymerase were aligned using Clone Manager 9 with VACV's promoter acting as the consensus (Sci-Ed Software). The promotor resides upstream of the polymerase gene therefore sequence analysis was done from the Adenine of the ATG site to 50 base pairs upstream. Amongst poxviruses of the *orthopoxviridae* family, there is a 100% match of nucleotides in each region (Fig. 4). Amongst poxviruses of different families there is a common sequence in the first section of the critical region up until the Guanine. The poxviruses promoter regions have a consistency within the critical region that has been identified in early promoters, but after the critical region is where variation occurs. Yet, the similarities between the promotors exist. Thus compounds that bind early promoters of VACV may cross react with other poxviruses.

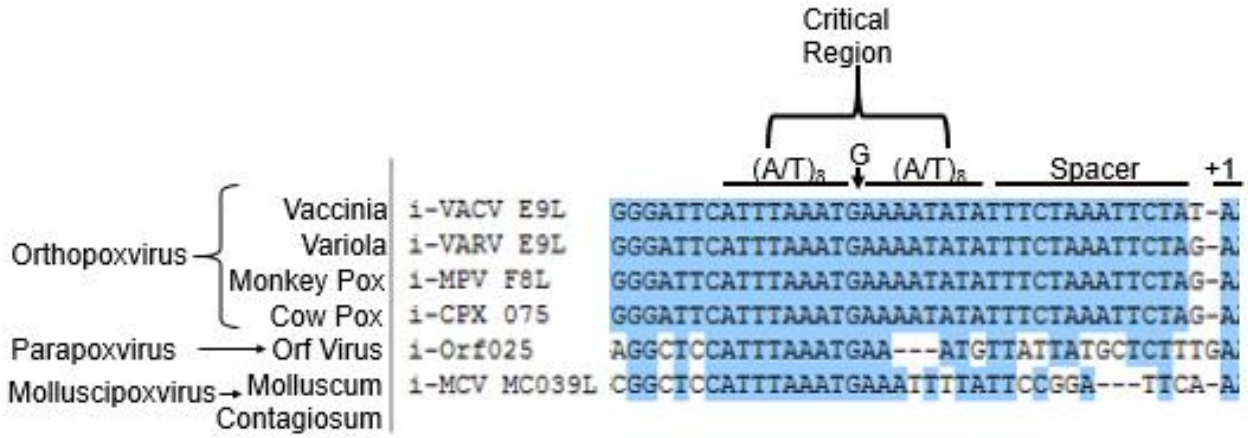


Figure 4: Sequence alignments of Poxvirus DNA Polymerase (E9L) promoter regions.

Promoters contain a critical region, and a spacer region before the DNA polymerase +1 A nucleotide.

Drug Cytotoxicity and Cell Viability:

H_2TMPYP was chosen to test binding due to preliminary data from Dr. Cosimo Antonacci and Taryn Heiser (Dept. of Chemistry, Seton Hall University, NJ). Circular Dichroism (CD) spectroscopy is a common biophysical technique utilized to assess protein and DNA conformation (Johnson, 1999; Jordan et al., 1972). In order to utilize CD spectroscopy, a molecule must absorb light and contain structural asymmetry. In the proper solvent and sequence, DNA contains both required elements. Of particular use are the specific spectroscopic signatures linked to specific DNA conformations. In a separate set of studies, Dr. Antonacci showed that the sequence adopted by these DNA promoters is non-canonical. It was apparent that non-canonical DNA may provide a more selective target versus typical Watson-Crick B-DNA. Therefore, Dr. Antonacci embarked on another set of studies, wherein he tested the binding of a known drug, 5, 10, 15, 20 tetrakis(1-methyl-4-pyridyl) 21H, 23H-porphine (H_2TMPyP) to the polymerase promoter sequence. When a drug binds DNA, the spectra of the drug can be significantly altered. By systemically increasing the DNA concentration and measuring the absorbance of a drug, one can assess the binding affinity and the total numbers of drug molecules bound to the drug. According to Dr. Antonacci's data, the drug bound with significantly greater affinity to the polymerase promoter sequences when compared to a scrambled polymerase promoter sequences (control). The next step was to determine if H_2TMPYP had any cellular cytotoxicity. To this end HeLa cells were treated with increasing concentrations of H_2TMPYP. After 24 hours of treatment with H_2TMPYP, cellular viability was determined using the MTS assay. Concentrations of H_2TMPYP of up to 50μM had no detectable effect on cellular viability as values were comparable to cells mock treated with DMSO alone

(Fig. 5). Two hundred micromolar of sulforaphane was used as a positive control to induce loss of cellular viability. Sulforaphane is a derivative of isothiocyanates, and previous literature suggests sulforaphane acts as a potent inducer of cell cycle arrest and apoptosis in cancer models (Ho et al., 2009; Li & Zhang, 2011).

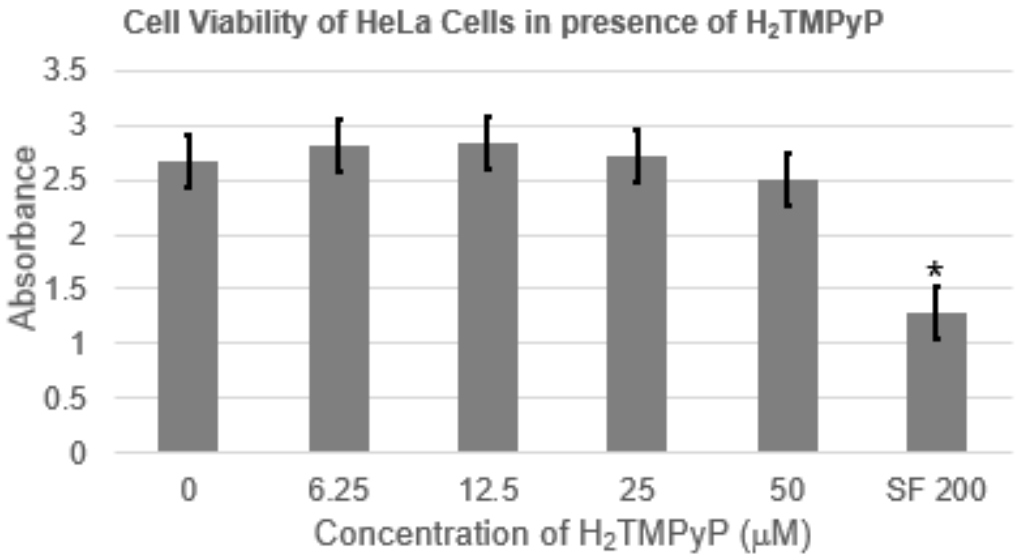


Figure 5: Cell Cytotoxicity of H_2TMPYP and tolerance of HeLa cells. Cells treated with increasing concentrations of H_2TMPYP. Sulforaphane (SF) was used as a positive control for cell death. Significance between treatments was determined by two tailed Student's T Test with significance (*) = $P<0.05$. Experiments were performed in triplicate, n=3.

PCR Amplification and Cloning:

Originally D6 and A7 were cloned into the mammalian expression vector pcDNA 3.1, thus allowing expression in mammalian cell lines. To express the VETFs in bacteria, D6 and A7 were PCR amplified and inserted into bacterial expression vector pET303 C-terminal His-tag at the XbaI and XhoI restriction sites (Fig. 3). The pET303 vector was chosen because it is inducible under the LacI repressor gene, providing control over expression. Further transcription is under the control of the T7 promoter. Only bacteria maintaining the T7 polymerase are able to be express the plasmid proteins. The vector also contains Ampicillin resistance gene facilitating selection for the plasmid. PCR amplification of D6 was successful providing a single amplicon of 1,900bp in length which is the predicted size of D6R (Fig. 6a). PCR amplification of A7 resulted in a single amplicon of the predicted size of 2,300bp (Fig. 7a). After verification, samples of D6 and A7 were purified and along with the uncut vector digested with XbaI and XhoI overnight, then run through gel electrophoresis and extraction. Once extracted, gel slices were purified and ligation was done. After ligation, DH5α were transformed with either A7L/pETCT or D6R/pETCT. DH5α is a *recA* deficient strand and is used to maintain plasmid DNA. Four colonies were picked, grown overnight, and harvested using the PureYield™ Plasmid Miniprep System. One microgram of plasmid DNAs was restriction digested using Xho I and Xba I restriction endonucleases to determine if A7 and D6 were successfully cloned into the bacterial expression vector. D6R/pETCT clones 1-4 were successfully transformed as the vector and insert were detected as bands at 1,900bp, for D6R, and 6,000bp, for the vector (Fig. 6b). D6/pETCT provides a linearized strand of the insert D6 and no detectable secondary products below or above the 1.9kb product.

