August 2005

HVS'S ORF12 and the Downregulation of MHC-I

Melissa Jo Mulla
Worcester Polytechnic Institute

MQP-BIO-DSA-6435

HERPESVIRUS SAIMIRI'S ORF-12 AND THE DOWNREGULATION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I

A Major Qualifying Project Report

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

Melissa Costello

August 22, 2005

APPROVED:

Robert Means, Ph.D.
Dept of Pathology
Yale University
Major Advisor

David S. Adams, Ph.D.
WPI Project Advisor

Abstract

K3 and K5 proteins encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) downregulate major histocompatibility complex class I (MHC-I) from the surface of host cells through ubiquitin-dependent endocytosis and degradation. The closest relative to KSHV is Herpesvirus saimiri (HVS) which naturally infects squirrel monkeys. The open reading frame (Orf) 12 of HVS shows a high structural homology to K3 and K5 proteins and thus, the functionality of Orf12 to downregulate MHC was tested. It was indeed found to be capable of downregulation.

Table of Contents

Abstract i
Table of Contents ii
Table of Figures iii
Table of Tables iv
Acknowledgements v
1 Background 1
1.1 An Overview 1
1.2 Project Purpose 5
2 Methods 8
2.1 Protocols, Equipment and Techniques 8
2.2 Polymerase Chain Reaction (PCR) 8
2.3 DNA Purification 9
2.4 Agarose Gel Electrophoresis 10
2.5 DNA Restriction Digests 10
2.6 DNA Cloning 11
2.7 Plasmid DNA Mini- and Maxipreps 11
2.8 Tissue Culture 12
2.9 Western Blotting 12
2.10 Flow Cytometry 13
2.11 Confocal Microscopy 14
3 Results 15
4 Conclusions 23
5 Appendix 25
5.1 HVS Orf12 amino acid sequence: 25
5.2 KSHV K3 amino acid sequence: 25
5.3 pTracer EF/V5-His A Vector Map: 26
5.4 pTracer EF/V5-His A Multiple Cloning Site: 27
5.5 pEGFP-N1 Vector Map: 28
5.6 PCR Primers: 29
5.7 K3 Structure Prediction: 30
5.8 Orf12 Structure Prediction: 30
6 Works Cited 31

Table of Figures

Figure 1: Homology within the K3 Protein Family (adapted from [4]) 3
Figure 2: Structure of Orf12 4
Figure 3: K3 and K5 Protein Motif Diagram (adapted from [9]) 4
Figure 4: Endocytosis and Degradation of MHC-I 5
Figure 5: Schematic of Orf12 and K3 6
Figure 6: Schematic of Chimeras and Point Mutant 7
Figure 7: PCR Primer Placement on Orf-12 and K3. 9
Figure 8: Restriction Digest Showing the Correct Size Orf12 wt Insert 15
Figure 9: Western Blot Showing Chimera 2 (mut #13) Fused to GFP 16
Figure 10: Flow Cytometry Analysis of MHC-I Expression in Transfected 293T and COS-7 Cells 17
Figure 11: Flow Cytometry Analysis of MHC-I Expression in Cells Transfeted with EGFP Chimera Two (mut, two clones) and Orf12 wt and mut 18
Figure 12: Flow Cytometry Analysis of MHC-I Expression in Cells Transfeted with Tracer Orf12 wt and mut (try two) and Chimera One (wt) 18
Figure 13: Flow Cytometry Data, 293T GMC 19
Figure 14: Flow Cytometry Data, 293T LMC 20
Figure 15: Flow Cytometry Data, 293T and COS-7 21
Figure 16: pEGFP Flow Cytometry Data 22
Figure 17: pTracer Flow Cytometry Data 22
Figure 18: E2 Binding Sites (adapted from [11]) 23

Table of Tables

Table 1: Flow Cytometry Data, 293T GMC 19
Table 2: Flow Cytometry Data, 293T LMC 20
Table 3: Flow Cytometry Data, 293T and COS-7 21

Acknowledgements

I would like to thank Bob and Sabine for welcoming me into their pathology lab at Yale University and guiding me through the ins and outs of what being a research scientist is all about. I learned so much from them this past summer, they were always ready to help me with my project, and the great times we had in the lab truly made this MQP a wonderful experience.

And of course this project never would have happened without the help of my advisor Dave Adams, who guided me through the initiation of it, and the final version of this report would not be what it is without his editing.

1 Background

1.1 An Overview

To delve into virology is to examine the many complex and clever means by which viruses evade the host immune response. Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), and its primate homologue Herpesvirus saimiri (HVS) are prime examples to show how viruses can thrive within a host system and remain undetected through a number of mechanisms.

To understand how viruses can survive in an environment designed to eliminate their presence, the various defenses in place within host systems must first be examined. The types of mechanisms involved can be placed into two broad categories; pathways which lead to lysis and cellular resistance through antibodies.

Interferons (IFNs), which have a hand in a number of metabolic, hormonal and signaling pathways, are also a part of the body's anti-viral immunity response. For example, IFN-alpha inhibits viral replication, which some viruses are able to halt by interfering at one of the many points in the cellular signal cascades, and some viruses can maneuver around the IFNs, like Epstein-Barr virus (EBV). Pertinent to this project, KSHV encodes an interferon response factor (IRF) which mimics the cells own IRFs to inhibit IFN function [1].

Another of the body's responses to infection are chemokines, which associate with 7-transmembrane (TM), G-protein coupled receptors. When a part of the body becomes inflamed, chemokines work to bring leukocytes to the point of inflammation. Similar to the evasion of IFNs, KSHV encodes three monocyte inflammatory proteins (MIPs) that can interject and competitively block chemokine receptors [1].

One method utilized to destroy cells is the complement system which, through interactions between certain cell proteins and foreign proteins, leads to the activation of a cascade

that triggers the membrane attack complex (MAC). By forming a MAC, targeted cells and pathogens can be lysed [1].

In addition to the complement system, cells can be destroyed through apoptosis, which is programmed cell death used to remove unhealthy or defective cells in the system. Apoptosis can be triggered by a signal from either a surface receptor or an intracellular molecule. The targeted cells, which are usually defective, old or infected by a pathogen, are sacrificed in order to prevent the spread of disease and to remove cells that are functioning incorrectly.

However, when certain viruses enter the system, the apoptotic cycle can be averted, giving rise to the possibility of creating an immortalized cell used to complete viral growth, an event which can lead to oncogenesis. KSHV is such a virus, with the ability to cause skin carcinomas and other lymphomas [1].

Yet another method employed to destroy unwanted cells is the use of natural killer (NK) cells found throughout the body. Their job is to find and destroy infected or defective cells. When a cell becomes infected, it expresses surface molecules to alert the immune system that there is a problem. The most important molecule for this project was major histocompatibility complex class I (MHC-I), which, when expressed on the cell surface, turns off the inhibition to kill of the NK cells, allowing them to either destroy the troubled cell or produce cytokines, which would work with the adaptive immune response against the cell [1].

KSHV has adapted to this uninviting environment of the host, finding ways to remain undetected as it replicates. They can avoid cytotoxic T lymphocytes (CTLs) by increasing the endocytosis and degradation of MHC-I surface molecules, as described above. As for B cell evasion, as of yet there is no data to say that KSHV directly interferes with their response, although indirectly KSHV’s K3 and K5, through their downregulation of the surface markers, may in fact limit the B cells’ ability to present antigens, which would mimic the effect of MHC-1 downregulation, and consequently slow T helper cell costimulation [1].

The main focus of this project was to work with KSHV's protein K3 (sometimes referred to in the literature as MIR1) and HVS's open reading frame 12 (Orf12), respectively, characterizing how Orf12 interacts with MHC-I surface proteins on host cells. Without MHC-I expression, along with downregulation of ICAM-1 and B7.2 by K5 (MIR2, [7,10,12]), NK and CTL cells are essentially blind to the difference between a healthy and infected cell [2].

