

**DEVELOPMENT OF A VACCINIA VIRUS EXPRESSION  
SYSTEM FOR STRUCTURAL PROTEINS OF ALEUTIAN  
MINK DISEASE PARVOVIRUS**

Submitted in Partial Fullfillment of the Requirements  
for Graduation with Honors to the Department of Biology  
and Chemistry at Carroll College, Helena, Montana

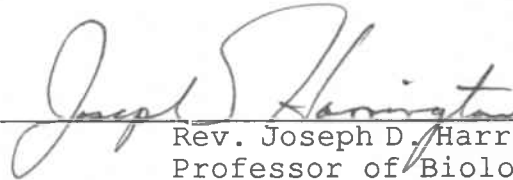
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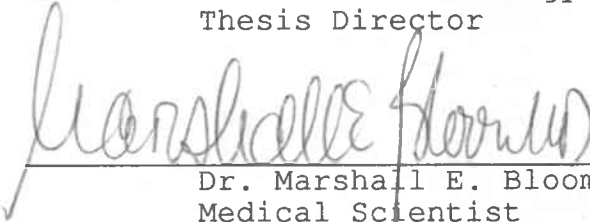


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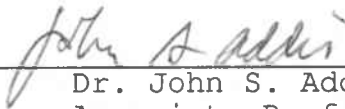
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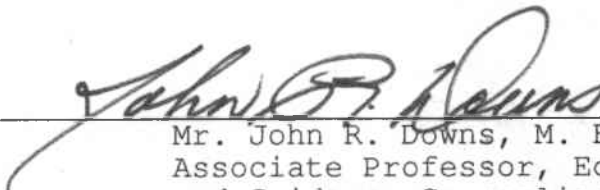
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## ABSTRACT

Aleutian Mink Disease Parvovirus (ADV) belongs to the parvovirus family. Although ADV can be grown in tissue culture and has been cloned in procaryotic systems, a eucaryotic expression system was desired. This would allow the opportunity to examine viral synthesis in absence of ADV replication. I attempted to develop a eucaryotic cloning system using vaccinia virus to express the ADV virion protein genes. My goal was to clone a segment of a cDNA clone (pIB21), that coded for the open reading frame containing both virion protein-1 (VP1) and virion protein-2 (VP2) of ADV, into pSC11, a modified vaccinia virus co-insertion vector. I attempted this cloning procedure with clones constructed from segments of two different strains of the ADV virus, ADV-G and ADV-Utah-1. The recombinant vaccinia virus co-insertion vectors generated from these clones were used by members of Dr. Marshall Bloom's research team at Rocky Mountain Laboratories. They homologously recombined them with live vaccinia virus by transfection into cells previously infected with vaccinia virus. The vaccinia system was successful in expressing the ADV genes and synthesizing the viral proteins. It was later demonstrated that these proteins self-assembled into

viral particles.

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## INTRODUCTION

Aleutian Mink Disease Parvovirus (ADV) is an autonomous replicating parvovirus capable of causing severe disease in mink (Bloom et al., 1980). It was first identified and described in 1956 as Aleutian Disease of Mink (AD) (Hartsough and Gorham, 1956). It was discovered in 1964 that the causative agent was a virus. It was found that the virus could infect all mink strains with varying degree of susceptibility, although it was originally thought only to infect the Aleutian genotype (Porter et al., 1969; Alexandersen, 1990). The virus was characterized as an autonomous parvovirus for the first time in 1980 by Dr. Marshall Bloom (Bloom et al., 1980). This disease possesses the ability to cause multiple separate chronic disorders related to the immune system. Some disorders characteristic of ADV infection by range from decreased fertility and abortion (Alexandersen, 1986) to other syndromes such as immune complex arteritis, hypergammaglobulinemia, plasmacytosis and fatal immune complex-mediated glomerulonephritis (Porter et al., 1972; Porter et al., 1973; Porter et al., 1980).

ADV, like other parvoviral diseases, causes a disease that is at least partially due to the genetic

background of the host organism. It was originally thought that ADV would only become manifest in mink of the recessive homozygous Aleutian genotype, corresponding to the Aleutian coat color (Pattison, 1988).

ADV is unique from other parvoviruses in that it also causes severe acute pneumonia in newborn kits. In this case it affects the Type II alveolar cells of the lung. So the disease is capable of causing chronic and acute disease syndromes in the same host (Alexandersen, 1990). Thus the age as well as the genetic background of mink affects the pathogenicity of the disease.

To express the structural proteins of this system would prove valuable for several reasons. Diagnostic tests could be done without having to grow live ADV in infected mink. Also there are vaccine possibilities from the expression of the structural proteins that could be developed. Another study would be to study proteins and how they act in the absence of DNA.

## LITERATURE REVIEW

### Parvoviridae

Parvoviruses, as a group, constitute one of the smallest DNA-containing vertebrate viruses (Rose, 1974). There are many different viral agents fitting into this family, constituted by three genera: *Parvovirus*, *Dependovirus*, and *Densovirus*. The virion particles of the autonomous replicating parvoviruses contain only two or three proteins and a linear single-stranded DNA molecule. The viruses are assembled in the cell nucleus and form nonenveloped icosahedral particles of 18 to 26 nanometers in diameter (Blacklow and Cukor, 1985). These particles have a molecular weight of  $5.5 \times 10^6$  to  $6.2 \times 10^6$  Daltons (75% to 80% protein). The viral capsids exhibit a large degree of heat and acid stability and are not susceptible to lipid solvents due to the lack of lipids found in the virus. They also have a high DNA content, ranging from 20% to 25%. Infectious viral capsids have high buoyant densities in cesium chloride gradients (from 1.39 to 1.42 g/cm<sup>3</sup>). Also large amounts of empty protein capsids (devoid of DNA) and defective particles containing submolar amounts of DNA can be grown in tissue culture (Cotmore and Tattersall, 1987). These

empty capsids generally have a lower buoyant density of approximately  $1.32\text{g/cm}^3$  (Ward and Tattersall, 1978).

The genome is a linear single-stranded chain of DNA, having a molecular weight (MW) of approximately  $1.5 \times 10^6$  to  $1.8 \times 10^6$  Daltons, approximately 4800 to 5000 bases. There is close conservation of genome length which suggests that there are fairly close constraints on the maximum length of DNA which can be encapsidated (Cotmore and Tattersall, 1987).

The autonomous parvoviruses encapsidate strands of only a single polarity (sense). The genomes of all the parvoviruses sequenced also contain non-identical palindromic or symmetric sequences at both the 3' and 5' ends. These are capable of folding into relatively stable hairpin duplexes in the single-stranded DNA form. The length of the palindrome at the 3' end of the virion strand are about 115 nucleotide bases (Berns et al., 1985). This segment of DNA is capable of folding back on itself, forming a hairpin structure which is stabilized by hydrogen bonding made possible by the self-complementary sequence. When the 3' palindromes fold over they generally fold into either a "T" or a "Y" shaped structure (Berns et al., 1985). The 5' end can also form hairpins, but the sequence is unrelated to the

3' end of the virion strand. The nucleotide sequence of the 5' palindrome is generally a little longer and exists in one of two orientations, termed either "flip" or "flop", which are the inverted complement of each other (Cotmore and Tattersall, 1987).