For A7, of the 4 colonies that were picked, 3 of the samples (lanes 3-5) provide a band at the predicted site, whereas lane 2 for A7/pETCT does not present a band at 2,300bp (Fig. 7b). The band that is seen in A7/pETCT lane 2 appears to be larger than the vector bands, suggesting the product may not have been digested properly or may not be the expected product. D6/pETCT and A7/pETCT sequences were confirmed by Sanger Sequencing by Genscript.

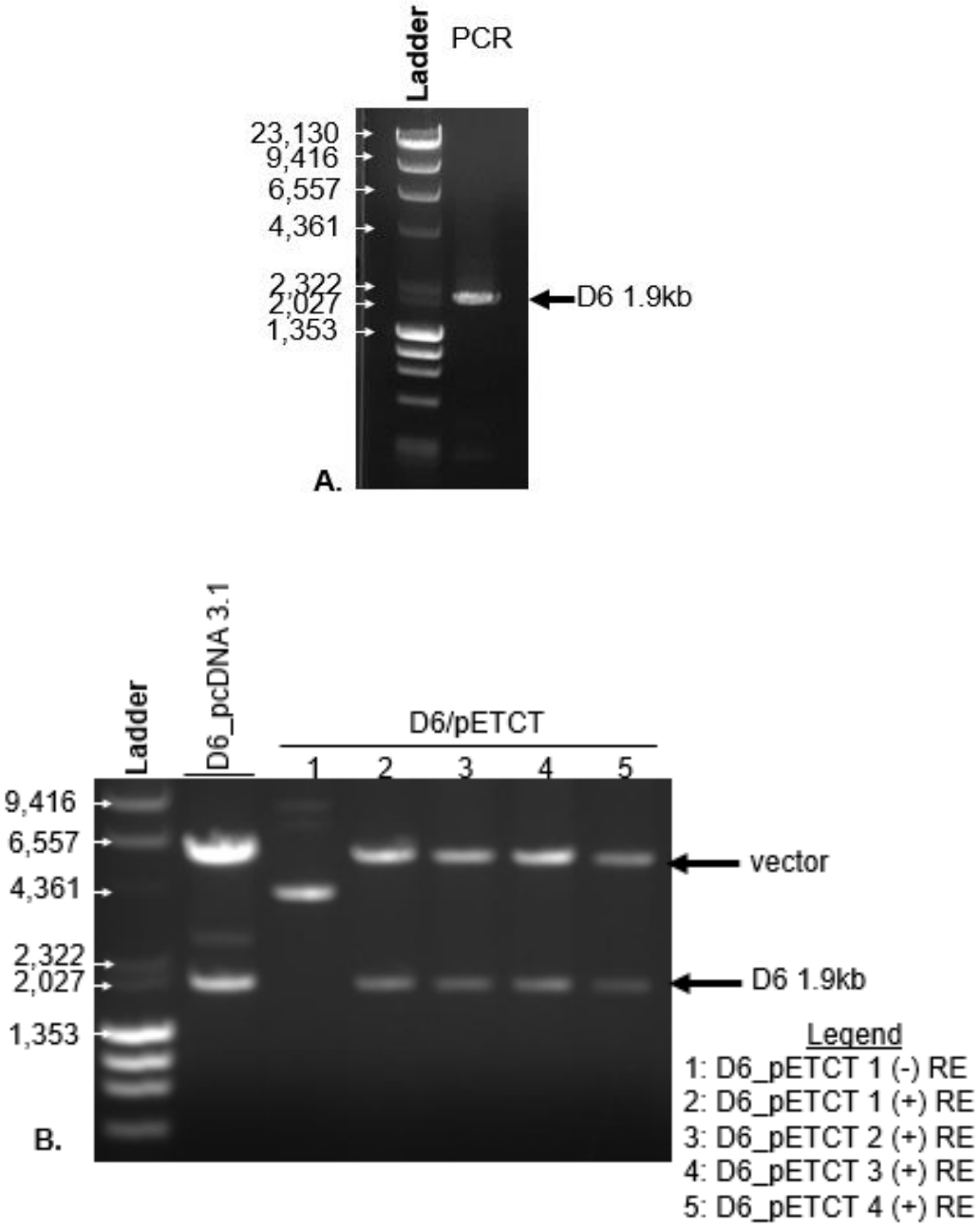


Figure 6. A) PCR and B) Restriction Digest verification of D6 CT. D6_pcDNA3.1 was digested using BamHI and XhoI.

