INTERACTION OF EQUINE INFECTIOUS ANEMIA VIRUS S2 PROTEIN WITH CELLULAR OS-9

A Senior Scholars Thesis

by

SARAH ELIZABETH HULSEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Molecular and Cell Biology

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Approved by:

Research Advisor: Susan Payne
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Interaction of Equine Infectious Anemia Virus S2 Protein with Cellular OS-9
(April 2008)

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Equine infectious anemia virus is a retrovirus that encodes four accessory proteins in addition to three structural proteins. One of the accessory proteins, designated only as ôS2ô is of unknown function. S2 has been shown to play an important role in viral replication and disease expression, however, specific functions of S2 are not known. As a first step to elucidate the function of S2, the protein was used as bait in a yeast two hybrid screen and was shown to interact with the cellular proteins TBP-1 and OS-9. My current studies probe the S2 amino acids important for interaction with OS-9. Three S2 mutants were designed with mutations generated in regions thought to be important for protein-protein interaction. These mutants were tested for interactions of S2 with OS-9 using a yeast two hybrid screen. None of the mutants significantly disrupted the interaction of S2 and OS-9, therefore it can be concluded that the three mutations created do not lie in areas of critical interaction of S2 and OS-9. Currently, an allele library containing random S2 mutants is being generated. These mutants will then be tested in a

yeast two hybrid screen. Mutants that are shown to cause a disruption in S2/OS-9 interaction will then be sequenced to determine where the mutations occur.

DEDICATION

This thesis is dedicated to my parents, Bruce and Pam Hulsey, and to my sister Chastine Hulsey. Because of my parents unwavering support and my sisterôs encouragement to attend Texas A&M University, I have been able to obtain an excellent undergraduate education that has prepared me for the future. Both my parents and my sister have each taken a part in my education and an interest in my research project in their own way, and for that I am indebted to them.

ACKNOWLEDGMENTS

I owe a great amount to all of the members of the Payne Lab for welcoming me into their lab and guiding me through my project for the past year. I owe a special thank you to my research advisor, Dr. Payne, and Lina Covaleda.

Dr. Payne, you welcomed me into your lab and trusted me with a project of my own so that I would learn what doing research is truly about. You also were tireless in editing posters, proposals, and this thesis, for that alone I can not thank you enough. Thank you for helping to prepare me for graduate school and for being a model of an outstanding research advisor.

Lina, you worked endlessly with me on projects big and small to help my project succeed. You were kind in answering any question I had, even when the answer seemed obvious to you. You also taught me to look at my project in a more objective way so that when something went wrong I knew what kind of steps to take to figure out the problem.

NOMENCLATURE

AD	Activating Domain
cDNA	Complementary DNA
DB	DNA Binding Domain
EIA	Equine Infectious Anemia
EIAV	Equine Infectious Anemia Virus
ELR1	Equine Lentivirus Receptor-1
HIF-1Ŭ	Hypoxia-inducible Factor
kDa	Kilo Daltons
NFəB2	Nuclear Factor kappa B p100/p52
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RRE	Rev-response Element
TBP-1	Tat-binding Protein-1

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CHAPTER I

INTRODUCTION

Equine Infectious Anemia Virus is a lentivirus of the family retroviridae that can be transmitted to equines either through insect vectors or unsterile needles (8). EIAV causes both acute and chronic disease states in horses. Within one to four weeks after infection, horses typically display an acute phase characterized by high viral loads and thrombocytopenia. In addition, the horse might have fever, central nervous system depression, or anorexia. A symptom free period usually follows an acute disease phase, in which there are no obvious signs of disease from the horse, however, low level viremia is still present. Animals that have been infected for a long period of time show limited virus replication and a lack of clinical symptoms of the disease, however, they are still carriers of the virus. The symptom free period may be interrupted by a period of chronic disease expression (6). It has been shown that the symptom free period is partially controlled by the immune system, because when the immune system of an infected horse is suppressed, disease episodes occur (11).

EIAV is the smallest and most simple lentivirus in regards to genome. EIAV is a positive sense, single stranded RNA virus (3). EIAVôs genome is only 8.2 kb. There are three genes that are found in all retroviruses, including EIAV; *gag*, *pol*, and *env*, which

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This thesis follows the style of Journal of Virology.

function as structural proteins. Additionally, four accessory proteins are also encoded by EIAV. They are Tat, Rev, Ttm, and S2. The Tat protein works with the long terminal repeat in the viral genome in order to allow expression of all viral genes. Rev acts with a region of the RNA sequence called the Rev-response element (RRE) which regulates structural gene expression in the virus (9). The function of Ttm is unknown (6). The EIAV genome can be seen in figure 1. Additionally, the function of the S2 protein is unknown. S2 is the focus of current studies.

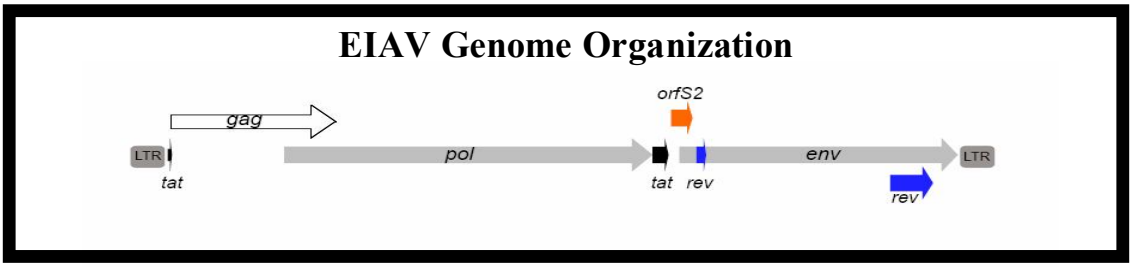


FIG. 1. EIAV Genome Organization. The EIAV genome consists of three structural genes: *gag*, *pol*, and *env*. Additionally four accessory proteins are encoded from other open reading frames in EIAV. They are Tat, Rev, Ttm, and S2.