All the parvoviruses sequenced so far contain two major open reading frames. A left open reading frame that is controlled by a promoter located at ~4 map units (MU). This specifies at least one nonstructural protein necessary for viral replication and gene regulation. A right open reading frame, controlled by a promoter located at ~38 to ~40 MU, which encodes for a set of structural (or capsid) proteins (Bloom et al., 1988). There are generally seven conserved elements found in the amino acid sequence of the autonomous parvoviruses, five of which are found in ADV (Bloom et al., 1988).

#### **ADV Sequence Background**

Early attempts to study ADV showed it consists of a single stranded DNA genome of approximately  $1.2 \times 10^6$  Daltons or around 5000 nucleotides. It was shown that when it was encapsidated the genome consisted of about 4800 nucleotides (Alexandersen and Bloom, 1988). In cell culture replication, replicative double-stranded DNA

molecules were formed (Bloom et al., 1983). The entire genome of ADV-G and central 88 map unit segment of ADV-Utah-1 strain was cloned by Dr. Bloom in 1988 (Bloom et al., 1988). This was crucial for recombinant work. In relation to other parvoviruses, ADV has < 50% DNA sequence homology which correlates with past serological studies that suggested that ADV is not closely related to other parvoviruses. It also only contains five out of seven short amino acid sequences that are conserved among the parvoviruses (Bloom et al., 1988).

## **Viral Strains**

### **ADV-G**

ADV-G (ADV-Gorham) is a strain of virus that has been adapted for growth in cell culture. It was derived in cell culture from ADV-Utah-1 but has lost its pathogenicity for mink (Bloom et al., 1980). The complete virus was molecularly cloned for this strain and the genome sequenced.

The virion DNA strand has several key features. First, its sequence has been found to be 4748 nucleotides and of negative polarity.

A second feature is that based on the sequence analysis and the transcriptional mapping, the two capsid

proteins VP1 and VP2 seem to arise from alternate initiation sites on a single mRNA transcript designated R3. The VP2 transcript is completely contained within that of VP1.

Third, there is a 117 nucleotide palindrome at the 3' terminus of the virion strand that has the potential to assume the "Y" configuration as was mentioned earlier.

Fourth, the known sequence was recently extended to include the 5' terminus, thus lengthening the ADV-G clone to include the entire viral genome (Bloom et al, 1990). This involved lengthening the clone by 155 nucleotides to 4748 nucleotides (Bloom et al., 1990). This segment included the 5' terminal palindrome. It was discovered that the 5' palindrome was 241 nucleotides long and oriented into a flop configuration similar to other sequenced parvoviruses (Bloom et al., 1990).

In a fifth feature, the full length clone contains the characteristic open reading frames of parvoviruses. There is a major left open reading frame of 622 amino acids. There is also a major right open reading frame of 702 amino acids that exists in a different translational frame than the left (Bloom et al., 1988). Despite minimal changes, this strain's pathogenicity is vastly different from ADV-Utah-1.

### **ADV-Utah-1**

ADV-Utah-1 is a strain of the virus very similar to ADV-G. Although no full length clone exists, a clone was constructed that contains the central 88 map unit segment of the clone. At the DNA level ADV-G and ADV-Utah-1 are 97.5% conserved. In the right open reading frame, which codes for the capsid proteins, there are only 22 amino acid differences found. These do not seem to affect the overall theoretical hydropathy of the proteins. A small region, however, known as the hypervariable region, has 8 of 11 amino acid residues changed. These changes may give rise to some of the determinants responsible for the pathogenicity differences found between ADV-G and ADV-Utah-1. Both ADV-G and ADV-Utah-1 code for similar proteins. When grown in vitro the capsid of both these viruses have two polypeptides consisting of 85 Kilodaltons and 75 Kilodaltons. These termed respectively VP1 and VP2 were mentioned earlier. These share overlapping polypeptide composition with the VP2 protein being slightly more abundant (Bloom et al., 1982).

### **VACCINIA VIRUS AS AN EXPRESSION VECTOR**

This member of the poxvirus family of DNA viruses,

which was developed and successfully used as a smallpox vaccine, now serves as a useful means of expressing genes within the cytoplasm of eucaryotic cells. Poxviruses are a family of large DNA viruses with the unique ability to replicate within the cytoplasm of the infected cell. The infectious virions are brickshaped and of 300 to 400 nanometers in diameter. They contain a genome that is composed of approximately 200,000 basepairs of duplex DNA (Moss, 1991). This large size makes usual recombinant DNA techniques impossible. However, because homologous DNA recombination occurs naturally during poxvirus replication it is possible to insert foreign DNA into the viral genome by manipulating this natural event (Fenner, 1985).

A foreign gene is first flanked by segments of vaccinia virus DNA, usually in the form of a plasmid. This plasmid is then transfected into cells that have been infected with live whole vaccinia virus. Within these cells homologous recombination occurs between the virus genome and the chimeric gene from the plasmid (Mackett and Smith, 1986). About 0.1% of the progeny genomes will undergo recombination of the foreign DNA (Moss, 1991).

The targeted area for recombination is generally a

nonessential region of the viral genome so that the recombinant virus will be capable of replication. One such area is the thymidine kinase (TK) gene. One can then screen for TK plaques which indicate a recombinant vaccinia virus (Chakrabarti et al., 1985).

#### **REVIEW OF PLASMIDS AND DNA USED:**

##### **pIB21**

pIB21 is a complementary DNA clone (cDNA) developed by Soren Alexandersen. cDNA is synthesized by copying the information of mRNA into cDNA. This occurs using an oligo-dT primer to anneal to the poly-adenylated (poly-(A)) tail of the mRNA and employing the RNA dependent, DNA polymerase known as *reverse transcriptase*. Thus, the mRNA is used as the template to synthesize the DNA (Watson et al., 1987). This clone represents the mRNA for a non-structural protein in the left open reading frame, but also contained the complete sequence for the start codons (ATGs) of both the virion proteins and the right open reading frame. This was the segment of the cloned gene that I was interested in using. The cDNA was ligated into the Xba I site of the multiple cloning region of a commercially available plasmid known as pGEM-3 for amplification of the DNA as well as further use.

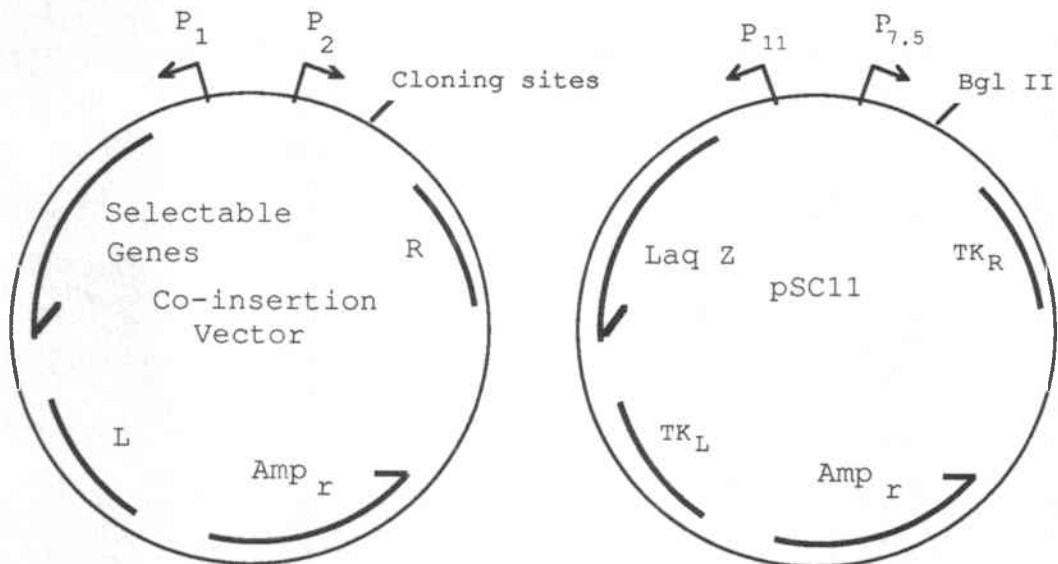
This construction was termed pIB21.