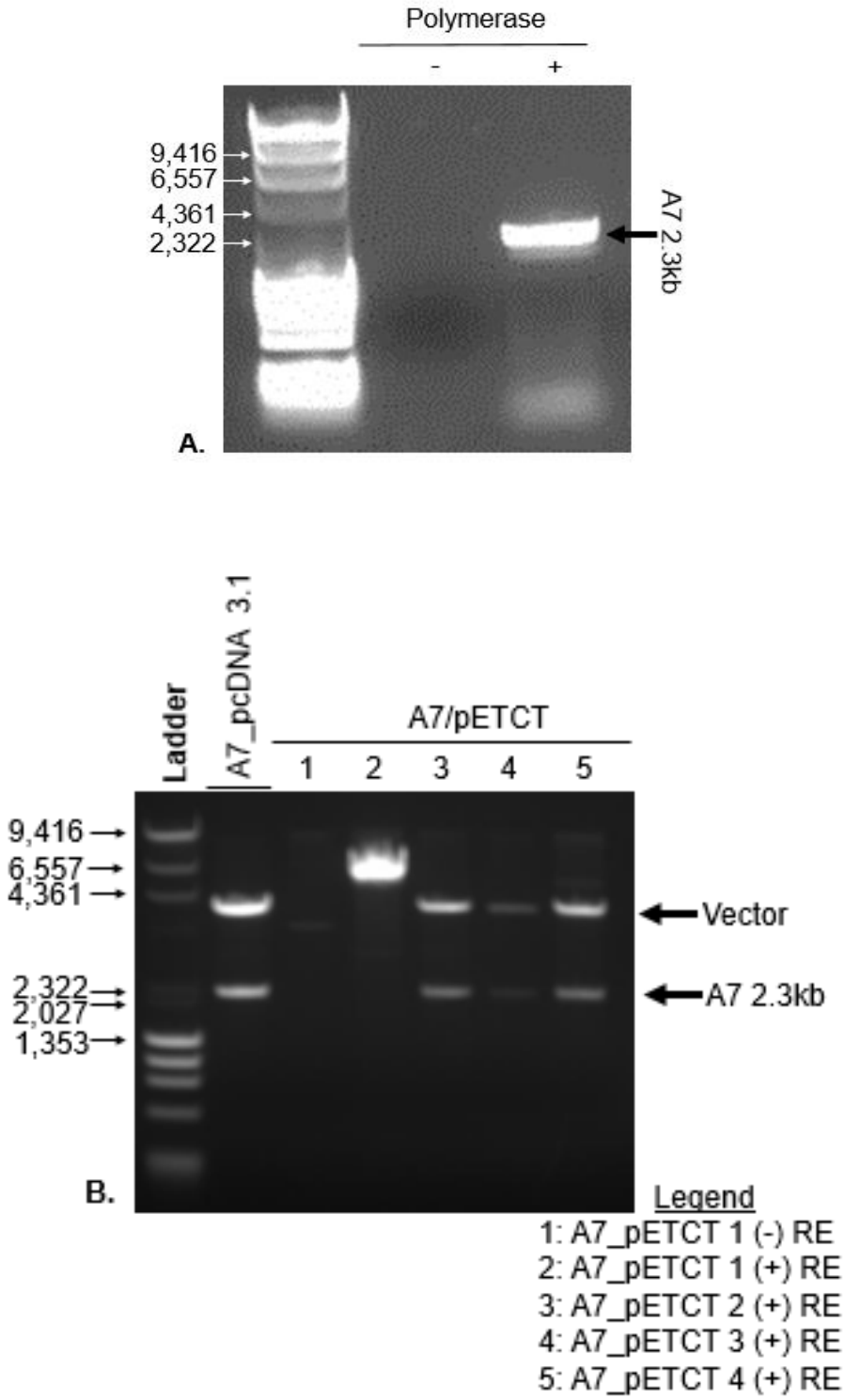
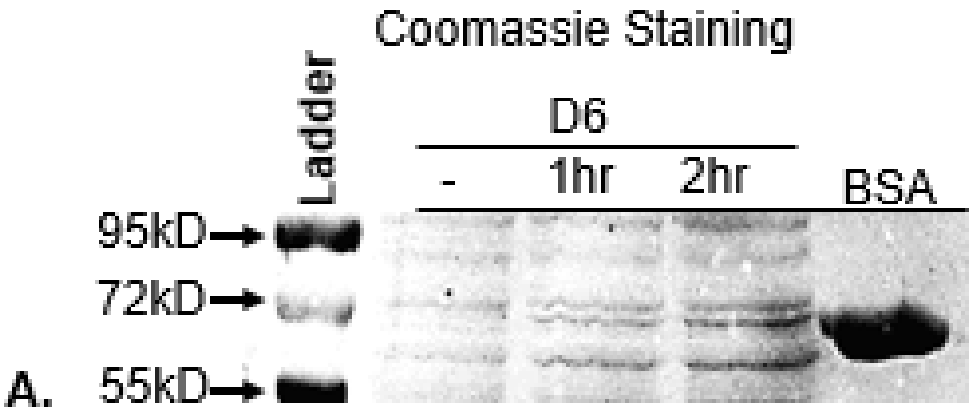


Figure 7: A) PCR and B) Restriction Digest of A7CT. A7_pcDNA 3.1 was used to verify band size.

Bacterial Expression:

DH5α are utilized for cloning and maintaining plasmid DNA of D6R/pETCT and A7L/pETCT. However, DH5α lack the proper T7 RNA polymerase to express the proteins of interest. To express D6R and A7L, plasmids D6R/pETCT and A7L/pETCT, were harvested from the DH5α and transformed into BL21 DE3. The BL21s have the T7 RNA polymerase. Protein expression is controlled under the *lac* operon at the LacI binding site which prevents transcription in the absence of IPTG. Transformed BL21 colonies were picked and grown overnight in the presence of *ampicillin* before being transferred to fresh media. Cells were induced for 2 hours after reaching an OD_{600}=0.8, and a time course between 0 to 2 hours for growth and expression of D6 and A7. Whole cell lysates were run through coomassie staining and western blotting (Fig. 8, 9 respectively). One hour following induction, bands of approximately 72kD and 82kD corresponding the predicted molecular weight of D6 and A7 were faintly detected (Fig 8a, and 8b). At 2 hours, both D6 and A7 expression were similar to levels detected at one hour. The lane for BSA, bovine serum albumin, was loaded as a control at 1mg/ml (Fig. 8a). Rac Kinase/pETCT was used as a control for expression and inducibility. Unlike D6 and A7, basal level expression was detectable as a band of approximately 57.7kD even in the absence of induction. Rac kinase expression increased significantly at one and two hours post induction.



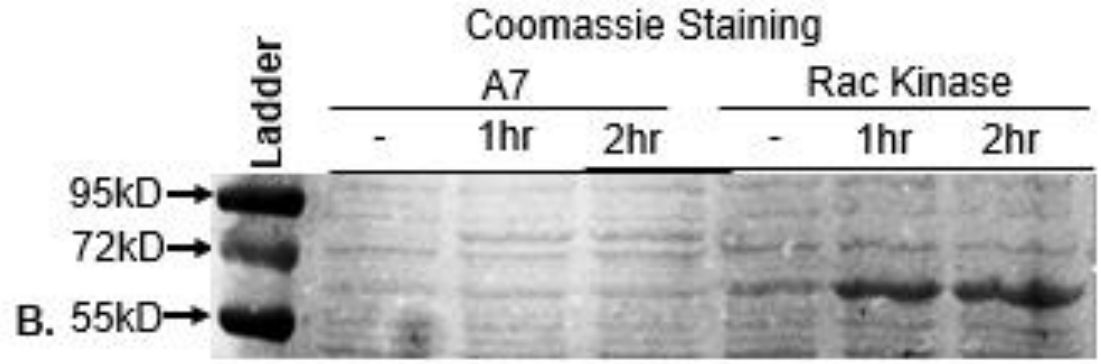


Figure 8: Coomassie stain of whole cell lysates for expression of D6 (72kD), A7 (83kD), Rac Kinase (57.7kD). A) Time course of D6 expression under IPTG (1mM) induction from zero hours to 2 hours. B) Time course of A7 and Rac Kinase expression under IPTG (1mM) induction from 0 to 2 hours.

Both D6 and A7 are His-tagged at the C-terminus (CT) providing a more specific method of detection. Immunoblotting confirmed that expression of D6 was not detectable in samples that had not been induced with IPTG (Fig. 9). However, upon induction, D6 was detected as a duplet, with a band at the expected 72kD size as well as a band below the 72kD marker (Fig. 9). The second D6 band is likely a result of an alternative initiation product. Expression of A7 and Rac kinase can be seen before induction with bands of approximately 83kD and 57kD respectively. Upon induction expression of both A7 and Rac significantly increased.

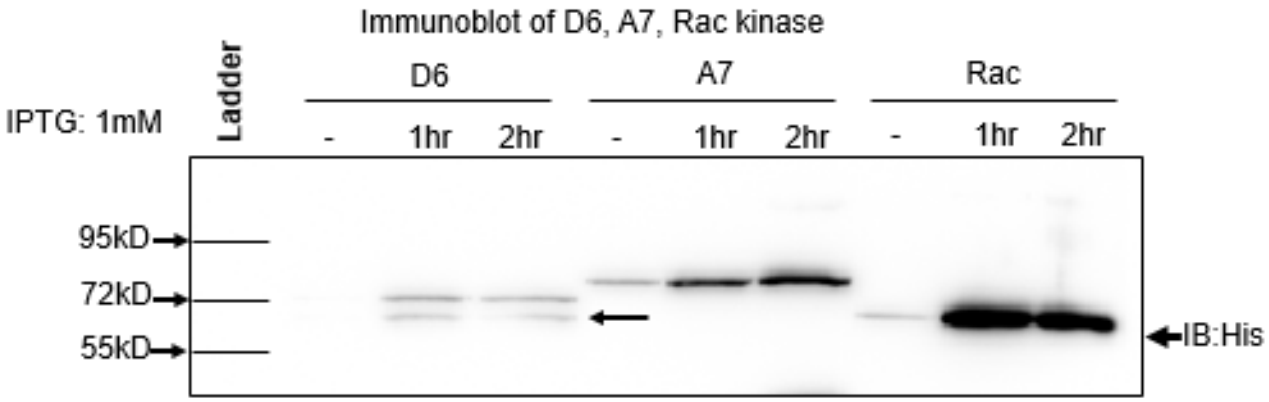


Fig 9. Immunoblot D6 (72kD), A7 (83kD), Rac Kinase (57.7kD). IPTG inductions from 0-2 hours for D6, A7, and Rac. Immunoblots were probed with αHis antibody and bands were visualized using chemiluminescence.