Another important aspect to the downregulation of MHC-I, besides being a viral evasion strategy, is the question of exactly how this downregulation occurs. Ubiquitin has been found to play an important role in this process, as K3, K5 and presumably Orf12 are E3 ubiquitin ligases [15]. Orf12 is thought to also be an E3 because of its high homology with other members of the MARCH protein family (Figure 1), which contain a plant homeodomain/leukemia-associated protein domain (PHD/LAP) at the N terminus, which includes a RING domain with a conserved series of cysteines and a histidine (C4HC3) involved in a zinc finger motif followed by a two TM (transmembrane) domain [4,9].

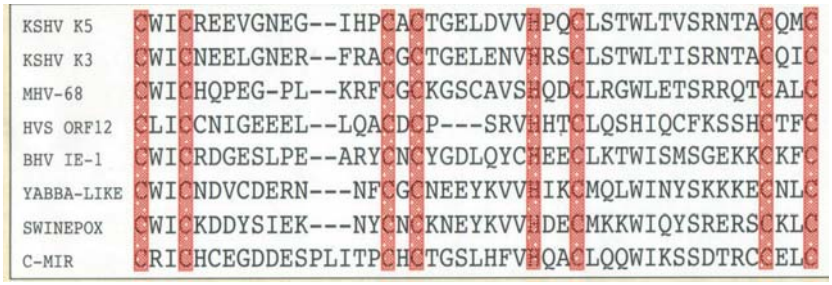


Figure 1: Homology within the K3 Protein Family (adapted from [4])

Also of note in Figure 1, besides the C4HC3 motif (highlighted red in the figure), is the tryptophan (W) to the right of C5, which all the members of this family show except for HVS Orf12, which has a histidine (H). The above amino acid sequence is part of the zinc binding finger, as seen in figure 2 below.

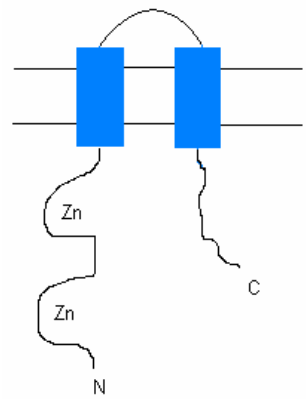


Figure 2: Structure of Orf12

The E3s associate with a specific substrate, in this case MHC-I, using their RING domain and their role is to facilitate the transfer of ubiquitin from an E2 enzyme to the substrate. Once tagged, MHC-I molecules are endocytosed and trafficked to the cell's lysosome, localizing to the trans-Golgi network (TGN) and late endocytic compartments before degradation [3,6,8,9]. Experiments performed on this trafficking pathway suggest that while the RING domain is responsible for internalization, the motifs highlighted below (Figure 3) in red and green in the C-terminus are essential for the directing of MHC-I to the lysosome from the TGN [1,9].

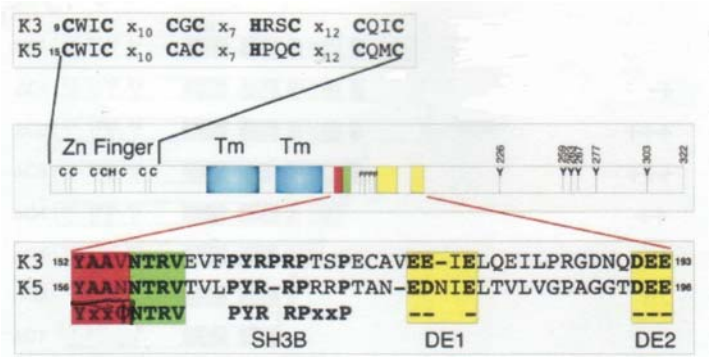


Figure 3: K3 and K5 Protein Motif Diagram (adapted from [9])

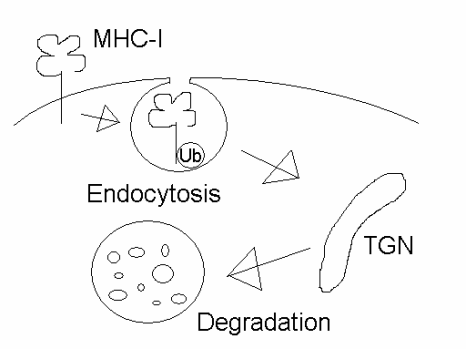


Figure 4: Endocytosis and Degradation of MHC-I

While studying the ubiquitination of MHC-I, researchers have also found that K3 does not require MHC-I to have a lysine in its intracytoplasmic tail in order for endocytosis to occur, as would be expected [14]. Instead, a cysteine is required, which in this case allows for ubiquitination structurally identical to that which is normally seen with K5 using lysine [15]. Orf12 should also be characterized based on this finding.

1.2 Project Purpose

I was given the task of working with a virus related to KSHV, Herpesvirus saimiri (HVS), whose natural host is the South American squirrel monkey [5]. When these primates are exposed to HVS, the virus is non-pathogenic, despite high virus titers. However, when introduced to other new world primates, they almost always develop fatal lymphomas. By comparing the differences and similarities between KSHV and HVS, we can better understand the mechanisms at work for pathogenesis and oncogenesis.

KSHV utilizes one of its proteins, K3, to downregulate MHC-I proteins on the surface of host cells. The homologue of K3 in HVS is the protein Orf12 (Figure 5), which is structurally very similar and may be functionally inactive. Besides having a truncated C-terminus, Orf12 has

a histidine at a key point in its PHD/LAP domain where K3 has a tryptophan, as was shown in figure 1 above.

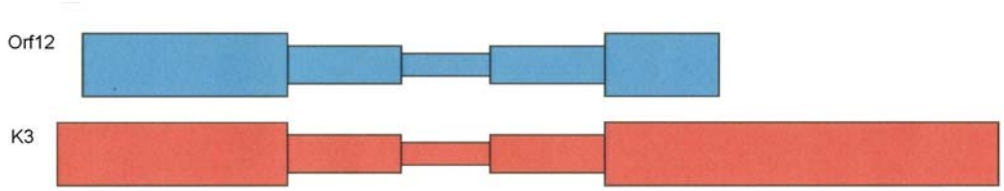


Figure 5: Schematic of Orf12 and K3

K3 and Orf12 belong to a family of E3 ubiquitin ligases, which means they are responsible for substrate specificity during ubiquitination. Because the PHD/LAP domain is thought to contain the active site for ubiquitination for K3, an Orf12 H->W point mutation was designed because every other homologous protein in the K3 family expresses a tryptophan where Orf12 expresses a histidine.

As described above, K3 is known to utilize ubiquitin to tag MHC-I and send it to the cells own lysosome, although the exact pathway has yet to be determined. The literature suggests that K3 acts to upregulate endocytosis of surface MHC-I molecules, and then directs them to the TGN before being sent to the lysosome for degradation [9]. The wild type (wt) and point mutant (mut) Orf12 constructs were packaged into micelles through the use of transfectin, and through membrane fusion the constructs were put into the cells in vitro. Flow cytometry was then used to look for downregulation of MHC-I, and confocal microscopy was used to see localization of Orf12 within the cell.

In addition to the construction of the point mutant, the wt and mut DNA were used as PCR templates to form chimeras of Orf12 and K3 as shown below in Figure 6 (Orf12 blue, K3 red). Unfortunately for this project, time permitted that only chimeras one and two could be made

and tested, although the PCR primers have already been assembled for the remaining combinations.