### pSC11

pSC11 plasmid is designed for homologous recombination with wild type vaccinia. It is a 7.88 kb cloning vector known as a co-insertion vector (Chakrabarti et al, 1985). It utilizes the capabilities of vaccinia virus to express genes that are cloned into unique sites within the vector. Due to the way it is

(a)

(b)



R & L = Vaccinia Flanking Genes

Figure 1 : Example of a generic co-insertion vector (a), compared to pSC11 (b).

constructed, a desired segment of DNA can be inserted into a cloning site downstream of the vaccinia promoter. When the plasmid is combined with the wild type vaccinia virus it will homologously recombine into the vaccinia genome. Then the cloned genes can be transcribed and translated via the vaccinia machinery.

The plasmid pSC11 has several components that allow the researcher to carry out these procedures. First of all there are two vaccinia early transcriptional promoters in the plasmid. These promoters are essential because a specific vaccinia promoter is needed. Vaccinia is a cytoplasmic virus, and these promoters are specific for RNA transcription in the cytoplasm. There are two such promoters in pSC11 known as P<sub>11</sub> and P<sub>7.5</sub>. Also, as is the case here, when working with cDNA clones there is not a promoter for the cloned gene. Once cloned into pSC11 the P<sub>7.5</sub> promoter controls transcription of the cloned gene.

The vector also has an ampicillin resistance (Amp<sup>r</sup>) gene. This allows the researcher to grow the cells transformed with the plasmid in broth that contains antibiotics. Only those cells that have been transformed with a plasmid will grow in the broth.

pSC11 contains the *E. coli* lac Z gene under the

control of the  $P_{11}$ . The lac Z gene is part of the lac operon. When this gene is present the recombinants can be screened for the expression of beta-galactosidase (B-gal). One performs an agarose overlay with agar containing X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside) which gets cleaved giving a blue product. Thus when picking colonies after transfection the researcher will initially screen for blue plaques.

One other crucial aspect of the pSC11 plasmid is that which endows it with the capability to homologously recombine with wild type vaccinia. This is the Thymidine Kinase (TK) gene. This gene is found in two parts of the pSC11, TK left ( $TK_L$ ) and TK right ( $TK_R$ ). These are the two portions that are excised out of pSC11 and recombined with the vaccinia virus, taking with them anything that lies between the  $TK_L$  and  $TK_R$  regions. This includes the promoters and the region that I attempted to clone into.

The pSC11 plasmid that I used was one that was somewhat modified. The only change was that the restriction endonuclease site that was generally used to clone foreign DNA into had been altered. Downstream of the  $P_{7.5}$  promoter there was initially a Sma I site. In the pSC11 that I used, this had been deleted and replaced by a Bgl II site (See Figure 1).

### **pXX-J-3**

The pXX-J-3 plasmid was a modification of another commercially purchased plasmid, pGEM-3Z. The 2.7 kb plasmid was modified to become a shuttle vector to subclone and modify Bgl II digested inserts (including VP1/VP2 insert I wished to use). It was modified by excising the multiple cloning region and adding Bgl II linkers leaving only a unique Bgl II site. This was performed by digesting pGEM-3Z with the restriction endonucleases Eco RI and Hind III. A fill-in reaction was done using Klenow fragment to fill in the 5'-overhanging ends. Then Bgl II linkers were ligated on. The plasmid was religated back together, propagated, and named pXX-J-3.

### **pK1**

The pK1 clone to isolate and purify the ADV-Utah-1 DNA from is Danish clone. This clone was obtained as a gift from Dr. Elisabeth Gottschalk. This contains the complete central 15 to 88 map unit segment of ADV-K (a Danish clone for ADV-Utah-1) cloned into a commercial plasmid.

## MATERIALS AND METHODS

### Materials:

The growth media used were NZY broth, NZY agar plates, SOB broth, and SOC broth for the transformations. Ampicillin resistant recombinant plasmids were screened for by growth on NZY agar plates and broth containing 250  $\mu$ g of carbenicillin per ml.

All restriction endonucleases and DNA ligases utilized were purchased commercially from Bethesda Research Laboratories (BRL), Rockville, Md., or New England Biolabs (NEB), Beverly, Mass. Calf Intestinal Phosphatase (CIP) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, In. The Klenow fragment used was purchased from BRL as well.

The deoxynucleotide triphosphates (dATP, dGTP, dCTP, and DTTP) used in the fill-in reaction were purchased from Pharmacia LKB Biotechnology, Alameda, CA.

The isotope ( $^{32}$ P) used in the Colony Hybridization Protocol was purchased from Du Pont-New England Nuclear Corporation (Boston, Mass.).

The commercially available plasmids, pGEM-3 and pGEM-3Z, were purchased from Promega Corp., Madison, WI. The pSC11 was a gift from Dr. Bernard Moss. Various

other ADV clones of different strains were all previously cloned by or gifts to Dr. Marshall Bloom and his colleagues.

The GeneClean kit used for isolation and purification of some DNA fragments from agarose gels was purchased from BIO 101 Inc., La Jolla, CA. The Elutrap used to isolate and purify by electroelution some DNA fragments from agarose gels was purchased from Schleicher & Schuell Inc., Keene, NH.

The radiolabeled probe used in the Colony Hybridization procedure was a RNA probe named 18-1. This probe is a plus sense probe radiolabeled with  $^{32}\text{P}$ . A 7- to-95 map unit segment of ADV DNA was cloned into the SP6 bacteriophage transcription vector and used to produce radiolabeled viral RNA transcripts (Bloom et al., 1987).

**Methods:**

The pIB21 clone was digested by Xho I and Xba I restriction endonucleases (Appendix C). The desired 2.3 Kilobase fragment that resulted was isolated by electroelution (Smith, 1980).

The 5' overhanging ends of this Xho I to Xba I restriction fragment were filled in using Klenow fragment (Appendix F). Bgl II linkers were ligated onto the blunt ends of the fragment using T4 DNA ligase (Appendix D). A Bgl II digestion was carried out to cleave off extra linkers and leave the appropriate ends for ligation into the modified pSC11 cloning vector. The pSC11 was similarly digested with Bgl II for cloning into the unique site downstream of the P<sub>7.5</sub> promoter.

The pSC11 was purified by electroelution using an Elutrap (Schleicher & Schuell). The purified linearized plasmid was subjected to an Alkaline Phosphatase reaction with Calf Intestinal Phosphatase to remove the 5'-terminal phosphates (Appendix E). These 5'-phosphates are required for ligation back to circular form (Maniatis et al., 1982). This makes it necessary to use the 5' phosphate from the insert for ligation to occur, eliminating the possibility of religation of the pSC11.

Ligation of the pSC11 and VP1/VP2 insert was

performed using T4 DNA ligase and the DNA was then transformed into *Escherichia coli* strain JM 109 (Appendix A). The transformants were plated on NZY agar plates containing 250  $\mu$ g of carbenicillin per ml (NZY/Carb).