The alternative His-tagged product formed just below the expected D6 band is likely the result of a secondary initiation product (Fig. 10). A second methionine is in frame with the His-tag. Therefore, the second methionine may act as a secondary start site allowing for translation and expression with the His-tag able to be detected upon immunoblotting (Fig. 9). The program ExPASy (Swiss Institute of Bioinformatics) was used to determine the molecular weight of the alternative product. From the second methionine through the His-tag, the program predicted the molecular weight to be approximately 66kD, consistent to the size of the band observed in the His-immunoblots. The secondary product could be detrimental for future expression and purification because it may inadvertently be purified along with intended D6CT and impact future binding assays. To prevent pulling down this alternative initiation product, D6R was cloned into the pET 302 N-terminal (NT) His-tagged vector (Fig. 11). Step 1, involved PCR, restriction digest, and gel extraction of linearized D6R. Step 2 involved restriction digest and gel extraction of linearized pET NT. Step 3 ligates the D6R into the pET NT vector, and step 4 involves transforming bacteria for plasmid maintenance and bacterial expression.

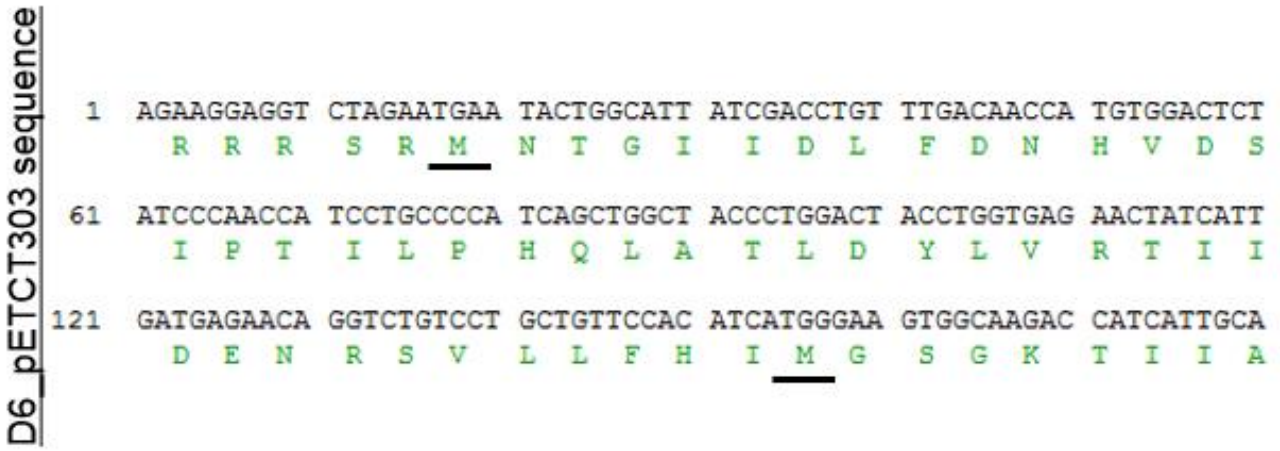


Figure 10: D6 secondary initiation site. Observance of a second Methionine (underlined) downstream and in frame with the first Methionine (also underlined).

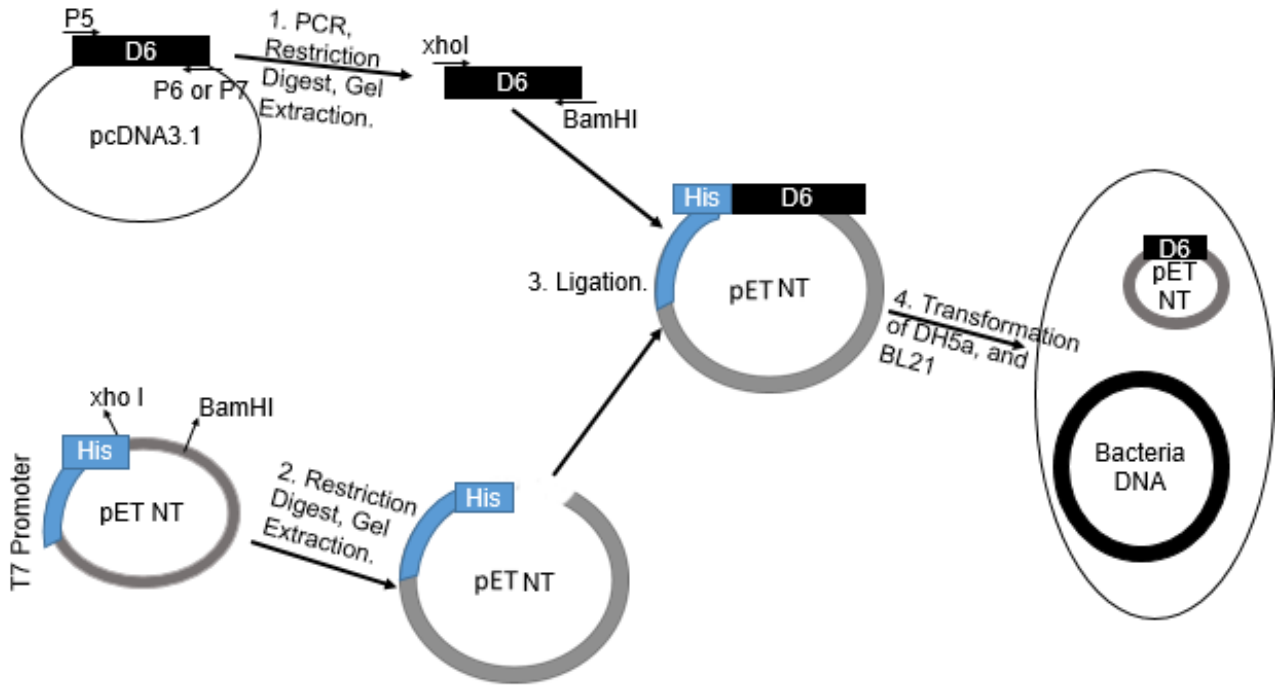


Figure 11: Cloning strategy for D6NT. Cloning of D6R into bacterial expression vector pET NT; PCR using primers (Table 1) was done to clone VETFs D6 into the pET NT bacterial expression vector.

Cloning of D6 into the N-terminal expression vector required removal of the actual start codon on D6, as the vector contained a start codon for N-terminal His expression. By making the start site farther upstream and pushing the second methionine further downstream, translation at the second site less likely to occur. Further, even if transcription occurred at the second initiation site, the secondary product would not be His-tagged and therefore, will not be purified in future purifications. Reverse primer was designed (P7) (Table 1) and used in place of P6 in order to produce singular amplicons (Fig. 12). Upon using P7 in place of PC a singular amplicon was easily detected, which was purified, restriction digested, ligated and transformed into DH5α. Four colonies were picked and grown overnight, D6R/pETNT was extracted, and 1 μg of plasmids were digested using XhoI and BamHI to verifiy cloning, and sequenced.