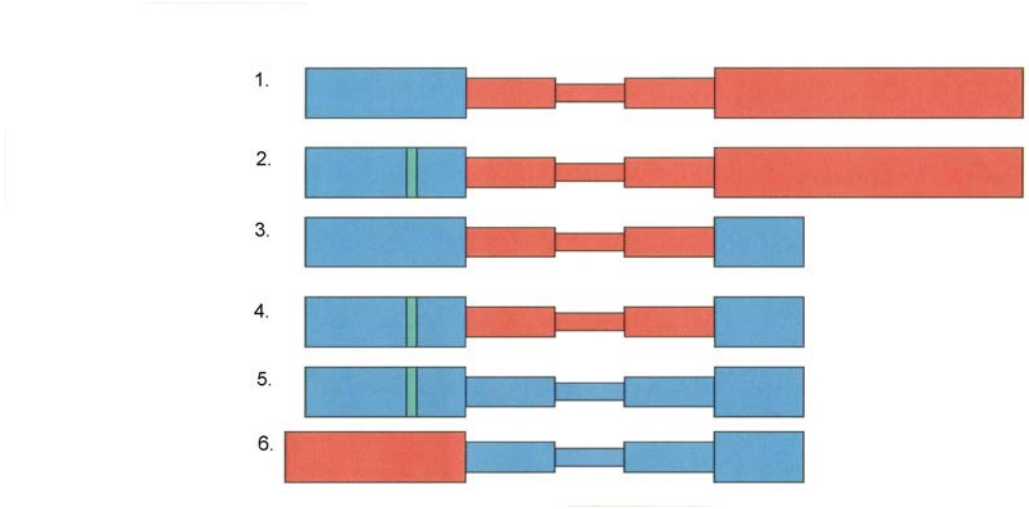


Figure 6: Schematic of Chimeras and Point Mutant

Because the TM domain of K3 is known to be responsible for localization, the first two chimeras include this domain and swap out the K3 PHD/LAP for the wt and mut Orf12. Using this construct, the protein should localize as expected from K3 experiments, but now a targeting of MHC-I by Orf12 should be observed. When assembled, chimeras three and four will determine whether or not the C-terminal portion of Orf12 affects downregulation of MHC-I, and chimera six will use the PHD/LAP of K3, which is known to be functional for downregulation, and the TM and C-terminal domain of Orf12 to characterize localization of Orf12.

The phenotypic assays performed included Western blotting, which was used to visualize protein expression with size markers, flow cytometry, which quantitates protein expression in cells with the use of antibody staining, and confocal microscopy, which shows localization of the protein within the cells.

2 Methods

2.1 Protocols, Equipment and Techniques

During the three months spent working in the lab I learned how to perform a number of techniques and procedures. Some I had been exposed to from lab courses during school, but most I had only read about in research articles. In this section I will describe the techniques I learned to perform and how I was able to utilize them. Each was critical for the completion of my research project.

2.2 Polymerase Chain Reaction (PCR)

PCR is a technique used to amplify specific portions of DNA. Primers are designed and added to a reaction along with the template DNA, dNTPs, water and a magnesium-containing buffer. The tubes are put into the PCR machine, which controls the temperature needed and the duration of each cycle. A typical program is an initial denaturation for two minutes at 94ºC, followed by 30 cycles of the following three steps:

1. 94ºC 10 seconds (denaturation)

2. 52ºC 1 minute (primer annealing)

3. 72ºC 1 minute (primer extension)

For this project I designed and utilized thirteen original primers to amplify the whole and parts of the Orf12 gene, and parts of the K3 gene. The list of primers and their sequences can be found in the appendix and a schematic of their alignment with the genes amplified is shown below in Figure 7. Primer names in parentheses represent primers used to clone into the pEGFP-N1 vector while the rest are for the pTracer EF/V5-His A vector.

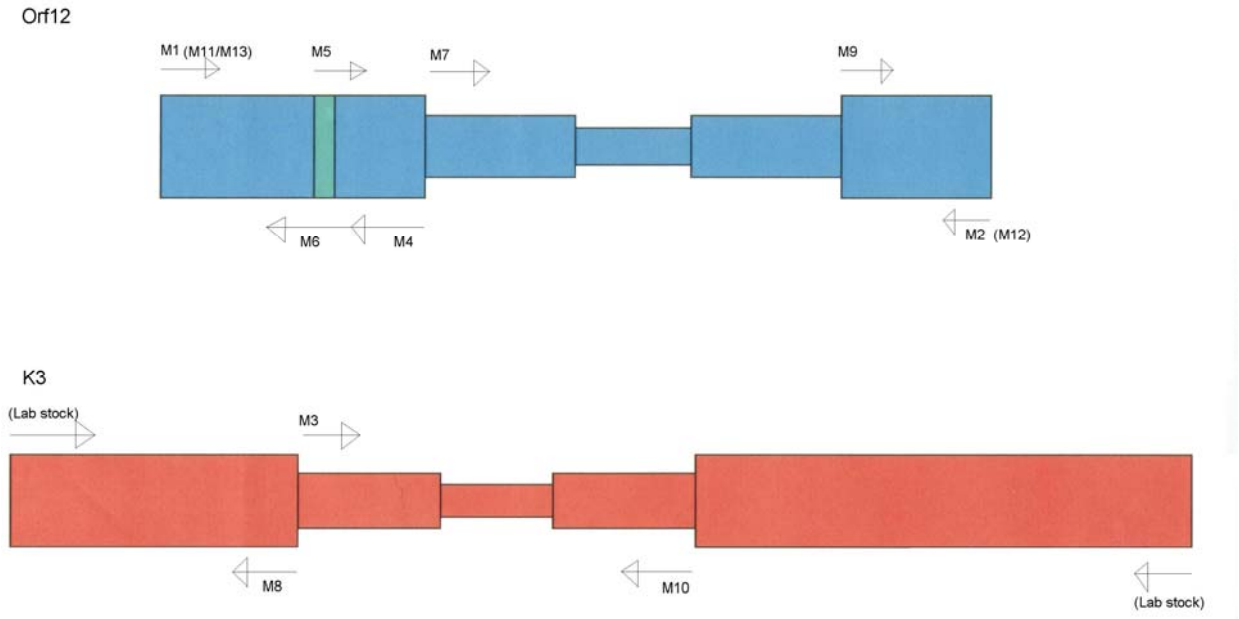


Figure 7: PCR Primer Placement on Orf-12 and K3.

First I amplified the entire Orf12 using the viral HVS DNA as a template and M1 and M2 as the primers. Next came the Orf12 H->W point mutant, which used primers M1, M4, M2 and M6. The mutant had to be done in two rounds of PCR, one to amplify the introduced mutation and the left half of the open reading frame and an overlap of the mutation with the right half of the open reading frame, then a second PCR to combine the two halves. The chimeras also had to be done in two rounds, the first round to amplify the Orf12 and K3 portions, then the second to stitch the two halves together to form one chimeric protein.

2.3 DNA Purification

To purify the PCR products, both before and after restriction digestion, and to purify the vectors after digestion, the Qiagen “QIAquick gel extraction kit” was used according to the manufacturer’s protocol. PCR product and buffer were spun in a spin column and washed with EtOH solution then eluted with warm water.

At one point in the project I had to purify bands of DNA from an agarose gel following electrophoresis, which entailed cutting out the bands with a razor blade, putting the gel into an eppendorf tube with buffer, melting the gel in the fifty-six degree Celsius water bath and then proceeding with the normal purification protocol of spinning, washing with EtOH and eluting with water.

2.4 Agarose Gel Electrophoresis

DNA concentration and restriction digest analyses were run often on either 1.0% or 1.5% agarose gels in 1X TBE buffer. A 1kB DNA ladder (Gibco BRL) was used as a size marker to locate the Orf12 fragment (~500bp). The electrophoresis was performed at 200V, 400mA and for twenty minutes usually, then the DNA stained with ethidium bromide was detected using a UV transilluminator.