The resulting colonies were screened for ADV-specific DNA sequences by colony hybridization (Appendix G) with the  $^{32}$ P radiolabeled 18-1 RNA probe (Bloom et al., 1987).

Positive colonies were inoculated into and grown in 2-ml mini preparations in NZY/carbenicillin (Carb) broth. The DNA from these mini preparations was purified by the IHB mini-prep protocol (Appendix B). Restriction endonuclease analysis was performed to verify the insert size and orientation were correct. Large scale plasmid DNA was performed as previously described (Maniatis et al., 1982).

Once these manipulations were complete, we began to attempt subcloning the Eco RI to Hind III fragment of the Utah-1 strain of the virus into the 2.3 kb fragment of ADV-G. This would act as a surrogate to a full Utah-1 clone for the viral protein region contained in the Right Open Reading Frame. We could then see if the ADV-Utah-1 capsid proteins could be expressed in vaccinia as well.

1 Xho I  
 \* \* \* \* \* T G C C A C A A C T G C C A C G A A G A A T A C T G G C A A C T C A C A A C C T A C T A C T G C A A A G A G  
 61 T G C A G A A A G T G T G A A C A C G G A A A A C T G C G A C A C A C C A A A A A G G A G T G C G A G C A G T G T G C C  
 121 T G C A A A G C A G C A C A A G A G A C C T C G G C A T G A G T A A A A T T C C T C A G C A C T A T C C T G G G A A A A  
 181 A G A G A A G T G C T C C A A G A C A C G T G T T T A T T C A G C A A G C A A A A A G A A G A A G C A A A C T A A C C  
 241 C T G C G G T C T A C C A C G G A G A G G A C A C C A T A G A G G A A A T G G A T T C T A C T G A A G C T G A A C A A A  
 301 T G G A C A C T G A G C A A G C A A C T A A C C A A A C T G C T G A A G C T G G T G G T G G G G G T G G T G G T G G T G  
 361 G T G G T G G G G T T G G T A A C A G C A C T G G C G G C T T A A T A A C A C A A C A G A A T T C A A A G T A A T A A  
 421 A C A A T G A A G T G T A T A T T A C T T G T C A G C T A C T A G A A T G G T A C A C A T T A A C C A A G C T G A C A  
 481 C A G A C G A A T A C T T G A T A T T T A A T G C T G T A G A A C T A C T G A T A C C A A A A C A C A T C A G C A A A  
 541 A A C T A A A C T T A G A A T T T T T T G T A T A T G A T G A T T T T C A C C A A C A A G T A A T G A C A C C T T G G T  
 601 A T A T A G T A G A T A G C A A C G C T T G G G G T G T A T G G A T G A G T C C T A A A G A C T T T C A A C A A A T G A  
 661 A A A C A C T G T G T A G T G A A A T T A G T T T G G T T A C T T T G G A A C A A G A A A T A G A C A A T G T A A C C A  
 721 T A A A A A C T G T A A C A G A A A C C A A C C A A G G T A A C G C A T C T A C C A A G C A A T T C A A C A A T G A C T  
 781 T A A C T G C G T C G T T A C A G G T T G C T T T A G A T A C T A A C A A C A T A C T G C C A T A T A C T C C A G C T G  
 841 C G C C G T T G G G G A A A C A C T G G G C T T T G T T C C T T G G A G A G C A A C C A A C C A A C C C A A T A T A  
 901 G G T A T T A T C A T C C A T G T T A C A T T T A C A A C A G A T A T C C T A A C A T T C A A A A G T T G C A A C A G  
 961 A A A C A C T A A C C T G G G A T G C A G T A C A A G A T G A T T A C C T T A G T G T G G A T G A C A G T A C T T T  
 1020 A A C T T T A T T A C T A T A G A G A A C A A C A T A C C T A T T A A C A T T C T C A G A A C G G G A G A T A A C T T T  
 1080 C A T A C A G G C T T G T A T G A G T T T A A C A G T A A A C C A T G T A A A C T A A C C T T A A G C T A T C A A A G T  
 1140 A C A C G T T G C T T G G G G T A C C T C C T C T G C A A A C C A A A G A C A G A T A C A A C A C A C A A A G T A  
 1200 A C C T C A A A A G A A A A C G G A G C T G A C C T A A T T T A C A T A C A A G G A C A A G A T A A T A C C A G A C T A  
 1260 G G T C A C T T T T G G G G T G A G G A A A G A G G T A A G A A A A C G C A G A G A T G A A C A G A A T T A G A C C T  
 1320 T A C A A C A T A G G T T A C C A A T A T C C T G A A T G G A T A A T A C C A G C A G G G T T A C A G G G T A G T T A C  
 1380 T T T G C T G G A G G A C C A A G A C A G T G G A G T G A C A C A A C C A A A G G T G C A G G T A C A C A C A G T C A A  
 1440 C A G T T A C A A C A G A A C T T T A G T A C T A G G T A C A T C T A T G A C A G A A A C C A G G T G G A G A C A A C  
 1500 G A G G T A G A C C T A T T A G A T G G A A T A C C C A T T C A T G A A A G A A G T A A C T A C T A C T C A G A C A A T  
 1560 G A G A T A G A G C A A C A T A C A G C A A A G C A A C C A A A G T T A C G T A C A C C A C C C A T T C A C C A C T C A  
 1620 A A A A T A G A C T C G T G G G A A G A A G A A G G T T G G C C T G C T G C T T C A G G C A C A C A C T T T G A A G A T  
 1680 G A G G T T A T A T A C C T A G A C T A C T T T A A C T T T A G T G G T G A A C A G G A G C T A A A G T T T C C A C A T  
 1740 G A A G T A T T A G A T G A T G C T G C T C A G A T G A A A A A G C T A C T T A A C T C A T A C C A A C C A A C A G T T  
 1800 G C T C A A G A C A A C G T T G G T C C T G T A T A C C C A T G G G G A C A G A T A T G G G A C A A G A A A C C T C A T  
 1860 A T G G A T C A C A A A C C T A G C A T G A A C A A C A A C G C T C C A T T T G T A T G T A A A A C A A C C C T C C A  
 1920 G G T C A A C T C T T T G T T A A A C T A A C A G A A A A C C T C A C T G A T A C A T T T A A C T A T G A T G A A A A T  
 1980 C C A G A C A G A A T A A A A A C C T A T G G T T A C T T T A C T T G G A G A G G C A A C T T G T A C T A A A A G G C  
 2040 A A A C T A A G C C A A G T A A C A T G C T G G A A T C C T G T T A A G A G A G A A C T C A T A G G A G A A C C T G G T  
 2100 G T A T T T A C T A A A G A C A A G T A T C A C A A A C A G A T A C C A A A C A A C A A G G T A A C T T T G A A A T A  
 2160 G G G T T A C A A T A T G G A A G A A G T A C T A T C A A A T A T A T C T A C A A A G T A A C C T G T G T A C T A T G  
 2220 T T A C T A T G T T A C T A T G A T A A T A T C T C A A T A A A A G T T A C A T G A A T A G T G A A C A A C C T A  
 \* \* \* \* \*

ADV-G is top strand & ADV-Utah-1 strand is below  
 Matches = 2231 Length = 2277 Matches/length = 98.0 %

Figure 2 : Comparison of ADV sequences of pXVIII-B-6-8 and pXX-R-14 ADV inserts with sequence differences, enzyme recognition sites, start codons, and the stop codon shown.