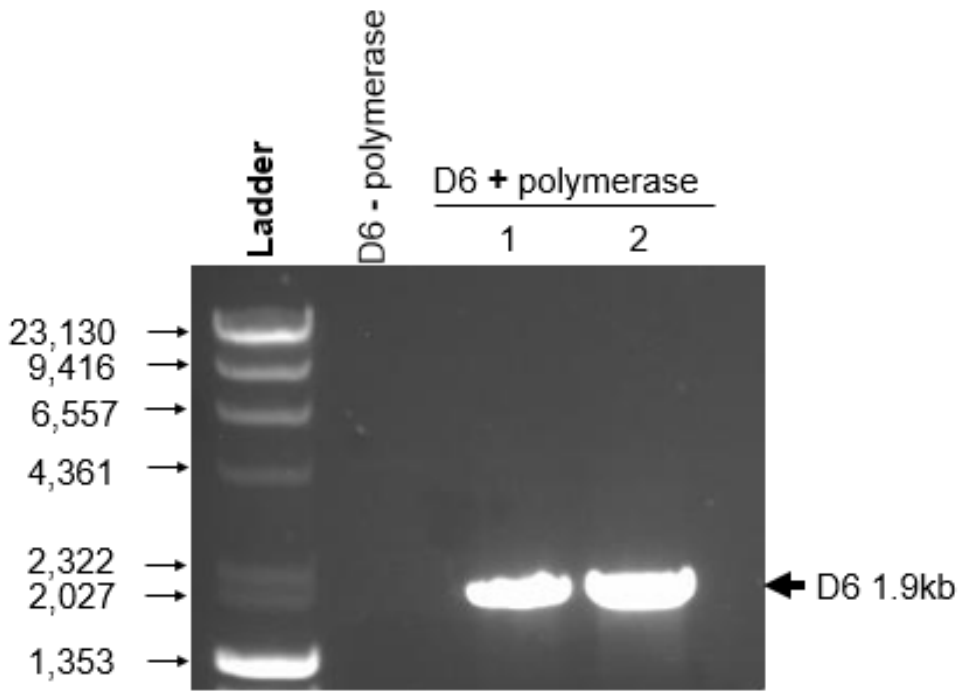


Figure 12: PCR amplification of D6R amplicons for cloning into pET NT. Amplicons produce a single amplicon of D6R (1.9kb) using the new primer P7 (Table 1).

To increase expression of proteins D6NT and A7CT, samples were induced with IPTG for 4 hours (Fig. 13, 14). Comparing 2 hours to 4 hours, D6 expression drastically improved. At 2 hours a faint band for D6 is detectable, but at 4 hours a significantly more intense band was detectable showing that induction up to 4 hours provides greater expression assuming similar OD 600 of inoculant (Fig. 13). The second band previously detected with D6CT (Fig. 9) was also solved as immunoblotting yielded no detection of the second band.

Overexpression of A7 was detected through coomassie staining (data not shown) and probing with Anti-His antibody (Fig 14). Basal expression is still visible even before induction is initiated. At 4 hours considerably greater expression of A7 occurs when compared with 1 hour and 2 hour. However, induction for periods extending past 4 hours would yield degradation products which could be problematic for purification. As a result future expression experiments were set for 4 hours of induction.

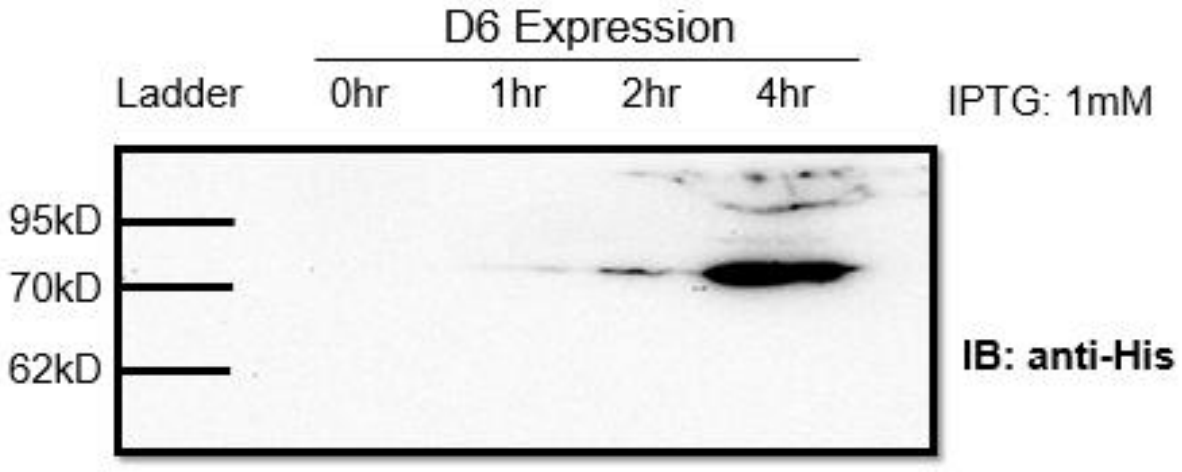


Figure 13: Whole cell D6NT (72kD) expression after 4 hours. Immunoblot of D6NT, depicts stronger banding at 4 hours when compared with 2 hours using 1mM IPTG for induction. No secondary product is detectable upon immunoblotting.

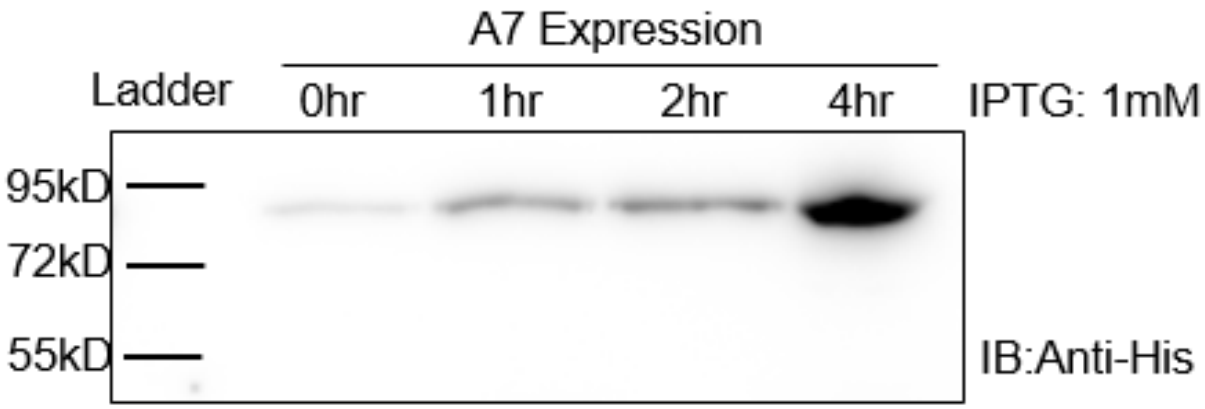


Fig 14: A7 (83kD) expression 4 hours. Detection of A7 before and after induction with 1mM IPTG shows increased expression from 0 hour before induction to 4 hours after induction.

Protein Extraction and Purification:

Once expressed at high concentrations, lysis and solubilization of D6 and A7 was necessary for purification. Samples of A7 and Rac kinase were grown and expressed under IPTG and a sodium phosphate buffer (buffer 1) was used to lyse the bacteria for purification (table 2). However, buffer 1 did not yield much solubilized protein let alone A7 (data not shown). Believing the protein was bound to the DNA, higher concentrations of NaCl and DTT were added to the solution to extract A7 (83kD) and the positive control Rac Kinase (57.7kD) (Fig. 15). From the coomassie stained gel (Fig. 15a) lysis was shown to be ineffective as the abundantly expressed proteins Rac kinase and A7 were stuck in the debris. Lysates also showed minimal lysis of cells, and even less than the control, BSA (0.5mg/mL). Both DTT and NaCl yielded minimal solubility of the two proteins (Fig 15b).