2.5 DNA Restriction Digests

Restriction digests allowed me to see whether or not my DNA fragment was inserted into the vector. I designed my PCR primers for cloning into the pTracer-EF/V5-His A vector using the restriction sites EcoRI and SpeI. This expression construct should produce the Orf12 protein fused to the V5 and His epitope tag to allow detection of the protein. Restriction digests using EcoRI and SpeI were incubated in the EcoRI buffer (New England Biolabs). For the EGFP-N1 vector I used the EcoRI and BamHI sites for cloning, and digests were also incubated in the EcoRI buffer. The original primer design for the EGFP-N1 vector used BglII and BamHI, however because problems developed with those two enzymes interacting with each other I was advised to make a new primer using EcoRI instead of BglII. This expression construct should result in the production of a fusion protein of Orf12 with green fluorescent protein (GFP).

2.6 DNA Cloning

Two vectors were used to clone into *E. coli*; pTracer-EF/V5 His A was the standard vector used for expression, and pEGFP-N1 was used for localization assays. Diagrams of each vector can be found in the appendix. First a ligation reaction was performed to combine the cut and purified vector with the cut and purified DNA fragment using ligation buffers and T4 DNA ligase. The ligated DNAs were then introduced into competent bacteria on ice.

After thirty minutes, the cells were heat shocked at forty-two degrees Celsius, and then recovered with S.O.C medium in the thirty-seven degree water bath for another thirty minutes. The transformed bacteria were then plated on an appropriately antibiotic LB agar plate along with a negative control plate (the ligation included water instead of insert). The plates were put into a thirty-seven degree incubator overnight and usually ten colonies were chosen to be grown up in LB broth for plasmid DNA minipreps.

2.7 Plasmid DNA Mini- and Maxipreps

Miniprep cultures were grown in 3mL LB media with the appropriate antibiotic, which was ampicillin for the Tracer vector and kanamycin for the EGFP vector. Colonies were picked from the cloning plates and following overnight incubation in medium, the DNA was extracted using the Marligen rapid purification kit. The DNA was then digested to see which colonies actually contained my insert, and from there sequencing reactions of promising clones were set up and sent to the Yale sequencing facility.

Once a clone with the correct sequence was determined, a maxiprep culture was grown in 500mL LB medium overnight. From this culture a high concentration of the correct plasmid DNA would be produced. Maxipreps of the wt and mut Orf12 were important for this project because Orf12 had never been cloned before so there is no stock of it. Also, to generate the chimeras these two sources of DNA would be used as templates during the PCR reactions instead of using up the entire stock of whole viral DNA.

2.8 Tissue Culture

For this project a 293T cell line was used. I was responsible for maintaining at least one culture flask, which I used to make six well plates when needed for transfections and I split them one to seven twice a week. The medium used was DMEM supplemented with ten percent FBS, glutamine, streptomycin and penicillin.

Transfections involved mini- or maxiprepped DNA, and as controls empty vector (negative) and vector with an inserted gB gene (positive). For minipreps, 8uL of DNA was arbitrarily chosen for transfections because of their low concentrations, however for the controls and for maxiprepped DNA 4ug was used. The method of transfection used transfectin, which forms micelles around the DNA, which then fuse to the phospholipid bilayers of the 293T cells, enabling them to deposit the DNA inside the cells.

While working with the tissue cultures, I had to become proficient at sterile technique and working in a biosafety cabinet. This skill included limiting the amount of time that containers were open and cells were exposed to air, not putting culture flask and media bottle caps on the surface inside the hood, and opening sterile plates, media containers and sterile pipettes behind the air flow and glass shield.

2.9 Western Blotting

Western blots are used to characterize proteins by visualizing their expression. Cell lysates are loaded onto an SDS polyacylamide gel along with a size marker, and then the gel is transferred using a semidry transfer blotter (BioRad) to a membrane. Antibodies specific to the protein of interest or epitope tags are added, followed by incubation with a secondary antibody that recognizes the first antibody, which can then be visualized using detection chemicals that cause fluorescence (enhanced chemiluminescence, ECL).

Because Orf12 is about twenty kD, I used a fifteen percent resolving solution to cast my acrylamide gels. I harvested my transfected 293T cells using PBS, then spun them down and resuspended the pellets in laemmli buffer. After boiling and vortexing the samples I loaded them into the wells along with a marker and filled any unused wells with laemmli buffer for equal salt distribution in the electric field.

The electrophoresis was performed at 150mA for one hour, and then the proteins were transferred onto membrane filter, which took about forty minutes. After the transfer the membrane was put into a five percent milk solution in PBS Tween for one hour, then the primary antibody was added for one hour and secondary antibody for forty-five minutes with PBS Tween washes in between. For pTracer expression constructs, an anti-V5 primary and anti-mouse secondary were used, and for pEGFP expression constructs an anti-GFP primary and anti-rabbit secondary were used. For these assays the secondary antibodies were preadsorbed to human antibodies.

2.10 Flow Cytometry

Flow cytometry is a technique used to quantitate how much protein is being expressed by cells. Paraformaldehyde-fixed cells are analyzed in the instrument in a single cell suspension, then hit with a laser to determine forward scatter (size), side scatter (granularity) and fluorescence (bound antibody presence). For this project, the amount of MHC-I expressed on the surface of 293T and COS-7 cells was measured using an antibody for MHC-I called W6/32 conjugated to a red fluorophor.

The cells, which were 293T and also COS-7 (primate), were grown in six well plates. For the first round of flow cytometry, the first well was left untransfected as a control, the second well was transfected with empty vector, the third well was transfected with K3 and the fourth with K5 as positive controls for the downregulation of MHC-I, and the fifth and sixth had my maxiprepped Orf12 wt and Orf12 mut DNA.

The cells were harvested with PBS plus EDTA, which separates the cells from the plate and from each other, and then they were screened through a mesh filter to ensure that the cells were not clumped together. They were added to a tube filled with 10% FBS DMEM to replace the calcium lost from the addition of PBS plus EDTA. The cells were stained with the appropriate antibody, pelleted, washed with PBS, pelleted again, then fixed with paraformaldehyde.

At the flow cytometer, the controls were used to set the gate parameters according to the cell populations and to make sure that the red (MHC-I) and green (GFP) signals did not cross into one another. Each sample was read until a fixed number of events had passed across the laser, and the mean fluorescence of the samples was recorded.

2.11 Confocal Microscopy

Confocal microscopy allows us to look at localization of proteins within cells. The microscope used for this project viewed cells in two dimensions instead of three in as small as 0.1 micron slices in order to best see where in the cell Orf12 localizes. COS-7 cells transfected with my clones were grown on the microscope slides, then fixed with paraformaldehyde after being stained with ethidium bromide as a nuclear cellular marker. The slides were then examined under the microscope, using a laser to make the stain and the protein fluoresce. In this case, the protein fused to GFP fluoresced green and the marker was red.

3 Results

The first major result of this project was the production of DNA stocks for Orf12 wt and Orf12H->W mutant in both pTracer-EF/V5 His A and pEGFP-N1 for future experiments. The Orf12 DNAs were amplified by PCR, and ligated into the plasmids. The correct insert size was determined by agarose gel electrophoresis (Figure 8) and the sequences were analyzed and found to be completely correct.

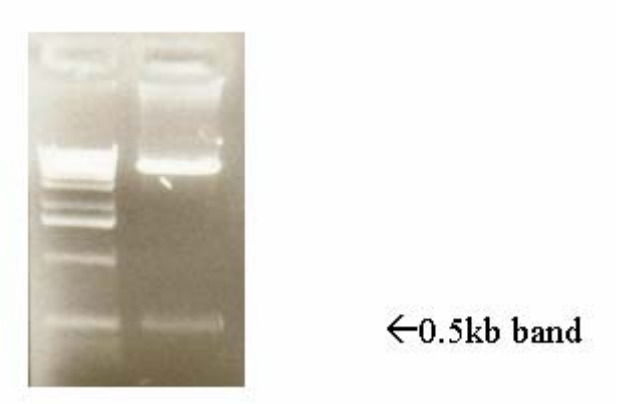


Figure 8: Restriction Digest Showing the Correct Size Orf12 wt Insert

Lane-1 represents a 1kB DNA ladder (Gibco BRL), and lane-2 denotes Orf12 wt in pTracer digested with EcoRI and SpeI restriction enzymes to release the insert.