This substitution would cause only 44 nucleotide base changes (Figure 1). Many of these base changes are silent, the changes coding for only a total of 16 amino acid changes.

In order to make this cloning procedure simpler the 2.3 kb Bgl II insert was cloned into the Bgl II site of a pXX-J-3. The insert was ligated into the pXX-J-3, transformed into JM 109, and positive colonies were selected. Once this was done I digested this recombinant with the endonucleases Eco RI and Hind III. The resulting 3.43 kb nucleotide fragment was purified. The purification of this fragment was performed by the GeneClean (BIO 101 Inc.) procedure (Appendix H), as opposed to using the Elutrap. Similar digestions were carried out on the ADV-Utah-1 Danish clone, pK1. The desired 1.6 kb ADV-Utah-1 Eco RI to Hind III DNA fragment was also purified by GeneClean. These were ligated together and transformed in *E. coli* strain JM 109. After growing colonies in 2-ml NZY/Carb cultures the plasmid DNA's were harvested by the IHB mini-prep protocol. After screening by restriction analysis a large scale plasmid prep was carried out as previously described (Maniatis et al., 1982).

The pXX-J-3 clone containing the new chimera of ADV-

G & ADV-Utah-1 was then digested with the restriction endonuclease Bgl II. This digestion yielded the 2.3 kb fragment that contained the Eco RI - Hind III ADV-Utah-1 insert, flanked by the ADV-G sequences. The piece was purified, then ligated with the previously Bgl II digested and purified pSC11.

Another Colony Hybridization procedure was carried out to screen for ADV positive colonies. These too were picked into 2-ml NZY/Carb cultures and the plasmid DNA was purified by IHB mini-prep protocol. Bgl II digestion was done to screen for colonies with 2.3 kb VP1/VP2 insert and the 7.88 kb vector (pSC11). A large scale plasmid batch preparation was done to purify the clone.

## RESULTS AND DISCUSSION

The first cloning attempt was unsuccessful at ligating the VP1/VP2 insert into the pSC11. The product of the ligation reaction was transformed into JM 109 cells. The transformants in JM 109 were plated on 3 NZY/Carb plates (330  $\mu$ l of cells each). Approximately 1200 colonies were evident after overnight incubation at 37°C. Due to the large number of colonies obtained and the unreasonable amount of IHB mini-preps required to screen these a colony hybridization was performed using the 18-1 probe. This probe would thus screen for ADV-G positive clones (ones with successfully cloned ADV-G segment). The autoradiograph showed no colonies were positive for ADV-G sequences. We started the experiment over using fresh materials.

The second cloning attempt of the ADV-G (VP1/VP2) insert proved successful. In this attempt the amount of insert was doubled to increase the likelihood of getting a recombinant. Again the ligation reaction was transformed in JM 109 and the transformants were plated on 5 NZY/plates (200  $\mu$ l of cells each). This yielded approximately 1000 colonies. Another colony

hybridization was carried out with the same probe. Of the 1000 colonies, 49 showed positive for ADV-G sequences on the autoradiograph with a four day exposure at -70°C.

Twenty-four of these 49 colonies were inoculated into 2-ml NZY/Carb cultures and incubated overnight. These were screened by Bgl II restriction endonuclease analysis. Fourteen of the 24 were positive for the correct 2.3 kb VP1/VP2 insert.

An Eco RI restriction endonuclease analysis was done to determine if the clones were "+" or "-" sense for the P<sub>7.5</sub> promoter. If "+" sense the digest would yield DNA fragments of 4.391, 3.010, 1.877, 0.781, and 0.099 kb. If "-" sense the results of the digest would be 4.391, 3.010, 2.472, 0.186, and 0.099 kb. Four of the 14 clones were found to be in the "+" sense orientation. Clone pXVIII-B-6-8 was the recombinant construction selected for plasmid batch preparation and future experimentation with the live vaccinia virus (see Figure 3 for construction).

The second part of my work was to subclone a segment of the ADV-Utah-1 genome into the clone pXVIII-B-6-8 construction I had completed. The Eco RI to Hind III region of ADV-Utah-I was the targeted segment for insertion into the corresponding sequence of

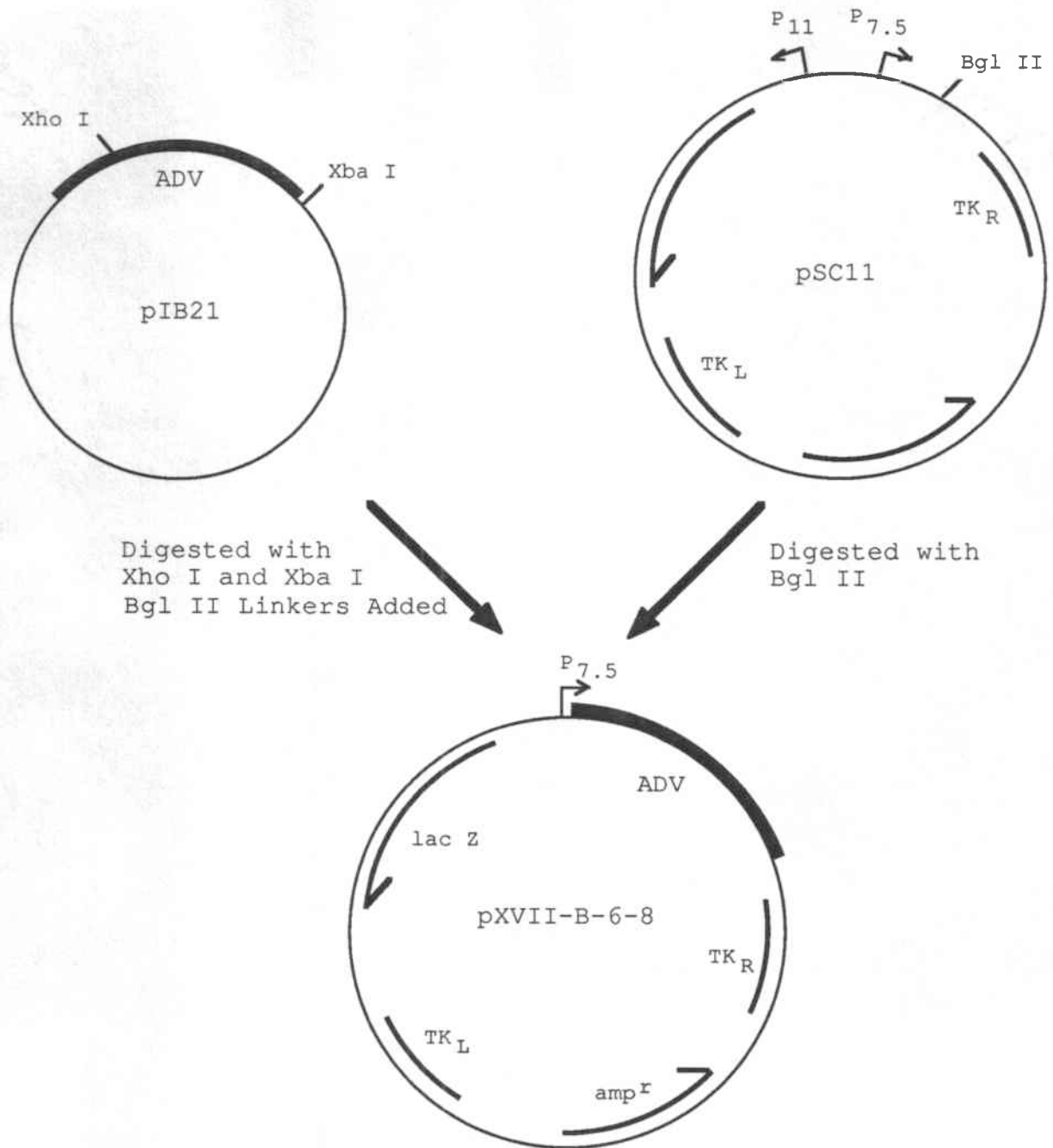


Figure 3 : Construction of the pXVIII-B-6-8 recombinant Vaccinia virus co-insertion vector from pIB21 and pSC11. The ADV sequence from pIB21 corresponding to the R3 transcript was digested then had Bgl II linkers added so as to correspond to the unique Bgl II site of pSC11.

pXVIII-B-6-8.