EIAV infects monocytes and macrophages but not lymphocytes in infected horses. The cell membrane receptor, equine lentivirus receptor-1, ELR1, is present on cells susceptible to EIAV, but absent on cells that are resistant to EIAV, such as human, simian, and rodent cells. ELR1 belongs to the tumor necrosis factor receptor family, based on amino acid sequence similarity. ELR1 is highly similar to the receptor for human herpes simplex virus-1 (HveA) (12).

S2 Protein

S2 encodes a 65-amino-acid protein that is approximately 7.2 kDa. The S2 gene is produced by ribosomal leaky scanning in the intergenic region of pol-env. The S2 gene overlaps the Env protein amino terminus. It has been shown that S2 is not necessary for virus infection or for replication in cultured equine monocytes, which are the target cells for EIAV (9). In vivo however, S2 expression is vital for replication of virus to high titers and for disease expression (6). A study reported by Craigo et. al. (5) showed the development of an attenuated proviral vaccine that mutated the S2 gene. This study showed horses to be completely protected from Equine Infectious Anemia when challenged with virulent EIAV strains. Although horses remained asymptomatic, further investigation showed that the horses did harbor some of the challenge virus. This study demonstrates the importance of S2 in viral pathogenesis.

The S2 sequence is highly conserved across EIAV strains sequenced to date (6). No protein with a similar overall sequence has been found among lentiviruses or cellular proteins. The unique sequence of S2 causes difficulty in determining the function of the protein. Also, since S2 is not necessary for replication in cultured cells, it is difficult to determine its function. Several functions for S2 have been suggested by comparison with other lentivirus proteins. The *vif* gene, which aids in virus assembly is found in every known lentivirus except EIAV, suggesting that S2 could possibly serve a similar function. Additionally, EIAV S2 is found close to the genomic location of the *vpu* gene found in other lentiviruses. Both *S2* and *vpu* share a high serine-threonine content,

showing that the S2 protein could potentially serve to aid in virion assembly and release, similar to Vpu (9).

One method that can be used to discern the function of S2 is to determine what host cell proteins it may interact with. By using a cDNA library from human spleen it was deduced that S2 interacts with several host cell proteins including OS-9, Tat-binding protein-1 (TBP-1), and Nuclear Factor kappa B p100/p52 (NFəB2). The equine homologues of these genes were then cloned and S2 was shown to interact with the OS-9 and TBP-1 equine homologues (4).

OS-9 Protein

OS-9 is a protein that is over-expressed in osteosarcomas. OS-9 has also been shown to be a negative regulator of hypoxia-inducible factor (HIF-1Ŭ), which regulates O_2 homeostasis. HIF-1Ŭis a transcription factor whose target genes produce proteins that help cells survive hypoxia (2). OS-9 participates in HIF-1Ŭdegradation, allowing the cell to regulate oxygen homeostasis. HIF-1Ŭis also thought to mediate immune responses. In order for macrophages (EIAV targets) to function and genes to be activated to counteract bacterial and virus infection, HIF-1Ŭmust be up regulated (7). Therefore, since OS-9 is known to interact with EIAV S2, looking at the specific interaction between the two proteins may help to elucidate the role of S2 in viral pathogenesis.

Yeast Two Hybrid Assay

A Yeast Two Hybrid Assay is an efficient way to screen mutated proteins (S2) for interaction with another protein, in this case, OS-9. S2 and OS-9 have been fused with a DNA Binding domain (DB) or an Activating Domain (AD), respectively. When S2 and OS-9 interact, the DNA Binding Domain and Activating Domain interact, turning on reporter genes. The interaction of DB and AD activates RNA Polymerase to produce gene expression. A model of the yeast two hybrid system can be seen in figure 2. *S2* was inserted into the pDEST32 vector, which contains the DNA binding domain of Gal4. *OS-9* was incorporated into the pDEST22 vector where the activation domain of Gal4 is located. When the two Gal4 domains are close enough to interact (when S2 and OS-9 interact), it will turn on gene expression of *his3*, *ura3*, and *lacZ* reporter genes. His3 and Ura3 are enzymes necessary to produce histidine and uracil, two key products in the cell. When yeast cells are plated onto media lacking either histidine or uracil, if growth is seen, interaction took place causing gene expression to be turned on. The *lacZ* gene produces ɓ-galactosidase protein. Screening for ɓ-galactosidase presence determines if *lacZ* was turned on.

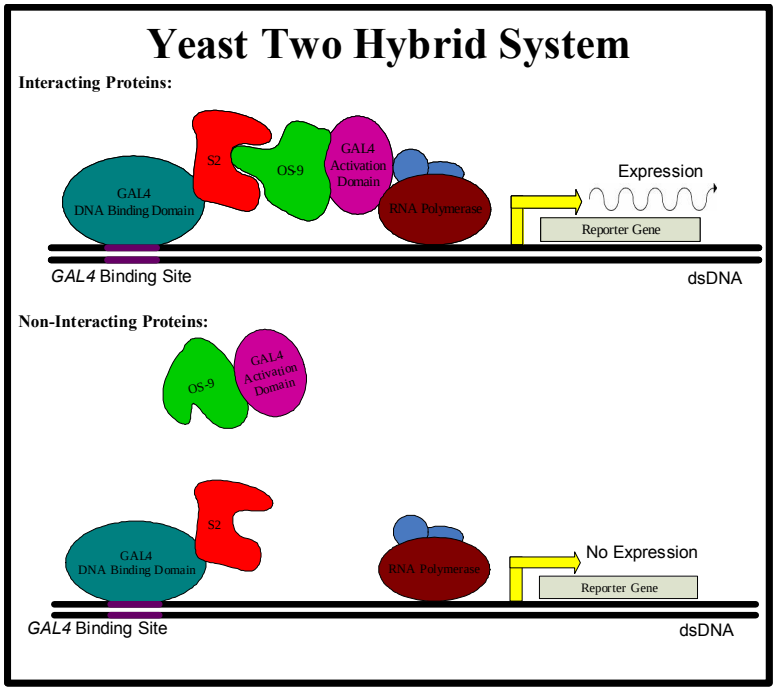


FIG. 2. Model of Yeast Two Hybrid System. S2 and OS-9 are fused to a DNA binding domain (DB) and an Activation Domain (AD), respectively. When S2 and OS-9 interact, DB and AD interact, turning on reporter gene expression.