Western blotting was performed several times but only one protein, chimera 2 (mut #13) was able to be detected, as seen below in Figure 9.

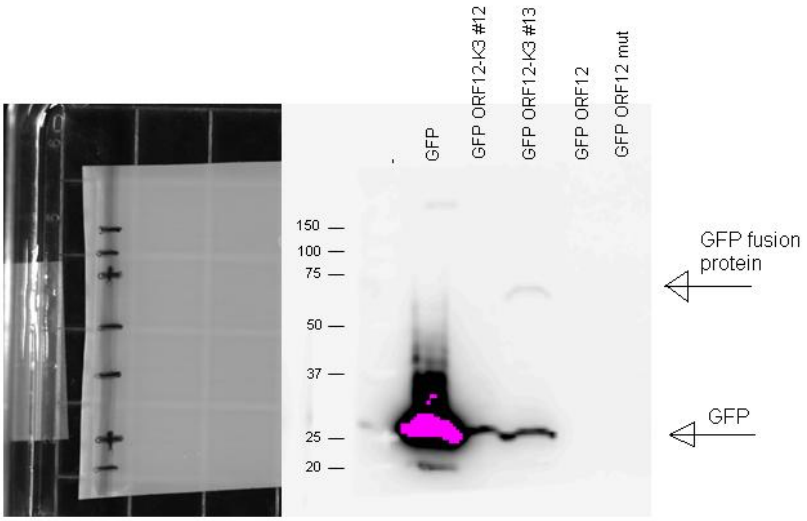


Figure 9: Western Blot Showing Chimera 2 (mut #13) Fused to GFP

The DNAs were transfected into 293T and COS-7 cells, and through the use of flow cytometry, Orf12 wt and mut were analyzed and found to indeed be able to downregulate surface MHC-I, and the mut seemed to work more efficiently than wt (Figure 9). Chimeras one and two were also characterized using flow cytometry and, as expected from the Orf12 wt and mut data, were able to downregulate MHC-I as well. Most samples were run alongside empty vector, using K3 and K5 as controls. K5 is known to be less efficient than K3 at downregulating MHC-I.

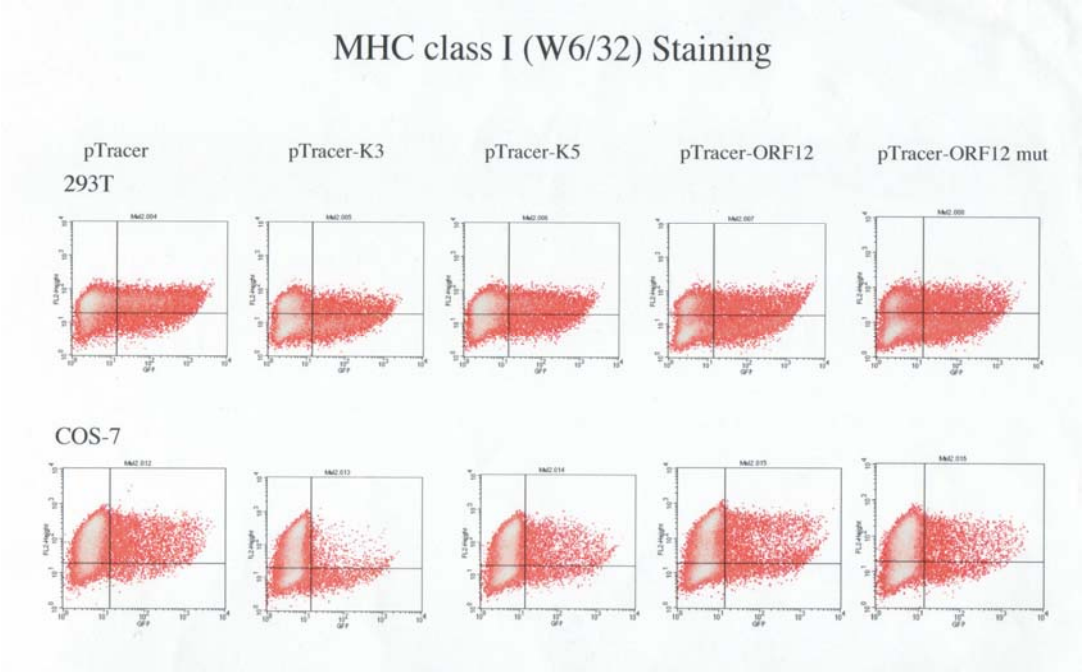


Figure 10: Flow Cytometry Analysis of MHC-I Expression in Transfected 293T and COS-7 Cells

The Y-axis denotes MHC-I staining, and the X-axis represents GFP staining.

In Figure 10 above, empty pTracer vector shows no downregulation, K3 shows relatively high downregulation, K5 shows slightly less downregulation, and Orf12 wt and mut show slightly less than K5, but there is still a significant difference in these from empty vector.

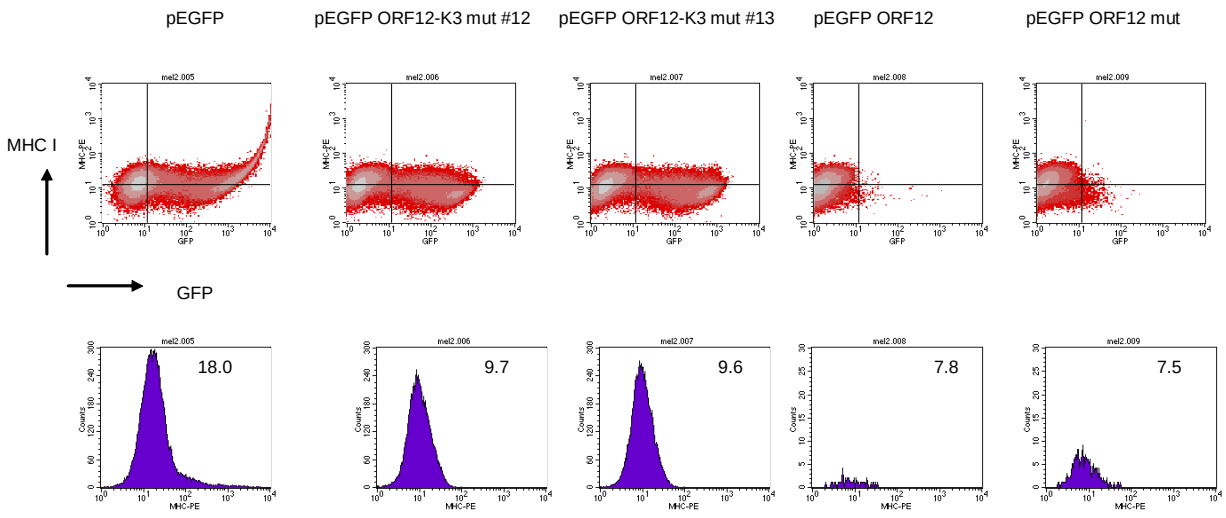


Figure 11: Flow Cytometry Analysis of MHC-I Expression in Cells Transfected with EGFP Chimera Two (mut, two clones) and Orf12 wt and mut