The first step of this experiment was to clone the VP1/VP2 ADV-G insert from pXVIII-B-6-8 into pXX-J-3. This cloning procedure proved immediately successful. Upon ligation and transformation of the purified DNA fragments, 82 colonies arose on five different NZY/Carb plates. Twelve of these were picked into 2-ml NZY/Carb cultures and incubated. The plasmid DNA purified by the IHB mini-prep method were screened by restriction analysis. The 12 DNA samples were screened by Bgl II digestion. Eight of the 12 had the correct 2.3 kb insert and 2.7 kb vector. Clone pXX-L-1 was selected, grown up in a large scale plasmid batch preparation, and purified for further cloning procedures.

The next step entailed cloning a piece of isolated pK1 (ADV-Utah-1) DNA into the pXX-L-1 Eco RI to Hind III region of the VP1/VP2 insert. The attempts at cloning the isolated purified DNA were successful. After transformation into JM 109 cells they were plated on five NZY/Carb plates and allowed to incubate overnight at 37°C. This yielded 161 colonies on the plates. Eleven of these were grown in 2-ml mini-prep cultures and the plasmid DNA was isolated. Upon Bgl II restriction endonuclease analysis there appeared to be the correct

2.3 kb insert (chimera ADV-G & Utah-1 VP1/VP2) and 2.7 kb vector (pXX-J-3).

Rsa I and Fok I endonuclease digestions were carried out to determine if the inserts were different from the original pXX-L-1 (ADV-G) insert. There are Rsa I and Fok I restriction site differences within the Eco RI and Hind III region. So upon digestion and agarose gel electrophoresis there should be DNA banding differences if the cloning switch was successful. If the original insert of pXX-L-1 was still present in the new clones there would be a unique band of 522 base pairs (bp) in the Rsa I digestion and of 278 bp in the Fok I digestion. Only clone pXX-Mc-10 had these characteristic bands. This was especially evident in the Rsa I digestion. The other 10 clones were the desired chimeras. pXX-Mc-1 was grown up in a large scale plasmid batch preparation to utilize in the final step of my experiment.

The final step involved cloning the chimera VP1/VP2 insert back into the Bgl II site of the pSC11 vaccinia co-insertion vector. This experiment was initially unsuccessful. The first ligation of the purified materials was again transformed into JM 109 cells. Upon plating on two NZY/Carb plates, only eight colonies appeared after overnight incubation at 37°C. These eight

were inoculated into 2-ml NZY/Carb mini-prep cultures and the plasmid DNA was purified. The plasmid DNA was screened for Bgl II inserts by Bgl II restriction digestion. All eight were negative for inserts. I started the experiment over by re-purifying the starting materials. I ligated, transformed, and plated on two NZY/Carb plates just as before. This second attempt yielded 22 colonies. I screened all of these from 2-ml NZY/Carb mini-prep cultures. The purified plasmid DNA was again digested with Bgl II restriction endonuclease. This time three of the 22 clones were positive for 2.3 kb Bgl II inserts. These three were Eco RI restriction endonuclease digested to check for the "+" sense orientation for the P<sub>7.5</sub> promoter. The product here, when run on an agarose gel by electrophoresis, should appear the same as the pXVIII-B-6-8. There should be bands of 4.391, 3.010, 1,877, 0.781, and 0.099 kb. All three clones positive for the insert proved to be "+" sense.

I selected pXX-R-14 as the clone to work up in a large scale plasmid batch preparation for Vaccinia recombination work. I digested this too with Rsa I to ensure the pXX-R-14 recombinant plasmid contained the correct ADV-Utah-1 strain Eco RI to Hind III insert. I compared this to pXVIII-B-6-8 to see if there was a

difference evident on the agarose gel after electrophoresis. The pXX-R-14 did not contain the 522 bp DNA fragment as did the pXVIII-B-6-8. There not being a DNA band in this region of the gel showed that the ADV-Utah-1 had in fact been successfully cloned into the pXX-R-14, thus creating the desired chimeric recombinant.

## EXTENDED LONG-TERM RESULTS

The pXVIII-B-6-8 was given to Dr. Dahn Clemens, another member of Dr. Bloom's research team at RML, to perform the vaccinia work. The pXVIII-B-6-8 was transfected into Green Monkey kidney cells (CV-1) previously infected with vaccinia virus (strain WR). A recombinant vaccinia virus named VV:IL-1 was isolated and purified. Both of the ADV-G structural proteins were detected, indicating that both VP1 and VP2 could be synthesized from the single cloned transcript of the VP1/VP2 insert (see Figure 3). Immunohistochemical examination of cells infected with VV:IL-1 showed that ADV specific antigen was primarily localized in the nucleus. Since vaccinia virus replicates in the cytoplasm the appearance of the antigen in the nucleus indicated that these antigens were transported to the nucleus, as is typical for parvovirus infection. Electron microscopic examination of the purified infected cell lysates demonstrated self-assembled viral particles. Thus, not only were VP1 and VP2 synthesized, but they also were transported to the nucleus and self-assembled into viral particles.