Allele Library Generation

In addition to using a yeast two hybrid system to screen mutants of the S2 gene, it is also possible to generate random mutants and then screen them in the yeast two hybrid system. This process makes use of allele library generation in which random mutants are created using mutagenic PCR. The mutant S2 genes are then cloned into an entry clone vector. By transforming those entry clones (the allele library) into competent cells and plating the cells on selective media, allele library DNA can be isolated. Once the full-length proteins have been selected for, further assays such as a reverse yeast two hybrid assay can be performed.

Purpose of Project

The interaction of S2 with equine OS-9 has been the central focus of my research. By using mutated versions of S2 protein which disrupt the interaction of S2 and OS-9, EIAV strains can be constructed. The mutated strains can then be tested in horses to determine if the S2 and OS-9 interaction is necessary for disease development. Understanding the proteins that S2 interacts with is vital for understanding how S2 impacts disease development.

CHAPTER II

METHODS

S2 Mutant Preparation

Creating S2 Mutants

Previously, three S2 gene mutants were created using PCR site directed mutagenesis. Each mutation was created based on regions in the S2 gene sequence that were thought to be important for protein-protein interaction. The first mutation changed glutamic acids at positions 18 and 22 to alanine, making the sequence ASQGA instead of ESQGE. The second mutation changed prolines 25 and 28 to alanine, changing the sequence from PLLP to ALLA. Finally, the third mutation changed valine at position 46 to aspartic acid, and isoleucine at position 48 to lysine, altering the sequence from VII to DIK. Figure 3, below, show the locations of each amino acid change for each mutant. Each mutant S2 gene was cloned into a pBT plasmid vector.

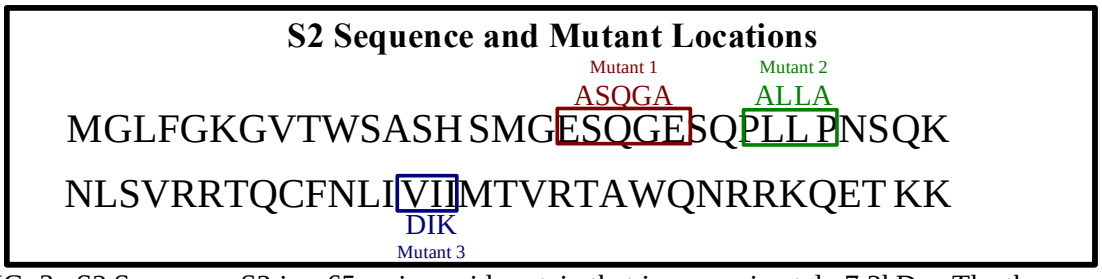


FIG. 3. S2 Sequence. S2 is a 65 amino acid protein that is approximately 7.2kDa. The three mutations are highlighted and their respective amino acid changes are shown above the sequence.

pBT S2 Mutant Analysis

In order to ensure that S2 mutant sequences had been inserted into the pBT plasmid, restriction enzyme analysis was performed. Plasmids encoding the three S2 mutants were digested with three different restriction enzymes known to give a specific banding pattern if the mutants had been inserted. ESQGE to ASQGA was digested with Spe1 (Invitrogen), PLLP to ALLP was digested with Xba (Promega), and VII to DIK was digested with EcoR1/Bam HI (Promega). Gel electrophoresis was performed on the digested mutants using 0.8% agarose gel for 15 min at 250 volts.

Amplifying S2 Mutants

Recombination sites (attB sites) were added to the S2 mutants using PCR. The Platinum Taq DNA polymerase, 10X PCR buffer, and 50mM $MgCl_2$ were obtained from Invitrogen. The following primers were used in the reaction EIAVS2NY2H (5ô-GGGGACAAGTTTGTACAAAAAAGCAGCCTTGATGGGAGTATTTGGT-3ô) and EIAVS2CY2H (5ô-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTCTTGGTCTCTTGC-3ô). The PCR cycling conditions were as follows: one initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 5 min. The PCR hold was set at 10°C. The PCR products were checked using gel electrophoresis on a 0.8% agarose gel run at 250 volts. Next, the PCR products were purified to remove primers and primer-dimers to avoid any primer or

primer-dimer recombination. A QIAGEN PCR Purification Kit was used to perform the PCR purification.

Creating Entry Clones

All three mutants were inserted into another plasmid, the pDONR plasmid. Using a BP Recombination Reaction, entry clones of the three mutants were inserted into the pDONR vector. All transformations were plated on LB + Kanamycin plates and incubated overnight at 37°C. The colonies were screened using PCR followed by plasmid purification. Plasmids were purified using the FastPlasmid Mini procedure from Eppendorf.

LR Recombination Reaction

The entry clones containing the mutated S2 genes were recombined with a pDEST32 (Invitrogen) destination vector which contains the Gal4 DNA Binding domain. The recombination reactions were each transformed into *Escherichia coli* host cells. The cells were plated onto prewarmed LB + Gentamicin plates and incubated overnight at 37°C. Four mutant colonies from each plate were screened using PCR followed by plasmid purification.