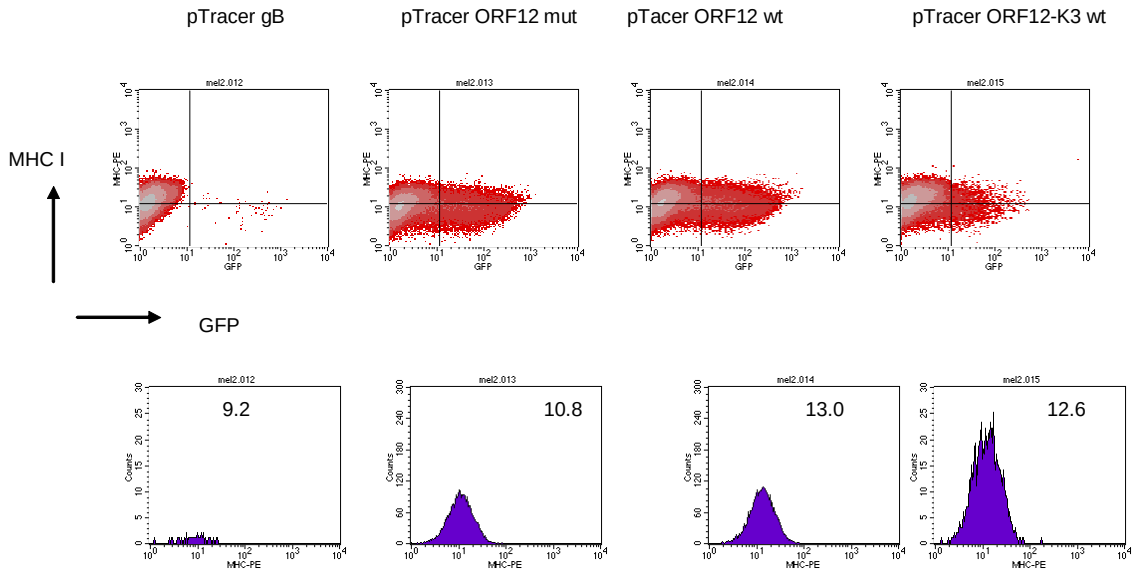


Figure 12: Flow Cytometry Analysis of MHC-I Expression in Cells Transfected with Tracer Orf12 wt and mut (try two) and Chimera One (wt)

In figures 12 and 17, the pTracer gB control had a surprisingly low transfection efficiency, as its fluorescence should be higher than the samples'. Therefore the three samples are better considered among themselves and not compared to the control, as all performed as expected relative to one another. Also of note is the closeness of data between the two chimera

muts (#12 and #13), which were run together because both their sequencing data were correct, and between Orf12 wt and Chimera 1 (wt).

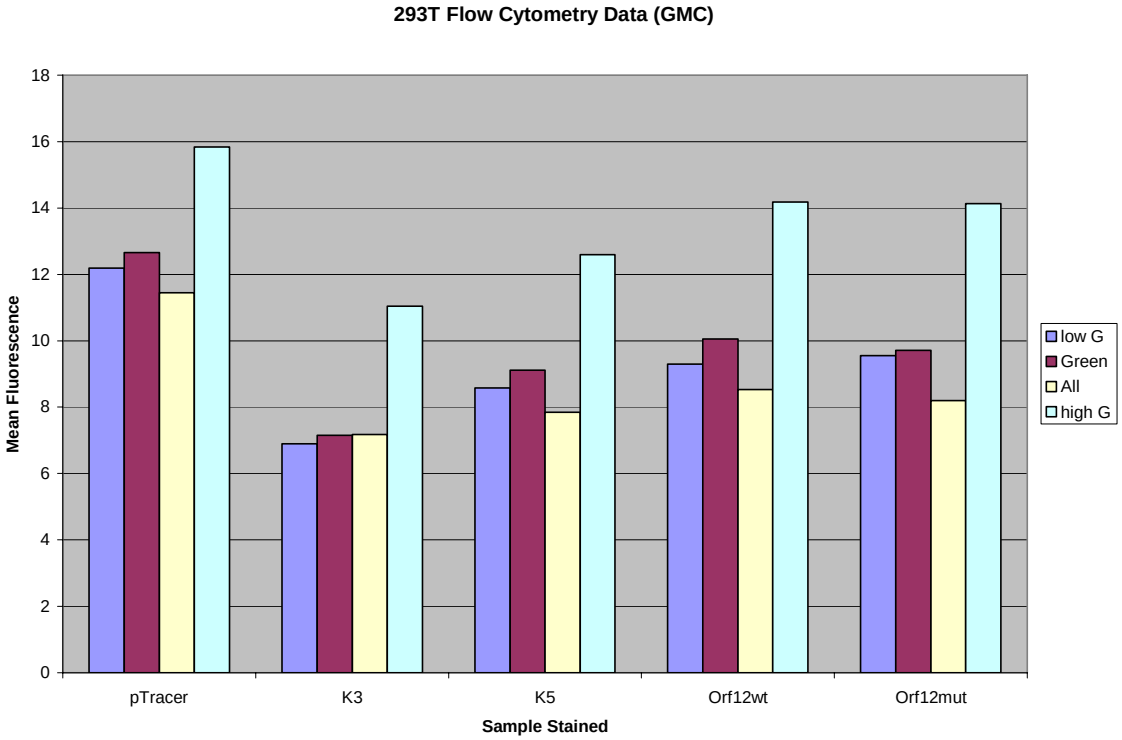


Figure 13: Flow Cytometry Data, 293T GMC

	low G	Green	All	high G
pTracer	12.19	12.66	11.45	15.84
K3	6.89	7.15	7.17	11.04
K5	8.58	9.11	7.85	12.6
Orf12wt	9.3	10.06	8.53	14.18
Orf12mut	9.56	9.71	8.2	14.13

Table 1: Flow Cytometry Data, 293T GMC

In the above chart and table (and following charts and tables), one of the key points to look at is how Orf12 mut always seems to be slightly more efficient at downregulating MHC-I than Orf12 wt. These data will have to be replicated to see if a pattern truly exists.

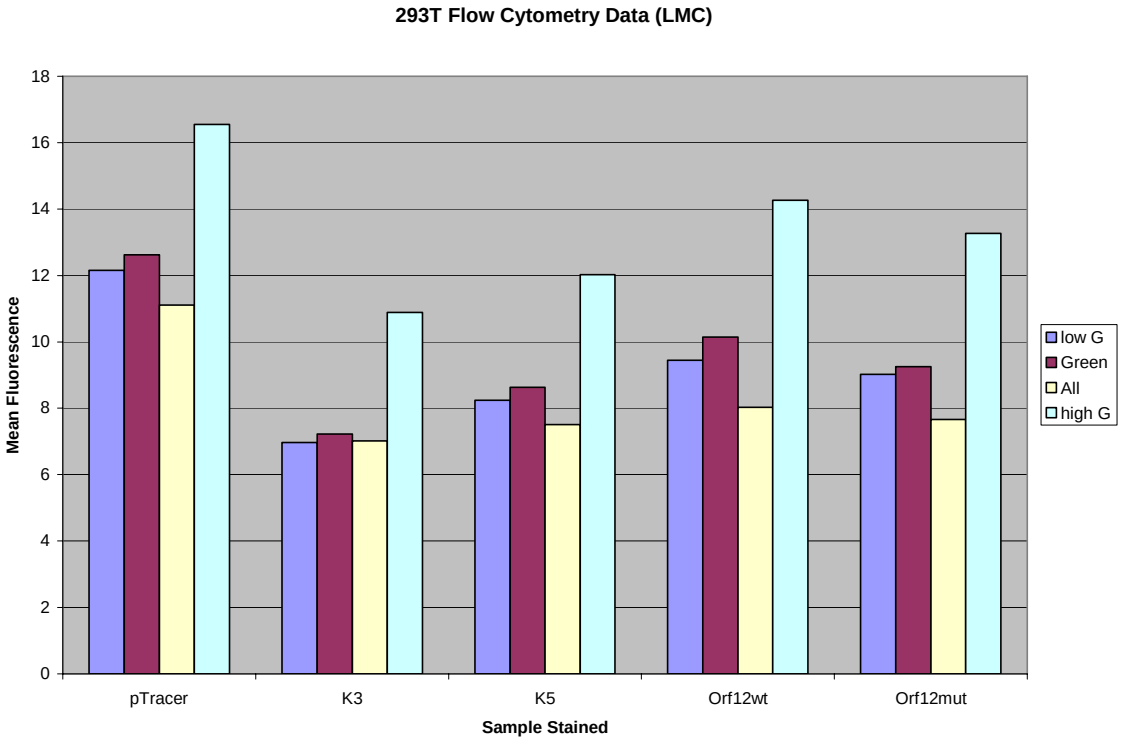


Figure 14: Flow Cytometry Data, 293T LMC

	low G	Green	All	high G
pTracer	12.16	12.62	11.1	16.55
K3	6.97	7.23	7.02	10.88
K5	8.24	8.63	7.5	12.02
Orf12wt	9.45	10.14	8.03	14.26
Orf12mut	9.02	9.25	7.66	13.27