The pXX-R-14 clone was used by Dr. Marshall Bloom at

VP1 start codon

1 RVPQLPRRILATHNLLLQRVQKVUTRKTATHQKGVRAVCLQSSTRDLGMSKIPQHYPGKK

VP2 start codon

61 RSAPRHVFIQQAKKKKQTNPAVYHGEDTIEEMDSTEAEQMDTEQATNQTAEAGGGGGGGG

121 GGVGNSTGGFNNTTEFKVINNEVYITCHATRMVHINQADTDEYLIFNAGRITDTHQOK  
(Eco R1) -----D-----A-K-

181 LNLEFFVYDDFHQQVMPWYIVDSNAWGVWMSPKDFQOMKTLTLCSEISLVTLQEIDNVTI  
-----F-----

241 KTVTETNQGNASTKQFNNDLTASLQVALDTNNILPYTPAAPLGETLGFVWRATKPTQYR  
-----

301 YYHPCYIYNRYPNIQKVATETLTWDAVQDDYLSVDEQYFNFITIENNIPINILRTGDNFH  
-----LGQ-Q-E-TGT-----

361 TGLYEFNSKPKCLTLSYQSTRCLGLPLCKPKTDTHKVTSKENGADLIYIQGDNTRLG  
-----

421 HFWGEERGKKAEMNRIRPYNIGYQYPEWIIIPAGLQGSYFAGGPRQWSDTTKGAGTHSQH  
-----V-----

481 LQQNFSTRYIYDRNHGGDNEVDLLDGIPHERSNYSDNEIEQHTAKQPKLRTPPIHHSI  
-----H-----

541 IDSWEEEGWPAASGTHFEDEVYLDYFNFSGEQELNFPHEVLDDAAQMKLLNSYQPTVA  
-----E-----

601 QDNVGPVYPWGQIWDKPKPHMDHKPSMNNNAPFVCKNNPPGQLFVKLTENLTDTFNYDENP  
-----D-----

661 DRIKTYGYFTWRGKLVKLGKLSQVTCWNPVKRELIGEPGVFTKDKYHKQIPNNKGNFEIG  
----- (Hind 3) -----

stop codon

721 LOYGRSTIKYIYU

16 Amino Acid residue changes coded for by the construction of pXX-R-14 from pXVIII-B-6-8.

**AMINO ACID SEQUENCE OF ADV-G EXPRESSED IN VACCINIA VV:IL-1  
AND  
COMPARISON WITH ADV-G/ADV-Utah-1 CHIMERA VV:XXU-1**

Figure 4 : Comparison of the amino acid residue sequence as expressed by VV:IL-1 and VV:XXU-1. These two recombinant Vaccinia viruses were developed by recombination with the two Vaccinia co-insertion vectors I constructed, pXVIII-B-6-8 and pXX-R-14. The ADV-G sequence of VV:IL-1 is shown above with the changes VV:XXU-1 expresses shown below (a [-] represents no difference).

RML in similar experiments to Dr. Clemens'. Dr. Bloom's experiments yielded much the same results. He too isolated and purified a recombinant vaccinia virus from infected CV-1 cell lysates. This recombinant virus, VV:XXU-1, was also shown to be capable of synthesizing the two structural proteins (this time of the chimeric VP1/VP2 ADV-G & ADV-Utah-1 insert). Immunohistochemical and electronmicroscopic work also showed the ability of these structural proteins to transport to the nucleus and self-assemble into viral particles.

Cesium chloride gradients were performed to determine virus particle bouyant density. Fractions were collected with the refractive index ranging from 1.27 to 1.45 g/cm<sup>3</sup>. Both the VV:IL-1 and VV:XXU-1 viruses were shown to have a buoyant density of 1.32 g/cm<sup>3</sup>, indicative of empty particles. From electron microscopic study it was determined that the particles were icosahedral and of approximately 27 nm in diameter, both characteristics indicative of parvoviral particles. See Clemens et al., 1992 for further detail of follow up experiments.

A useful diagnostic test was developed by Mr. James B. Wolfenbarger and Dr. Marshall Bloom from VV:IL-1 infected cell lysates containing the empty particles. The test involved counterimmunoelectrophoresis (CIEP) to

test cell lysates versus ADV-infected mink sera. The test utilized the antigen from the VV:IL-1 infected cells to provide a quick test for mink sera containing anti-ADV antibody. The test antigen utilized from the recombinant vaccinia virus, developed from my pXVIII-B-6-8 clone, provided a quick means to test mink for possible ADV infection by simply taking a blood sample.

## SUMMARY AND CONCLUSION

My project was successful in the sense that the objective of expressing the ADV structural proteins in vaccinia virus, a eucaryotic expression system, was achieved. There is much more continuation work that can be done based on the work I completed. Based on the research I completed as well as the research of other members of Dr. Marshall Bloom's research team, several conclusions can be drawn.

1. Recombinant vaccinia viruses are suitable for the expression of parvovirus proteins.
2. Both ADV capsid (viral) proteins VP1 and VP2 are expressed in both recombinant vaccinia viruses, VV:IL-1 and VV:XXU-1.
3. All of the information required for the synthesis and nuclear transport of both VP1 and VP2 is contained in the cDNA transcript R3.
4. The structural proteins of ADV expressed by VV:IL-1 and VV:XXU-1 assemble into ADV virions.
5. The antigens produced by VV:IL-1 and VV:XXU-1 are suitable for use in diagnostic assays (e.g. CIEP).

There are many future experiments that are planned for this vaccinia system. Some of these have already been started by Dr. Bloom's research team at RML. Some of these experiments I myself was able to begin work on while still at RML. One such experiment is to clone only the VP2 gene into the pSC11, vaccinia co-insertion vector. This would allow us to see if expression of only the VP2 gene is capable of forming virus particles, as has been shown with some other parvoviruses. Another experiment that I started involved using the Polymerase Chain Reaction (PCR) for site directed mutagenesis. I utilized this technique to induce a single nucleotide mutation in the VP1/VP2 insert designed to delete the VP2 start codon. I changed the start codon sequence from ATG, coding for Methionine, to TTG, which codes for Leucine. I made a mutant insert that covers approximately 420 nucleotides of the VP2 region of the VP1/VP2 insert. This will be cloned back into the full 2.3 kb insert in the pSC11. It will then be tested to determine if it can express itself and produce capsid proteins in vaccinia virus through the expression of only the VP1 gene.

## APPENDIX A

### Supertransformation Protocol

1. Prepare a fresh overnight of bacteria (i.e. JM 109) in 5-ml NZY.
2. Dilute overnight 1/50 in 25-ml of SOB broth in a 250 ml sidearm flask.
3. Shake in 37°C at 250 rpm until 100 Klett Units are reached.
4. Sit on ice until culture is chilled.
5. Spin at 2500 rpm for 5-8 minutes at 4°C. Discard media.
6. Suspend cells on ice in 8-ml chilled TFB. Let sit on ice for 10-15 minutes.
7. Spin at 2500 as in step 5.
8. Suspend cells in 2-ml of TFB.
9. Add 70  $\mu$ l of dimethylformamide (DMF). Let sit on ice for 5 minutes.
10. Add 70  $\mu$ l of 2.25 M dithiothreitol (DTT) (stored at -20°C). Let sit on ice for 10 minutes.
11. Add 70  $\mu$ l DMF. Let sit on ice for 5 minutes. The cells are now "competent" for uptake of plasmid DNA.
12. Add 210  $\mu$ l of competent cells to DNA samples in tubes.
13. Let sit on ice for 30 minutes.

14. Incubate at 42°C for exactly 90 seconds.
15. Let sit on ice for at least 2 minutes.
16. Add 1-ml of SOC broth.
17. Shake at 37°C at 250 rpm for 60 minutes.
18. Spin 2500 rpm for 5-8 minutes. Discard media.  
Resuspend in 1-ml fresh SOC broth.
19. Plate desired quantity on plates and incubate  
overnight at 37°C. Store remainder of SOC suspension  
at 4°C.

## APPENDIX B

### IHB plasmid mini-prep protocol

1. Starting with a overnight 2-ml culture, save 1-ml and mix with 1-ml of 50% NZY/Glycerol.
2. Spin other 1-ml in a 2-ml microfuge tube for 60 seconds. Aspirate supernatant.
3. Suspend pellet in 100- $\mu$ l of IHB #1 solution. Store here at -20°C or incubate 5 minutes at room temperature and proceed.
4. Add 200- $\mu$ l of fresh IHB #2 solution. Mix well, incubate 5 minutes on ice.
5. Add 150- $\mu$ l of cold IHB #3 solution. Mix well, incubate 5 minutes on ice.
6. Spin in microfuge for 3 minutes.
7. Transfer 500- $\mu$ l of supernatant to labeled 1.5 ml microfuge tube containing 1-ml of 95% Ethanol.
8. Spin 10 minutes in microfuge. There should be a visible pellet. Dry 5 minutes in speed vacuum.
9. Dissolve pellet in 50- $\mu$ l of 1X TE. Analyze by restriction analysis and agarose gel electrophoresis (using a gel loading buffer containing RNAase).