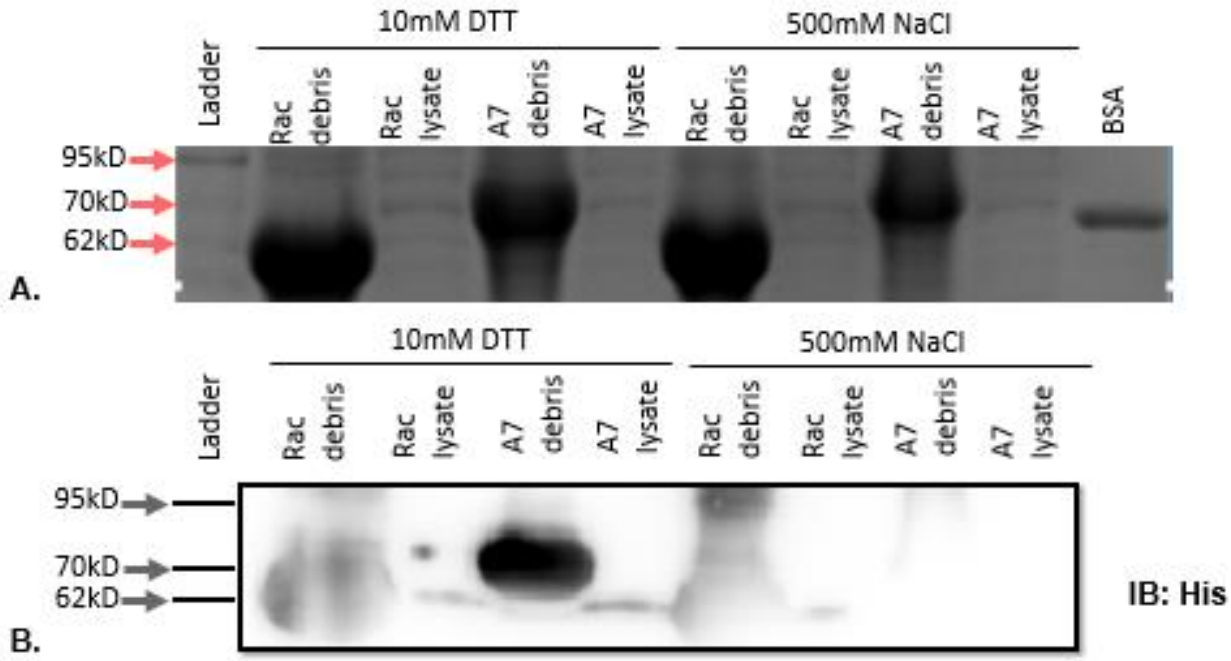


Figure 15: Lysis of Rac Kinase (57.7kD) and A7 (83kD) without Lysozyme. A) Coomassie staining of PAGE gels showing that expression of A7, Rac Kinase, and BSA (0.5mg/mL); B) Immunoblot of Rac and A7 probed with anti-His, bands were visualized using chemiluminescence.

With greater concentration of protein detectable in the debris, the question came to whether or not the bacteria were being lysed properly. *E. coli* maintain a peptidoglycan wall which can be difficult to breakdown, preventing the compounds in buffer 1 from effectively lysing the cells. Therefore, chicken egg white lysozyme (Amresco) was added to the lysis buffer in order to enzymatically break down the peptidoglycan wall. However, upon adding the lysozyme to the buffer there was still no effect on solubility of A7 (data not shown). By optimizing the pH and components of the new buffers, Buffer 2 and 3, the effectiveness of lysis was increased (Fig. 16), when compared with the images from Figure 15. A7 is finally detectable in the supernatant for lysis conditions using Buffers 2 and 3, (STET Buffer (10mM Tris-HCl, 0.1M NaCl, 1mM EDTA, 1% Triton X-100)). The effect of pH in extracting A7 was effective in lysing the bacteria but not solubilizing the majority of A7. Thus denaturation of A7 using 8M Urea (Buffer 4) was initiated and provided a greater solubilization of A7 (Fig 16, Buf 4, pH 8.0). Denaturation followed lysis, and only denaturant supernatants were visualized (Fig. 16). STET Buffers (10mM Tris-HCl, 0.1M NaCl, 1mM EDTA, 1% Triton X-100) with pH 8.0 (Buf 2) provide better lysis than 6.2 (Buf 3) when comparing lysis buffers. Denaturing buffer (50mM $NaPO_4$, pH 8.0, 300mM NaCl, 8M Urea) were used to further solubilize A7 from bacterial pellets after lysis with Buf 2 or Buf 3. Upon denaturation with Buffer 4 more A7 was able to be solubilized, especially under pH 8.0 conditions. However further repetitions provided suboptimal extraction with buffer 4. Thus, buffer 2 was used for further lysis experiments in protein extractions and purification while further denaturation conditions were tested.

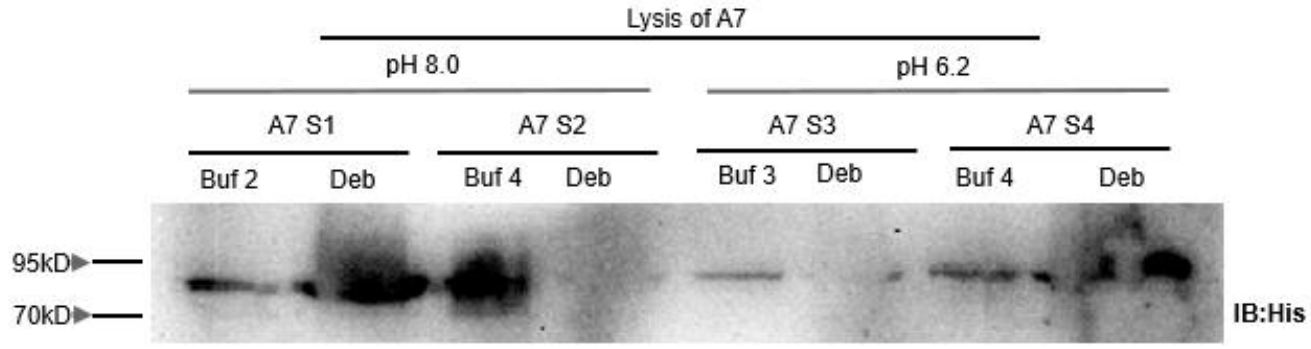


Figure 16: Lysis of A7 (83kD) with lysozyme and optimized buffer conditions. Probing of specific conditions to detect A7 solubilization. Lysis and denaturation was done with different samples of A7 (S1 through S4) shows greater detection of solubilized A7 in the supernatant of Buf 2) (10mM Tris-HCl, pH 8.0, 0.1M NaCl, 1mM EDTA, 1% Triton) and denaturing Buf 4 (50mM NaPO4, pH 8.0, 300mM NaCl, 8M Urea).

To obtain greater concentrations of A7 in the previous buffers, a stronger denaturant was used, Buffer 5, containing SDS, Urea, and DTT (Fig. 17). At different lengths of exposure using the new buffer. Solubilization of A7 was increased and effectively extracted from the bacterial pellet. Coomassie staining indicated that samples of A7 were fully solubilized after 1 hour and that a drastic time length such as 10 hours was unnecessary (Fig 17b.) However the immunoblot in figure 17a notes that although A7 is being extracted at 1 hour, A7 is still detectable in the debris. Ten percent SDS was effective in solubilizing A7, however, the percentage of SDS in the solution was found to be drastically greater than the acceptable concentration necessary for purification. Thus the concentration of SDS was greatly reduced from 10% to 1% (buffer 6) which was the maximum allowed for purification, and finally to 0.5% (buffer 7) (Fig 18). Further buffer 7 consisted of components similar to that of the purification buffer components in order to reduce variability during purification (Table 2).