Table 2: Flow Cytometry Data, 293T LMC

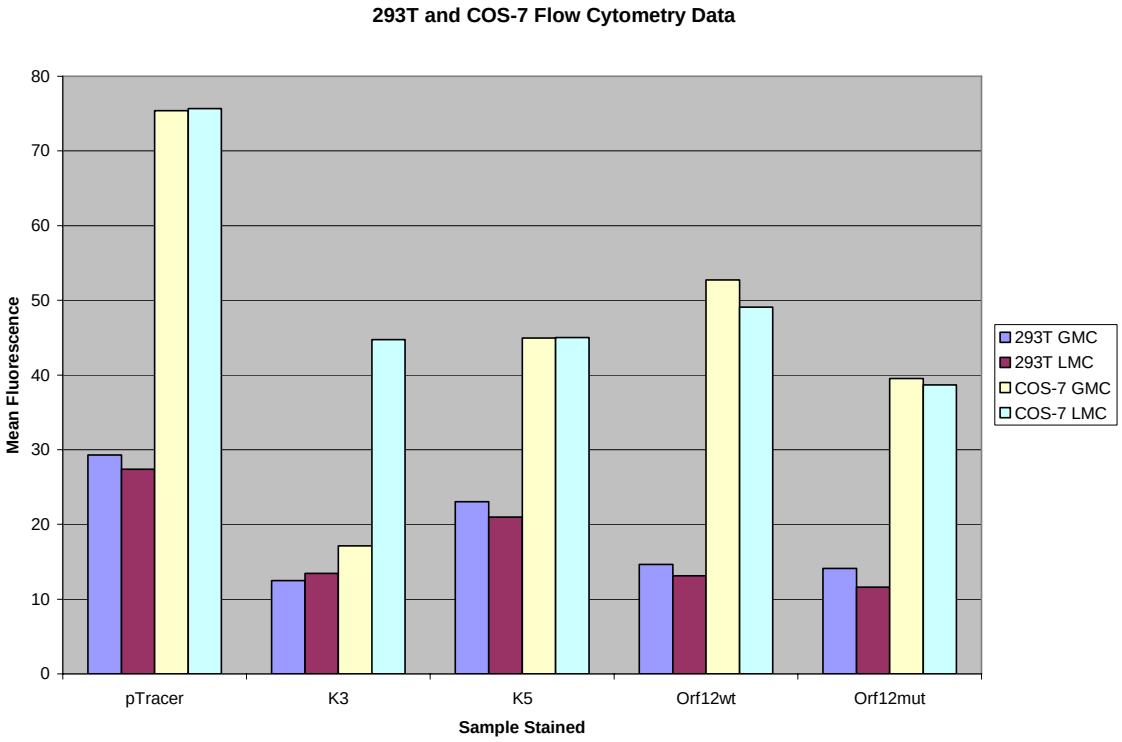


Figure 15: Flow Cytometry Data, 293T and COS-7

	293T		COS-7	
	GMC	LMC	GMC	LMC
pTracer	29.29	27.38	75.38	75.68
K3	12.45	13.45	17.14	44.74
K5	23.06	20.98	44.98	45.04
Orf12wt	14.65	13.12	52.71	49.09
Orf12mut	14.12	11.61	39.54	38.69