**Stock solutions for IHB mini-prep protocol**

IHB #1: 50 mM = 12.5 ml of 2 M glucose  
25 mM Tris (pH 8.0) = 12.5 M 1.0 M Tris  
10 mM EDTA = 10 ml of 0.5 M EDTA  
Quantity Sufficient with distilled water (dH<sub>2</sub>O)  
to 500ml.  
Sterile filter and store at room temperature.

IHB #2: Prepare Fresh  
0.2 N NaOH = 40 ml 1.0 N NaOH  
1% Sodium Dodecyl Sulfate (SDS) = 10 ml 20% SDS  
dH<sub>2</sub>O = 150 ml  
Add NaOH to dH<sub>2</sub>O, then add SDS.

IHB #3: 5 M Potassium Acetate (KOAc) = 147g of KOAc  
Glacial Acetic Acid (CH<sub>3</sub>COOH) = 57.5 ml  
Quantity Sufficient to 500 ml with dH<sub>2</sub>O.  
Sterile filter and store at 4°C.

Reference: Ish-Horowicz and Burke. 1981. Nucleic Acids  
Research. 9: 2989

## APPENDIX C

### Restriction Endonuclease digestion protocol

To DNA sample:

1. Add 2- $\mu$ l of 10X digestion buffer + BSA (adjusted to particular enzymes characteristic requirements)
2. Add 2- $\mu$ l of desired restriction endonuclease (between 40 to 60 units usually).
3. Quantity sufficient to 20- $\mu$ l with sterile milli-Q water (SMQ).
4. Incubate at 37°C for 2 hours.

For all restriction endonucleases used, the 10X Eco RI buffer was adequate:

10 X Eco RI:	0.5 M Tris (pH 8.0)
	0.1 M MgCl <sub>2</sub>
	1.0 M NaCl
	60 mM 2-mercaptoethanol
	1 mg/ml Bovine Serum Albumin (BSA)

Stored at -20°C

## APPENDIX D

### Ligation reaction protocol

1. Add 100 ng desired vector DNA (so approximately 10 ng/ $\mu$ l final concentration).
2. Add desired insert DNA to a final ratio of 2:1 (insert:vector) of digested ends.
3. Add 1- $\mu$ l 10X Ligase Buffer.
4. Add 1- $\mu$ l T4 DNA Ligase.
5. Quantity sufficient to 10- $\mu$ l with SMQ.
6. Incubate overnight at 14°C.

10X Ligase Buffer:	0.5 M Tris HCl (pH 7.5)
Low ATP mixture	0.1 M MgCl <sub>2</sub>
	0.1 M DTT (dithiothreitol)
	0.5mM rATP
	1mg/ml BSA

Stored at -70°C

## APPENDIX E

### Alkaline phosphatase reaction (CIP reaction)

To DNA sample:

1. Add 5- $\mu$ l of 10X CIP Buffer.
2. Add 1- $\mu$ l of Calf Intestinal Phosphatase (CIP).
3. Quantity Sufficient to 50- $\mu$ l with SMQ.
4. Incubate at 37°C for 30 minutes.
5. Add second 1- $\mu$ l aliquot of CIP.
6. Incubate at 37°C for 30 minutes.
7. Add 70- $\mu$ l of dH<sub>2</sub>O, 1.2- $\mu$ l 100X TE, and 2.5- $\mu$ l of 20% SDS.
8. Incubate at 68°C for 15 minutes (to heat inactivate).

10X CIP Buffer:	0.5 M Tris Cl (pH 9.0)
	10 mM MgCl <sub>2</sub>
	1 mM ZnCl <sub>2</sub>
	10 mM spermidine

Stored at -20°C

## APPENDIX F

### Fill-In reaction protocol

To DNA sample (usually 0.5 to 1  $\mu$ g of DNA):

1. Add 3.0- $\mu$ l of 10X Fill-In Buffer.
2. Add 1.2- $\mu$ l of 0.5 mM pool of dATP, dGTP, dCTP, and dTTP.
3. Add 0.5 units Klenow Fragment.
4. Quantity sufficient to 30- $\mu$ l with SMQ.
5. Mix and incubate at room temperature for 15 minutes.
6. Add 1- $\mu$ l of 0.5 M EDTA.
7. Add 70- $\mu$ l of 1X TE.
8. Perform phenol-chloroform extraction, chloroform extraction, and ethanol precipitation using ammonium acetate with oyster glycogen.
9. Dissolve in 10- $\mu$ l of SMQ and verify concentration on agarose gel.

10X Fill-In Buffer: 0.5 M Tris (pH 7.5).  
0.1 M  $MgCl_2$   
0.01 M DTT  
1mg/ml BSA

Stored at -20°C

## APPENDIX G

### Colony hybridization protocol

#### I. Blotting and prehybridization

1. Place nitrocellulose membrane filters on NZY/Carb. plates with colonies on them. Dry the membranes in vacuum oven.
2. Warm premixed prehybridization solution to 50°C and add 100X tRNA solution.
3. Place filters in bag with prehybridization solution and seal.
4. Prehybridize the filters at 50°C for 2 to 5 hours.

#### II. Hybridization

1. Add the 18-1 probe to the hybridization solution (same as prehybridization solution except has probe in it).
2. Aspirate the prehybridization solution and add the probe with hybridization solution.
3. Hybridize at 50°C overnight.

#### III. Washing

1. Aspirate the probe.
2. Rinse the filters in the bag with 1XSSPE-0.1%SDS.
3. Put all the filters in one large bag.

4. Perform 2 washes at 65°C for 30 minute with 0.1XSSPE-0.1%SDS.

#### IV. Ribonuclease treatment.

1. Rinse the filters twice with 2XSSPE for 5 minutes.
2. Incubate the filters at room temperature for 5 minutes.
3. Incubate the filters at room temperature for 15 minutes on the shaker with 1.0 µg/ml RNAase A in 2XSSPE.
4. Wash twice at 50°C for 15 minutes with 0.1XSSPE-0.1%SDS.
5. Remove filters to clean bags. Seal with a minimal amount of wash fluid, expose to film as needed.

Prehybridization solution:      50% formamide  
                                         6X SSPE  
                                         0.1% SDS  
                                         5X Denhardt's solution  
                                         200 µg/ml salmon sperm DNA

Store at -20°C

## APPENDIX H

### Geneclean protocol

1. Excise desired DNA band from agarose gel.
2. Weigh the agarose block. Add 3 volumes of NaI stock solution to the volume of agarose (i.e. to 0.10 mg of agarose I would add 0.30-ml of NaI).
3. Incubate at 55°C until agarose has melted.
4. Add 5- $\mu$ l Glassmilk suspension (provided with Geneclean kit).
5. Incubate on ice for 5-10 minutes.
6. Pellet DNA in microfuge for 10 seconds. Aspirate NaI-agarose solution.
7. Wash pellet 3 times with 500- $\mu$ l of NEW WASH (also provided with Geneclean kit). Each wash consists of re-suspension pellet with NEW WASH, pelleting the DNA by 5 seconds in microfuge, and aspirating the supernatant.
8. Add 5- $\mu$ l of SMQ to Glassmilk pellet and resuspend. Incubate 3 minutes at 55°C.
9. Spin 1 minute in microfuge and elute off SMQ with the now purified DNA.
10. Repeat elution with a second 5- $\mu$ l of SMQ.

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