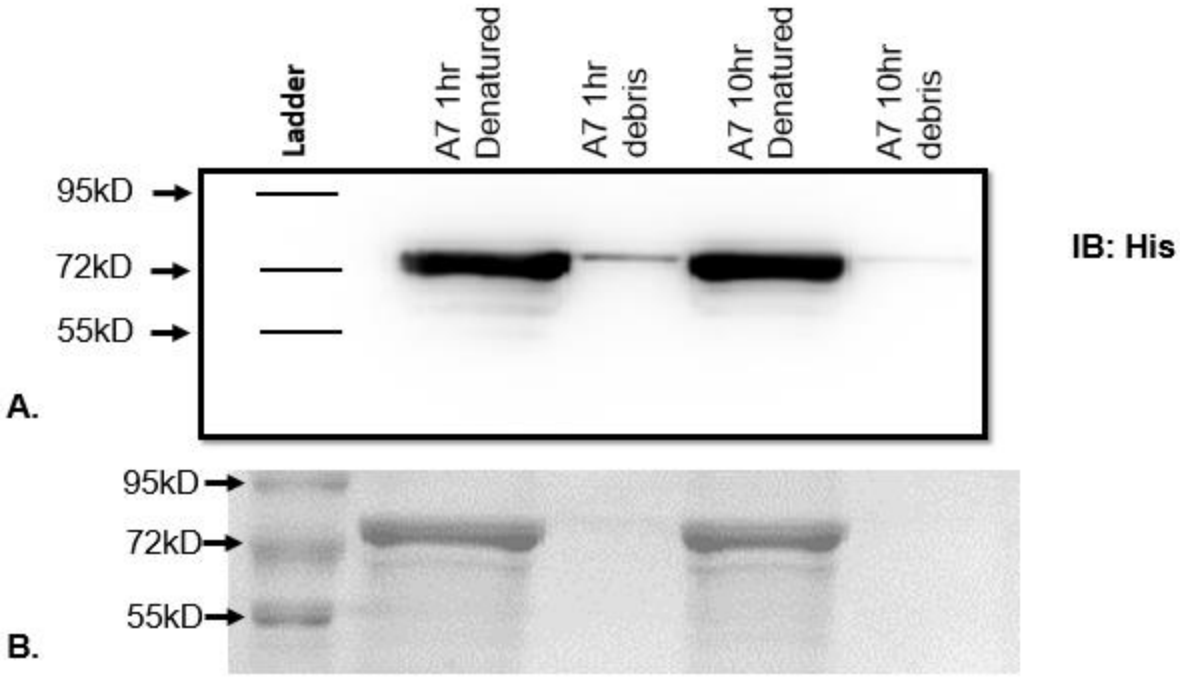


Figure 17: Denaturation of A7 (83kD) with 10% SDS Denaturing Buffer. A) Immunblot detection of A7 over time course denaturation using 10% SDS buffer. Denaturation for 1 or 10 hour yields solubilization of the majority of A7 from the debris. B) Coomassie staining of samples shows detection of the majority of bacterial proteins and A7 within the lysates.

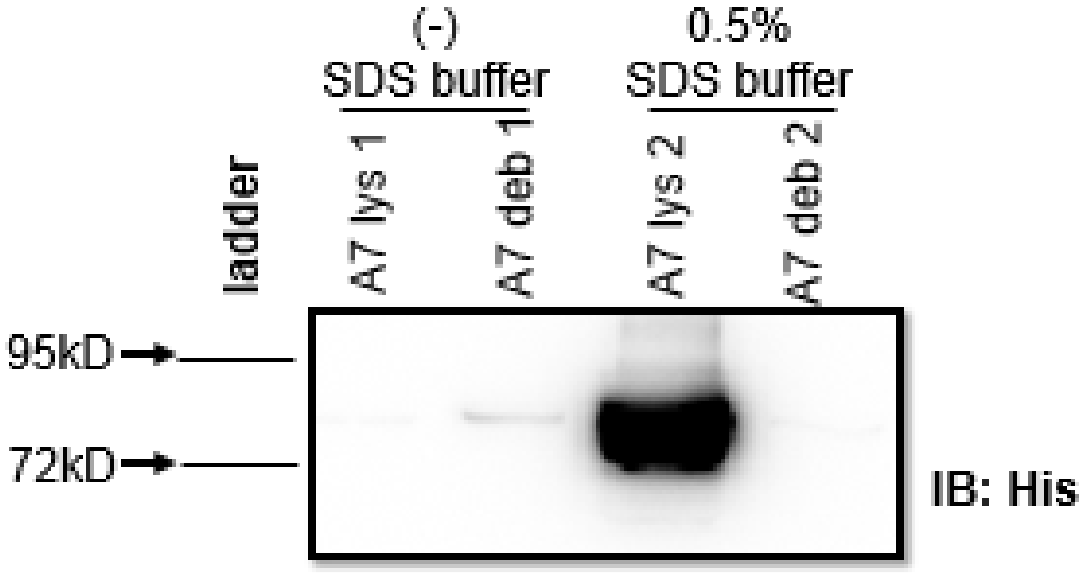


Figure 18: Compared denaturation of A7 (83kD) with non-SDS buffer vs. 0.5% SDS denaturing buffer. Samples of A7 were incubated with buffers to determine the capability to solubilize A7 at lower percentages of SDS.

Purification entailed binding the His-tagged proteins to a nickel based resin, which would then be eluted using imidazole to displace the bound A7 and provide an eluted purified sample. Expressed A7 samples were denatured and run through the miniprep kit to determine if the denaturing conditions would provide a high yield product. The kit allows quick purification of small scale samples, and is capable of yielding roughly 0.1mg/mL to 0.5mg/mL of protein. The kit comes with columns preloaded with a nickel mesh bed which binds the His-tagged proteins. The first attempt yielded no purified A7 and during the purification, a discoloration of the resin was evident. Upon further literature search, it appears that the resin may have been reduced by DTT and prevented A7 from being able to be bind to the column. The second attempt at purification was successful as evidenced by Figure 19. Probing PVDF membranes with Anti-His antibody indicated the presence of A7 in the first elution (lane 4) (Fig. 19), despite significant losses in flow-through (lane 2) and wash (lane 3), most likely due to column capacity and/or poor binding kinetics in the presence of SDS.

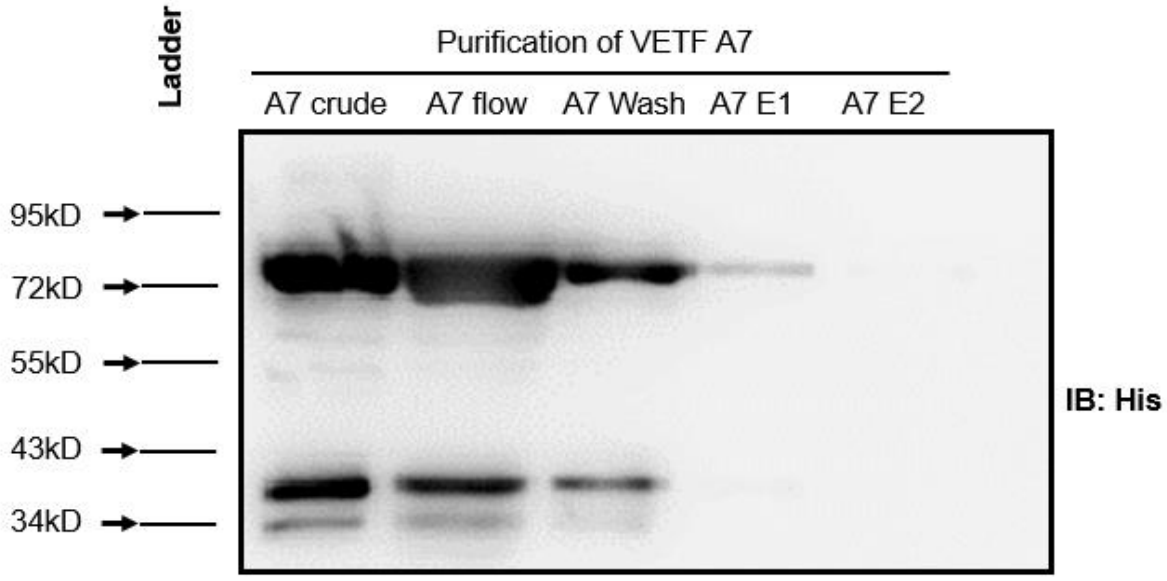


Figure 19: Purification of A7 (83kD) after denaturation with 1.0% SDS. Immunoblot detection of A7 purification products.