Sequencing pDEST 32/S2 Mutants

In order to confirm that mutant S2 genes had been inserted into the pDEST32 destination vector, sequencing analysis was performed. A sequencing reaction of pDEST32/S2 was

set up. The following reagents were added to a microcentrifuge tube: 3εl of dH_20, 2εl betaine, 1εl forward primer, 1εl reverse primer, 1.5εl 5X buffer, 0.5εl BigDye, and 2εl of plasmid DNA. Four samples of each mutant were prepared using the Spin-50 Mini-Column from USA Scientific, Inc. Each microcentrifuge tube containing the mutants was centrifuged at 2700 rpm for 3 min. After loading the gel bed surface of the microcentrifuge tubes with the samples, they were again centrifuged at 2700 rpm for three min. The purified samples were then removed from the collecting tube. Samples were sequenced using the ABI 3100 Automated Sequencer at the Gene Technology Lab at Texas A&M University.

Transformation of Vectors

The S2 mutants and OS-9 were then delivered to competent *Saccharomyces cerevisiae* yeast cells so that the yeast two hybrid screen could be performed. Competent cells were made by inoculating 10ml of YPDA with a colony of *S. cerevisiae* strain Mav203, and incubated overnight while shaking at 30°C. The OD was measured and the culture reseeded at OD_{600} equal to 0.5 in 50ml of YPDA. After measuring the OD to ensure that OD_{600} was 2.0 after approximately 7 hours, the cells were spun at 3000x g for 5 min. Pellets were resuspended in 25ml of dH_20 and centrifuged again. Next, the pellets were resuspended in 8ml of 100mM Lithium Acetate (LiAc), centrifuged again, resuspended in 4ml of LiAc, centrifuged and then resuspended in 1.2ml of 100mM LiAc + 188εl of 100% glycerol. Cells were frozen in 100εl aliquots at -80°C. Individual transformations were performed by aliquoting 50εl samples of the newly made competent cells and then

pelleting the cells to remove excess LiAc. Cells were resuspended with 240εl of 50% PEG 3350. To the resuspended cells, 36εl LiAc, 25εl denatured calf thymus DNA, 48εl dH_20, and 2εl of PDEST32/S2 and 2εl of PDEST22/eqOS-9 was added. The solutions were incubated in a water bath for 30 min at 30°C, and then heat shocked for 25 min at 42°C. The cells were pelleted and then resuspended in 200εl of dH_20.

Yeast Two Hybrid Screen

The pDEST32/S2- pDEST22/eqOS-9 transformed cells were plated onto non-selective media and incubated for 24 hours at 30°C. Following incubation, cells were transferred from non-selective media to selective media. Cells were plated onto the following selective media plates: SC-Leu-Trp+3AT, 3AT-his, and FOA. The cells were allowed to grow for 24 hours at 30°C. Replica cleaning was performed on the selective media plates. Additionally, a ϐ-galactosidase reaction was performed using mutant colonies from the non-selective media. A Yeast ϐ-galactosidase Assay Kit from Pierce Biotechnology was used to perform the ϐ-galactosidase reaction.

Allele Library Construction

An allele library is in the process of being created using polymerase chain reaction. By using mutagenic PCR conditions, mutated S2 proteins can be made. To ensure that only full length mutant S2 proteins are selected for in *E. coli* cells, S2 was fused with the kanamycin resistance gene. The allele library has been generated using the SureFrame Allele Library Construction Kit from Invitrogen.

Generating S2 Expression Clone without Stop Codon

The S2 gene was modified to remove its stop codon to allow expression of the kanamycin resistance gene when S2 is expressed. To do this, S2 was amplified using PCR with gene-specific primers. Two reactions were performed to remove the S2 stop codon and add attB sites to S2. The first reaction removed the stop codon, and the second reaction added attB sites. The following components were added for the first reaction: 1εl template DNA (pDEST2/S2), 38.5εl dH_20, 5εl 10X buffer, 1εl dNTP, 2εl $MgSO_4$, 1εl pDEST32/S2 attB1 primer (5ô-AAAAAGCAGCCTTGATGGGAGTATTTG-3ô), 1εl S2 Reverse attB2 pDONR-Express primer (5- AGAAAGCTGGGTTTTTCTTG-3ô). PCR cycling parameters for the first reaction are as follows: 95°C for 2 min, 20 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min. The final cycle was 72°C for 5 min, with a 4°C hold. Next, attB sites were added to the S2 gene without a stop codon by taking 10εl of the first PCR reaction and adding it to a second PCR reaction mixture that used a different set of primers, SP AttB1 adapter (5ô-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3ô) and ATTB2 Outer (5ô- GGGGACCACTTTGTACAAGAAAGCTGGGT-3ô). PCR cycling parameters were as follows: 95°C for 1 min, 5 cycles of 94°C for 15 sec, 45°C for 30 sec, and 68°C for 1 min. In a second stage the PCR was subjected to 25 cycles of 94°C for 45 sec, 55°C for 30 sec, 38°C for 1 min, and 72°C for 5 min. After these final cycles, the PCR products were held at 4°C . These PCR reactions used Invitrogen HiFi Taq polymerase. Using the Gateway System, the final PCR product, after being

purified, was inserted into pDONR-Express vector. Next the insert was transferred into the destination vector, pDEST32 using an LR recombination reaction.

Testing the S2 Expression Clone

In order to ensure that the altered S2 gene would function properly in the yeast two hybrid system, the expression clone was tested. This was done by performing a yeast two hybrid screen with the S2 expression clone and wildtype OS-9. In addition, a positive control was tested in the yeast two hybrid screen using wildtype S2 and wildtype OS-9. Five controls were also tested to ensure the accuracy of yeast two hybrid results.

Kanamycin Titration

Fusing the S2 gene with the kanamycin resistance gene, neomycin phosphotransferase, allows selection of S2 expression on plates containing kanamycin. Since fusing a gene with neomycin phosphotransferase can alter its function, a kanamycin titration was done to determine the optimal concentration of kanamycin in media plates.

The new expression clone was amplified using PCR. In this reaction, a different set of primers that are shorter, were used to ensure that deletions or modifications would not occur during replication. The forward primer that was used was attB1-5ô(5ô-ACAAGTTTGTACAAAAAAGCA-3ô) and the reverse primer that was used was attB2-3ô(5ô- ACCACTTTGTACAAGAAAGCT-3ô).

The PCR product was run on an agarose gel for 20 min at 250 volts to confirm attB site addition. Next, a BP recombination reaction was performed to insert the PCR product into the pDONR-Express vector. The BP Recombination Reaction was incubated for 20 hours at 25°C.

Next, the recombined product had to be transformed into *E. coli* cells so that they could be plated on various kanamycin concentrations and the optimal concentration determined. To do this, the BP Recombination Reaction was transformed into TOP10 Electrocompetent *E. coli* cells using an electroporation procedure. The electroporator was set at 1700 volts, 200q , and 25εF. The electroporated cells were then recovered in 920εl of SOB medium. The solution was then divided into two 500εl aliquots. To one of the aliquots, 5εl of IPTG was added, inducing the expression of the fusion protein. The other aliquot was left unaltered and served as the uninduced sample. To allow the kanamycin resistance genes to be expressed, both tubes were incubated at 37°C for several hours. Both the induced and uninduced transformations were plated onto various concentrations of kanamycin as well as on spectinomycin plates. The spectinomycin plates displayed how efficient the BP Recombination reaction was. Each transformation was diluted to 10^{-2}, 10^{-3}, and 10^{-4} dilutions and then plated onto different concentrations of antibiotic. Various concentrations of kanamycin and spectinomycin are shown in table 1.

TABLE 1. Antibiotic concentrations for kanamycin titration.

	Spectinomycin	**Kanamycin**				**Kanamycin + 1mM IPTG**			
Concentration of antibiotic (εg/ml)	100	20	30	40	50	20	30	40	50

Allele Library Generation

After determining the optimal kanamycin concentration, the allele library was generated. The allele library contains S2 genes with random mutations. Those random mutants were cloned using the Gateway System so that an allele library is created in the pENTR-Express vector.

To create the mutant S2 genes, mutageneic PCR was used to add attB sites so that the Gateway System could be used. The PCR reaction contained the following components: 5εl of 10X PCR Buffer-Mg, 1εl of 100mM dCTP, dGTP, and dTTP. Also, 1εl of 10mM dATP, 15εl of 50mM $MgCl_2$, 4εl of 5Mm $MnCl_2$, 1εl attB1-5ôprimer, 1εl attB1-3ôprimer, 1εl template DNA, 1εl of 5 U/εl Platinum Taq DNA Polymerase, and 18εl of dH_20 were added to the mixture.

The mutagenic PCR products were then checked using agarose gel electrophoresis and then purified to remove primer-dimers and primers using a QIAquick CR Purification Kit. Since the first mutagenic PCR reaction did not show any product, a $MnCl_2$ titration was performed to determine the concentration of $MnCl_2$ that would mutate S2 while still producing an adequate amount of product.

Following mutagenic PCR and purification, a BP recombination reaction was performed to insert the mutant genes into pDONR-Express. To do this, the same BP recombination procedure was used as described previously.

CHAPTER III

RESULTS

In this study mutant EIAV S2 proteins were prepared and tested using the yeast two hybrid system to determine if the mutant versions of the proteins disrupted the interaction between EIAV S2 and equine OS-9. In order to perform the yeast two hybrid analysis, S2 mutants were prepared.

S2 Mutant Preparation

The three S2 mutants were successfully transferred first into the pBT vector. In order to test that the mutants had been transferred into the vector, restriction enzyme digestion analysis was performed. Several enzymes including Xba (Promega), Spe1 (Invitrogen), and EcoR1/BamH1 (Promega) were used to observe banding patterns of the vector. One band was seen on the agarose gel containing the vector digested with Xba, indicating that the three mutants had been successfully inserted. Additionally, the Spe enzyme verified the presence of one fragment on the gel, thus one piece of DNA and a change in the Xba cut site. Finally, the EcoR1/ BamH1 digestion, showed two fragments on the gel, again confirming the insertion of the S2 mutants in the vector.

Recombination sites had to be added to the S2 mutants using PCR. It was seen that attB sites were successfully added to the mutants by running the PCR product on an agarose gel. It was determined that the product was larger (did not run as far on the gel) than S2 without added recombination sites.

The S2 mutants were inserted into a pDONR vector to create entry clones, using a BP recombination reaction. Mutant plasmids were transformed into competent cells and then plated onto LB+ Kanamycin plates. Growth on the plates indicated successful transformation. To check this, a PCR screen was performed and analyzed by agarose gel electrophoresis.