Due to the difficulty with solubilizing A7, expression and solubility of D6 (72kD) was also tested as shown in Figure 20. Buffer 2 was utilized for lysis but there is no distinguishable band for D6 after probing with anti-His in lysate 1, lysate 2, and lysate 3 (Fig. 20). Denaturation of D6 is shown to have been very effective in the Buffer 7, yielding strong bands in the immunoblot (Fig. 20), with accompanied greater detection of degradation products. These degradation products may cause problems during purification, as each distinguishable band maintains the His-tag epitope. Regardless, D6 solubilization is quite evident, by the high concentration of solubilized protein when compared with the all 3 sample debris.

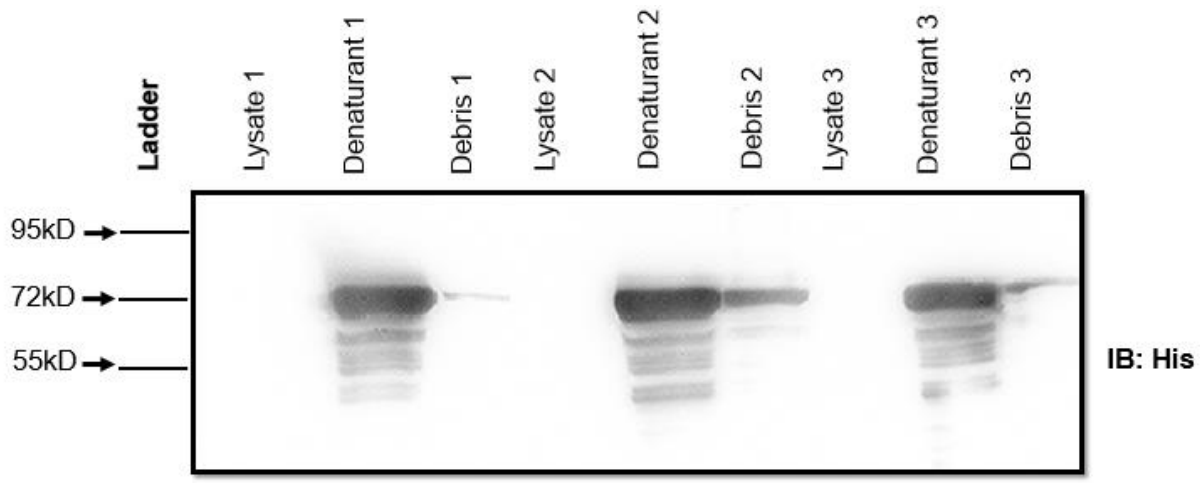


Figure 20: Lysis and Denaturation of D6 (72kD). Samples of D6 were lysed with STET buffer (Buffer 2); pellets were further denatured with 0.5% SDS buffer (Buffer 7) and then run through SDS-PAGE for anti-His probing

Discussion:

The goal of this thesis was to clone and develop a method to express VETF D6 and A7 in bacteria. Previous literature has not shown bacterial expression of VETFs, therefore this thesis represents the first attempt at expressing VETFs in bacteria. These novel methods are the first step in developing an assay to test compounds for potential anti-viral therapy that is both cheap and safe to be done in a BSL-1 setting. Expressing VETFs A7 and D6 in bacteria allows overexpression and maintenance of bacterial stocks for multiple rounds of purification (Fig. 13, 14). This method is far cheaper and safer than maintaining stably transfected mammalian cell lines, because glycerol stocks of transformed BL21 DE3 are easier to maintain and expression can be produced overnight. Bacterial selection is cheaper than mammalian cell selection, further expression can be scaled up to produce more protein than tissue culture or viral cultures would be able to produce.

The compound, H_2TMPyP, which is being used for developing this assay shows high promise as a candidate for anti-poxviral applications. Preliminary data done by Dr. Cosimo Antonacci and Taryn Heiser has shown the compound has high specificity for the VACV DNA polymerase promotor sequence and that sequences are non-canonical. Dr. Antonacci confirmed this data by using circular dichroism and UV-Vis drug titrations. There is no significant cytotoxic effect to mammalian cell lines in the presence of H_2TMPYP with HeLa and HEK 293 cells tolerating the compound up to a 50μM concentration.

By modifying induction conditions, control over the expression of D6 and A7 was able to be established (Fig. 8, 9, 13, and 14). At 1mM IPTG induction expression of A7 was successful, resulting in greater expression of A7 (Fig. 13, 14), while at 0.125mM induction detection of A7 within the supernatant of lysed samples was observable (Fig. 16). Further, establishing proper

induction time courses, conditions, and the concentration of IPTG allowed for better expression levels. Induction may need further optimization to prevent D6 and A7 from becoming insoluble, allowing future endeavors to extract the proteins without the need for denaturation.

Purification of VACV transcription factors D6 and A7 is difficult, with A7 being the more difficult of the two to purify alone (Broyles 1993, Davison & Moss 1989). To increase the capability for purification at greater concentrations, dialysis, selective precipitation and size exclusion chromatography of solubilized proteins can be performed. Further purification by these methods can separate proteins that may interfere with binding.

Future Directions

Further optimization needs to be done in order to increase binding to the nickel mesh columns of the Capturem^{TM} His-Tagged Purification Miniprep Kit (Clontech) (Fig. 19). Binding interference may be solved by diluting the supernatant with a buffer similar to Buffer 7, however not containing SDS, to maintain the amount of protein extracted.

Optimization of the buffer conditions is still necessary to better solubilize A7 without the need for harsh denaturants. By washing the cell pellet with different buffers, it may be possible to extract A7 slowly at low levels, and the resulting samples can be concentrated by purification or purchased concentrators. Although requiring more time, it may prevent degradation and allow for A7 to bind without interference to the nickel resin. If the need for denaturation still arises, refolding the proteins A7 and D6 will be necessary, involving dialysis to slowly fold the VETFs into their proper structures. A second method could involve extracting with 1% SDS then using Ammonium precipitation to crash A7 or D6 out of solution. Once precipitated the VETFs can be solubilized in a less harsh buffer for purification.

Bacterial expression provides a safer cost effective means to express A7 and D6 and can further be scaled to increase the amount of protein expressed. By using bacteria, rather than mammalian cell lines or viruses, researchers reduce expenditure on equipment and reagents. Previous literature provides a method of purifying VETFs from HeLa cells (Broyles 1993). Thus, transfected HeLa cells represents an alternative method for expressing A7 and D6. However, Broyles had similar problems with purification of A7, but by co-expressing A7 and D6 was able to detect both purified A7 and D6. MVA is safe for BSL-1 settings, thus purification of A7 and D6 can be done through viral expression, until bacterial purification has been completely optimized. Therefore, if necessary, co-expression in mammalian cells or viral expression may provide a means for completing this assay until bacterial protein purification has been optimized.

To this point, bacterial expression of VETFs D6 and A7 using the pET vector has been established and optimized to provide abundant expression of protein. Further extraction and solubilization of the proteins A7 and D6 was rigorously tested, and a purification of A7 serves as the foothold for furthering the development of this novel assay. In order for the assay to be effective, A7 and D6, will need to maintain their binding activity in order to determine the efficacy of potential compounds that can competitively bind A7 and D6 sites. Once preliminary testing and binding *in vitro* is completed, one could potentially establish if the compounds have anti-viral properties in live virus. Once developed, this novel assay provides modern anti-poxviral research with a method of testing compounds for broad spectrum anti-viral therapy. This endpoint would improve the efficiency of anti-poxviral research by reducing the cost of research, and may increase the amount of school programs and companies involved in poxviral research.

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