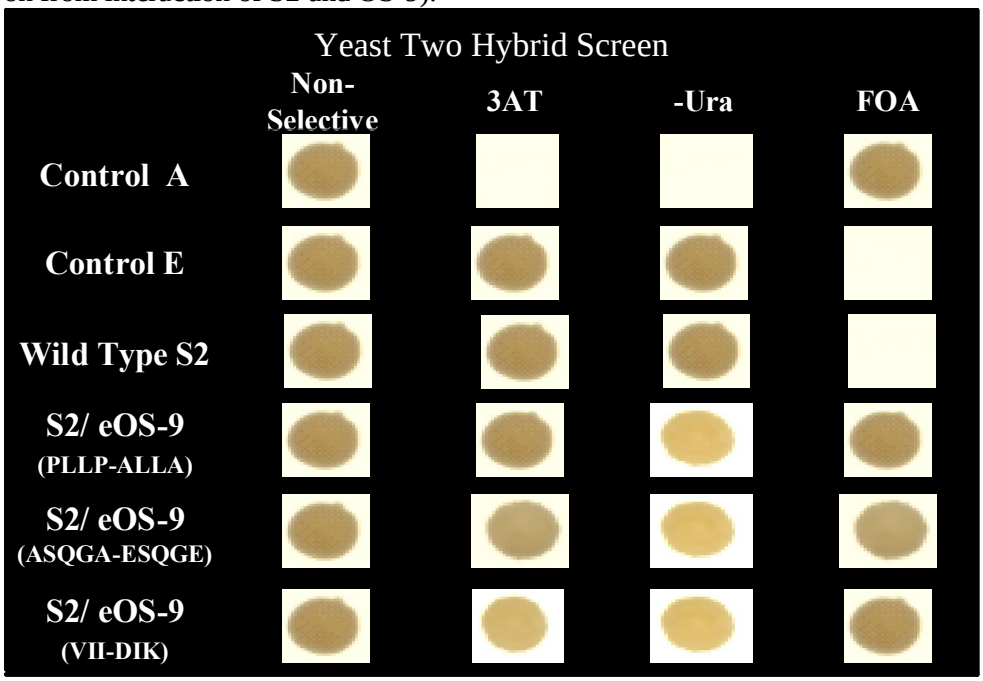
The entry clones (pDONR vector) were recombined with the pDEST32 vector in order to insert the mutants into pDEST32, creating the fusion protein of S2 and the GAL4 DNA Binding Domain. The recombination reactions were then transformed into competent *E. coli* cells and plated on LB+ Gentamicin plates. Four colonies from each plate were screened using PCR and the expected size products were seen by electrophoresis gel. In order to further check that the mutant S2 genes had been inserted into the pDEST32 destination vectors, the mutants were sequenced. Analysis of the sequencing results confirmed successful insertion of the mutant genes.

In final preparation for the yeast two hybrid screen, pDEST32/S2 and pDEST22/OS-9 were transformed into yeast cells. The transformations were plated onto non-selective media. Growth indicated that both plasmids had been transformed into the yeast cells.

Yeast Two Hybrid Screen

A yeast two hybrid screen was performed with the transformed yeast cells that contained the pDEST32/S2 and pDEST22/OS-9 plasmids. By plating the yeast cells onto selective media, it could be determined if a disruption in interaction occurred. Several controls were used that showed both positive interaction and lack of interaction. Table 2 shows results obtained from a negative control (control A), a positive control (control E), wildtype S2, and the three S2 mutants, of the yeast two hybrid assay.

TABLE 2. Results of yeast two hybrid interactions shown as growth or no growth on indicator plates. Control A is a negative control (no growth on 3AT and -Ura plates), Control E is a strong interaction control (growth on 3AT and -Ura plates, no growth on FOA). Wildtype S2 showed strong interaction, thus growth on 3AT, and ï Ura plates, but not FOA plates. All three S2 mutants grew on 3AT, -Ura and FOA indicating a weak interaction with OS-9 (reporter genes turned on from interaction of S2 and OS-9).



When wildtype S2 and equine OS-9 was transformed into yeast cells and plated onto indicator plates, a strong interaction was seen, as expected. This is indicated by growth on 3AT and ï Ura plates, and no growth on FOA plates. Mutant number one, ESQGE to ASQGA, grew on all three plates, indicating a weak interaction between the mutant S2 protein and OS-9 protein. Mutant number two, PLLP to ALLA, grew on all three plates, including FOA, indicating that the interaction between S2 and OS-9 was weakened, but not disrupted. Finally, mutant three, VII to DIK, grew on all three plates, but not as much growth was seen as in the first two mutants. Although growth was less, the S2/OS-9 interaction was not disrupted enough to inactivate gene expression.

In addition to plating the transformations on selective media, a ɓ-galactosidase assay was performed using a Pierce Biotechnology ɓ-galactosidase assay kit. Several controls, as well as the mutants and wildtype S2 transformations were assayed. Results showed that interactions took place in mutant one (ESQGE to ASQGA) and mutant two (PLLP to ALLA) as seen by a color change in the solution from clear to yellow. Six colonies of various sizes from each mutant were tested. Mutant 3 (VII to DIK) did not show a color change, indicating that a disruption in interaction had occurred. Results from the ɓ-galactosidase can be seen in figure 4.

FIG. 4. ϐ-galactosidase Reaction of Three S2 Mutants. The top row shows controls A-E. Although control A is a negative control and control E is a positive control, conclusive results from all controls was not seen. Controls A, C and D do show correct results. Mutants one and two indicate that interactions between mutant S2 and OS-9 took place. A lighter color indicates a weakened interaction. Mutant three showed no color change, indicating a disrupted interaction between S2 and OS-9.

Allele Library Generation

The generation of an allele library allows for the creation of randomly mutated S2 genes that are full-length. By creating the mutants, they can then be selected for on kanamycin plates and further assays can be done to identify mutations which caused a disruption in interaction of EIAV S2 and equine OS-9. Following that, the mutants that did affect

interaction can be sequenced and the specific amino acids involved in S2/OS-9 interaction can be determined.

In order to create the allele library containing mutated S2 genes, the stop codon had to be removed from the S2 expression clone and attB sites were added. This was done using PCR. When the PCR product was checked using agarose gel electrophoresis, a single band of 250-280bp was observed, confirming successful PCR amplification. The entry clones were then subjected to an LR recombination reaction to insert the gene into the destination vector. The LR recombination reaction was then transformed into competent cells and screened using PCR. Again, gel electrophoresis confirmed correct insertion of the gene and fusion with the kanamycin resistance gene (Kan^R). Figure 5 below shows the sequence of the S2/Kan^R fusion protein. The new fusion protein containing S2 and Kan^R were tested in a yeast two hybrid system with wildtype OS-9 protein. Wildtype S2 and wildtype OS-9 were used as positive controls. Wildtype proteins interact, showing positive results in the yeast two hybrid screen. Additionally, the fusion S2 and equine OS-9 showed positive results in the yeast two hybrid screen, indicating that the S2 fusion protein would be suitable for S2 expression. Results from the S2+Kan^R yeast two hybrid screen can be seen in figure 6.

N-MGVFGKGVTWSASHSMGESQGESQPLLPNSQKNLSVRR
TQCFNLIVIIMTVRTAWQNRRKQETKKNPAFLYKVGIIRKHCLSIC
CNEQVTISQNKIIICHPADIASIEQDGLHAGS-C

FIG. 5. Sequence of S2/ Kan^R Fusion Protein. The S2 sequence is shown in blue and the Kan^R sequence is shown in red.

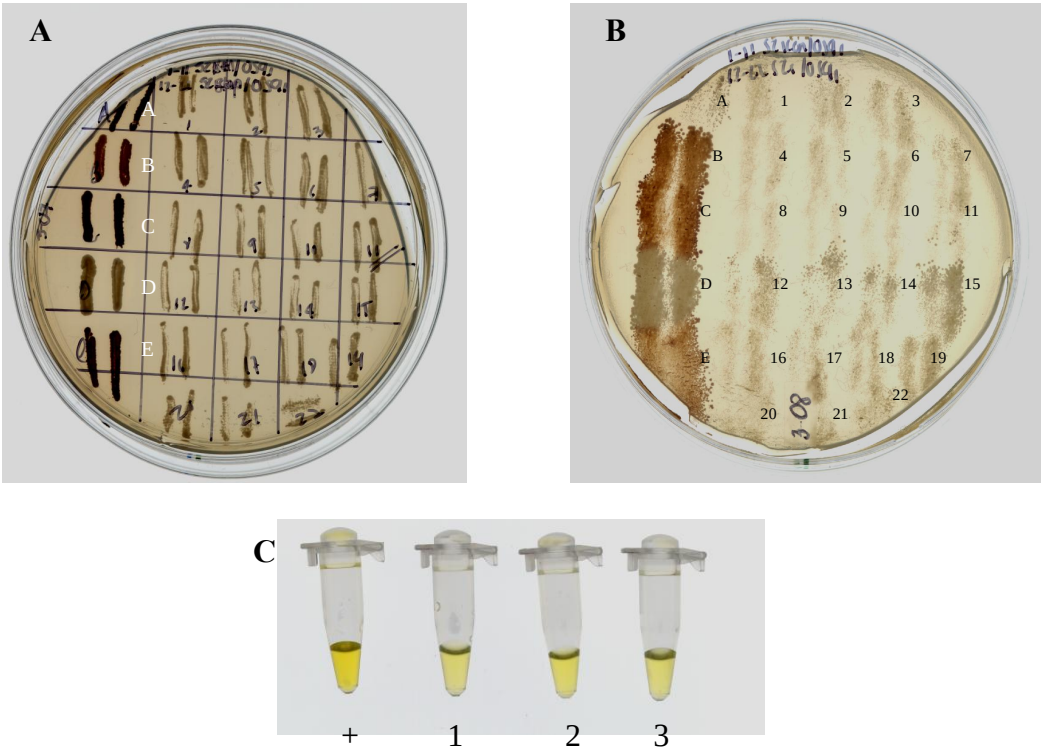


FIG. 6. Yeast Two Hybrid Screen for S2/Kan^R Fusion Protein. (A) S2/Kan^R fusion/OS-9 (streaks 1-11) and wildtype S2/OS-9 (streaks 12-22) interaction on non-selective media. Controls are labeled A-E. Control A is a negative control, and Control E is a positive control. Both reactions grew sufficiently on non-selective media. (B) S2/Kan^R fusion/OS-9 (streaks 1-11) and wildtype S2/OS-9 (streaks 12-22) interaction on 3AT selective media. Wildtype S2/OS-9 reaction showed slightly more growth on the selective media than the S2 fusion protein. (C) ɓ-galactosidase reaction of S2/ Kan^R fusion/OS-9. Three colonies from non-selective media were assayed as well as one control. Yellow solution color indicates that S2/ Kan^R fusion/OS-9 produces an interaction.

A kanamycin titration was also performed to determine what concentration of kanamycin would produce optimal results when the transformed cells containing the fusion protein were plated. It was determined that plates containing 40ε g/ml of kanamycin produced the best results. Many colonies, too many to count, were seen in the induced samples, and only three colonies were seen on the uninduced sample.

After determining the optimal kanamycin concentration, the allele library generation was begun. Mutagenic PCR was used to create the allele library with S2 mutants. When the mutagenic PCR was first checked using gel electrophoresis, no product was seen. This was suspected to be a result of a high concentration of $MnCl_2$, the mutagenic agent. To determine what amount of $MnCl_2$ would produce mutated genes but also give an adequate yield of product, a $MnCl_2$ titration was performed. When this was done, it was product was seen when concentrations of 0.3mM, 0.2mM, a 0.05mM of $MnCl_2$ was added to the PCR reaction. PCR products using 0.3mM and 0.2mM were combined into one tube to perform the PCR purification and BP recombination reaction.

CHAPTER IV

SUMMARY AND CONCLUSIONS

EIAV is important to study not only because of its clinical symptoms inflicted on infected horses, but also because it serves as an important model for a lentivirus that infects macrophages. Determining what causes viral replication and disease progression in EIAV will help to model mechanisms for controlling and eliminating the disease, EIA. S2 protein is known to play a role in viral replication in vivo because horses infected with viruses not expressing S2 show reduced viral virulence and low viral replication levels. Additionally, S2 protein is highly conserved during infection, shown by sequencing analysis during disease progression. The highly conserved nature of S2 may indicate its critical role during viral replication (10).

One method to begin to elucidate the role of S2 in viral pathogenesis is to determine what equine proteins S2 interacts with. Although S2 was shown to interact with several proteins (4), my study focused on S2ôs interaction with equine OS-9 protein. I used three mutant S2 proteins to determine if mutations in specific regions of S2 caused a disruption in interaction of S2 protein and OS-9 protein. All three mutations were created for specific purposes. Mutant number one, ESQGE to ASQGA, was chosen because of its high acidic content. It was thought that the high number of acidic amino acids concentrated in that specific region might be important for the two proteins to interact. The yeast two-hybrid assay results revealed that this mutation did not have a

critical affect on the interaction of S2 and OS-9. Mutant number two, PLLP TO ALLA, was not expected to disrupt S2/OS-9 interactions because it is widely accepted that the PLLP motif interacts with SH3 domains, which are not present in OS-9. The PXXP motif has not been shown to interact with other protein domains (10). As expected, the PLLP to ALLA S2 mutant interacted with OS-9. Finally, mutant number three was chosen because the region was hydrophobic, a property that is sometimes seen in regions of protein- protein interaction. The yeast two-hybrid analysis showed that this region was also not critical in determining the interaction of the two proteins.

Wildtype S2 protein and the positive control grew on the plates lacking histidine and uracil but not the plate containing FOA. Proteins that interact strongly display this kind of growth pattern. Growth on FOA plates indicates a disrupted interaction (or at least a weakened interaction) because when the uracil reporter gene is turned on it causes the conversion of 5FOA to 5-flurouracil which is toxic to the cells, thus preventing growth on FOA plates. It is important to note that although some of the mutants did not grow quite as efficiently on the plates (such as on the ï Ura, and FOA plates), the most reliable indication of interaction is by growth on 3AT plates. All mutants grew effectively on the 3AT plates. This demonstrates that although the interaction between S2 and OS-9 might not be as strong with the mutant S2 proteins, the interaction still took place. When ϐ-galactosidase reactions were analyzed it was seen that mutants one and two showed interaction, although weakened (as indicated by the lighter yellow color of solution). These results are concurrent with the 3AT screen. Unlike mutants one and

two, mutant three showed different results than that of its 3AT plate. In the ϐ-galactosidase test, mutant 3 did not change solution color, indicating that the interaction between S2 and OS-9 had been disrupted. Since growth on the 3AT plates was lightest when mutant 3 was plated, and since the ϐ-galactosidase test was negative, it can be assumed that mutant 3 lies in a region that influences interaction between S2 and OS-9 in some manner. Further tests are needed to confirm this assumption.

Additionally, an allele library has been created so that further tests can be done to determine what specific mutations in the S2 gene cause a disruption in interaction between EIAV S2 and equine OS-9. Although this work is still in progress, it has been shown that S2 is able to be fused with neomycin phosphotransferase (kanamycin resistance gene) so that full-length S2 proteins can be selected for. S2 has been inserted into the pDONR- Express vector which contains Kan^R. By adding recombination sites to the S2 gene that align with the recombination sites with the pDONR vector, two genes in pDONR are replaced with S2. One of these genes is *ccdB*. Since *E. coli* is sensitive to CcdB effects, plasmids containing the ccdB gene can be selected against (1). This ensures another way of selecting only *E. coli* cells that contain the gene of interest, S2. A map of the pDONR vector can be seen in figure 7 below. It has also been shown that even though S2 was fused with the Kan^R gene, the Kan^R gene did not disrupt the expression of S2, as seen in testing the fusion protein in the yeast two hybrid system.

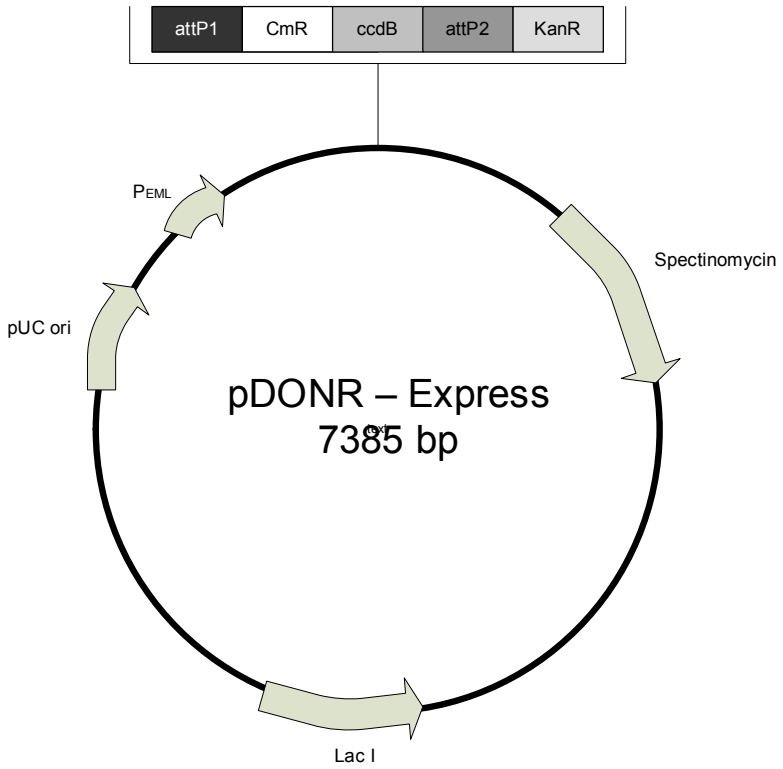


FIG. 7. pDONR-Express. The donor vector contains genes important to allele library generation. The cassette containing *ccdB* and Cm^R is spliced out when S2 is recombined with the vector. CcdB aids in selection against *E. coli* cells that contain the cassette, and therefore do not contain the S2 gene. The Kan^R gene is located downstream from S2 and is expressed when S2 is expressed. The Spectinomycin resistance gene also allows for selection in *E. coli*. The *LacI* gene codes a lac repressor protein which helps silence the EML promoter when IPTG is not present. The vector also contains a pUC origin to allow for high-copy maintenance.

Current Work

Currently, selection for full-length mutated S2 proteins is being performed. Once that has been finalized, the allele library DNA can be isolated and further assays can be performed. These mutants will then be tested in a yeast two-hybrid system to identify mutations that disrupt the interaction of the two proteins.

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