Table 3: Flow Cytometry Data, 293T and COS-7

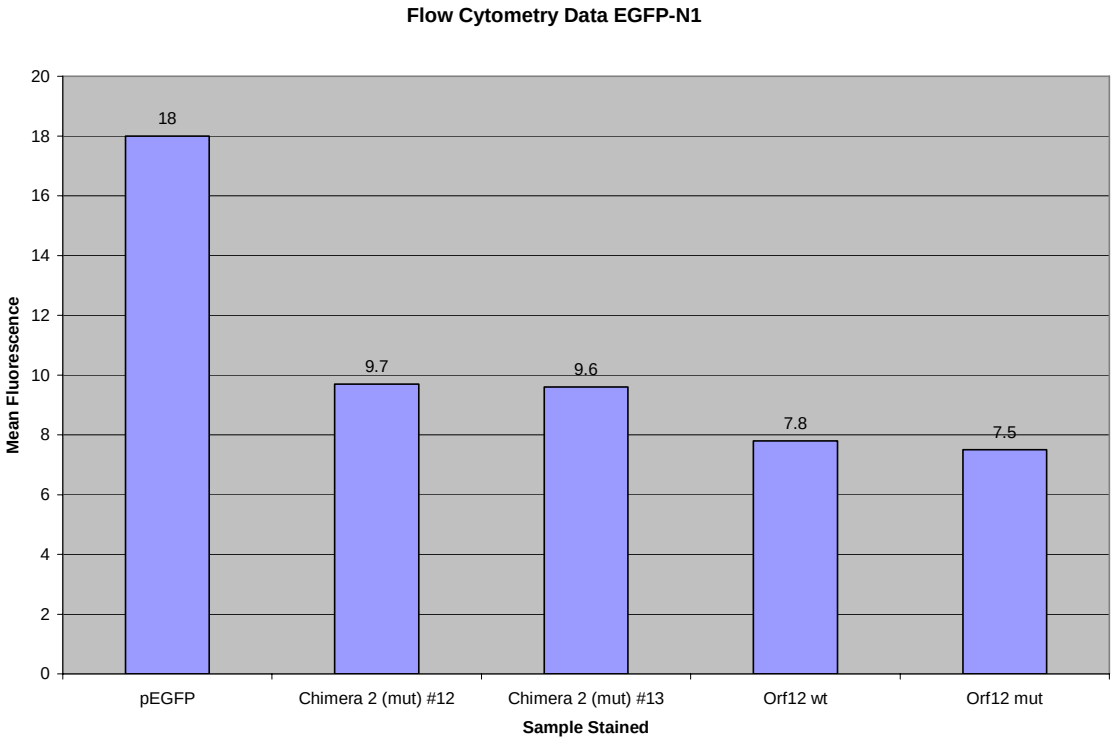


Figure 16: pEGFP Flow Cytometry Data

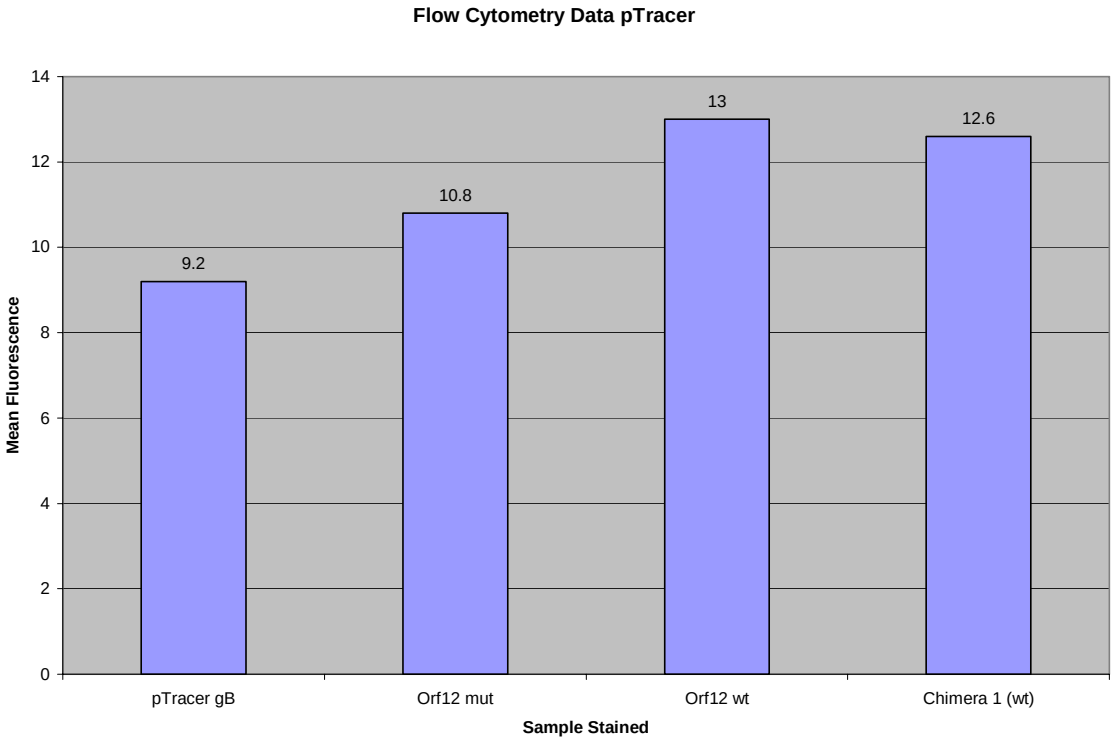


Figure 17: pTracer Flow Cytometry Data

4 Conclusions

The completion of this project has begun the scientific characterization of the Orf12 protein of HVS. From the data collected, Orf12 wt, Orf12 H->W point mutant and chimeras one and two can downregulate MHC-I from the cell surface of transfected 293T or COS-7 cells. This project also hoped to look at localization of the proteins inside host cells using immunostaining and confocal microscopy, however the correct conditions for the slides were difficult to meet and time restrictions allowed for only a couple tries before the project ended.

According to the flow cytometry data, Orf12 wt can downregulate MHC-I, however the Orf12 mut seems to downregulate MHC-I more efficiently. This data brought up an interesting question, because while other proteins in the same family as Orf12, namely K3 and K5, use a tryptophan as the connecting point for the E2 enzyme, Orf12 has a histidine. Assuming that the histidine would need to be changed to a tryptophan in order to produce functionality, we were surprised to find that both the wt and mut downregulated MHC-I.

Assuming the findings from this single FACS experiment duplicate, these data may indicate that the Orf12 protein, when mutated to mimic the key tryptophan residue of other family members, has surrounding domains that facilitate a stronger interaction with E2 than K3 or K5. Or alternatively, further research into the nature of E2s may demonstrate that while other members of the K3 family of E3 proteins use the E2 UbcH7, Orf12 could possibly break away from the pattern and use Rad6, an E2 normally involved with DNA replication corrections [11].

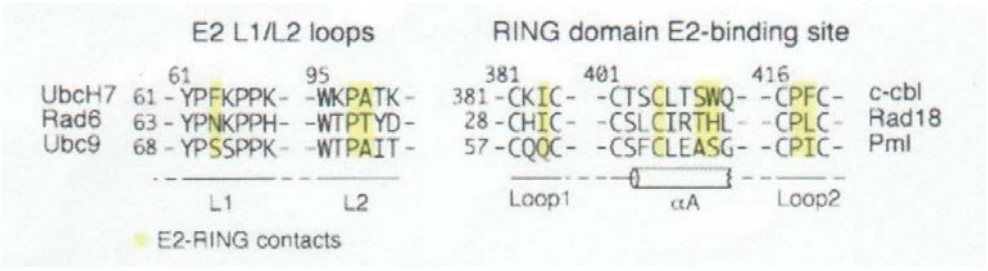


Figure 18: E2 Binding Sites (adapted from [11])

Because 293T and COS-7 cells were used, there is the possibility that these cell lines have more UbcH7 than Rad6 E2s, which would explain why the point mutant would appear more efficient at downregulation of MHC-I. The next step will be to examine Orf12 wt and mut in the presence of either UbcH7 or Rad 6 to further characterize this interaction.

As far as Western blotting and confocal microscopy, the proteins and cells were uncooperative. Expression of Orf12 was difficult to visualize on Western blots, the most likely possibilities being its small size (~18.5kD), low translation rate within cells, instability, aggregation or degradation inside cells. However expression could be seen under a fluorescence scope and flow cytometry data confirms that the proteins are present because of downregulated MHC-I expression. At the very end of the project chimera 1 (wt) was finally visualized by Western blot, probably because this protein is twice as large as Orf12. Confocal microscopy also posed a problem, as the cells used for the transfections did not adhere very well to the slides and thus could not be visualized correctly.

Unfortunately time did not permit for me to perform thorough phenotypic assays for all of the chimeras, although just as my time in the lab ended correct clones were constructed for chimeras one and two. The chimeric fragments were not inserting well into the vectors and needed several repeats in order to get the few clones acquired. The data collected and chimeras eventually formed as a result of this project are the beginning of a continuing characterization that will hopefully result in a publication. Another will use the rest of the primers I developed to better characterize Orf12 beyond my preliminary findings, so the final result of my time in the lab was to setup for future experiments.

5 Appendix

5.1 HVS Orf12 amino acid sequence:

MSSIQKKCLICCNIGEEELLQACDCPSRVHHTCLQSHIQCFKSSHCTFCEKKYKIMVMCNS
LKKCSSPVLEQANWIVLCVCVSTLLCILCILLDICLTIRLWQSSVLCYEVYNTFYFLVLCG
TFSIAFYLAAWYDIFFEFHSLCSFIWNLKKISQSYPCEASKNALKIL*

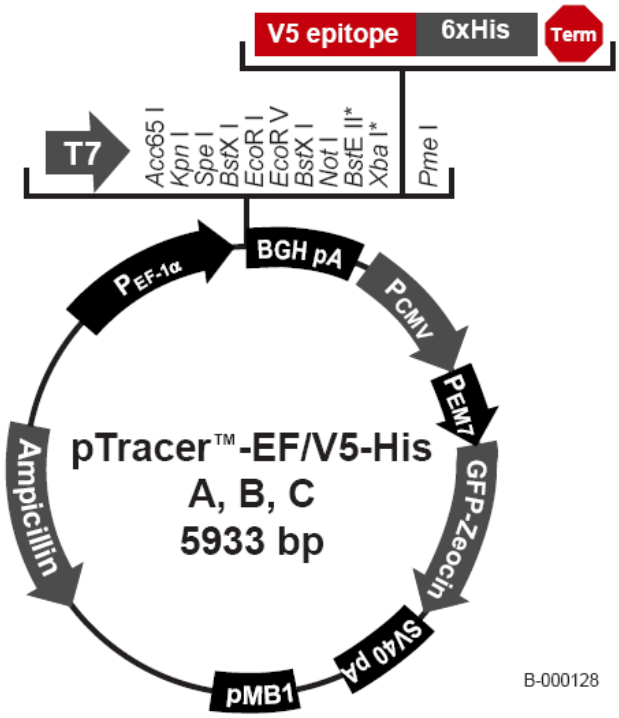
5.2 KSHV K3 amino acid sequence:

MEDEDVPVCWICNEELGNERFRACGCTGELENVHRSCLSTWLTISRNTACQICGVVYNT
RVVWRPLREMTLLPRLTYQEGLELIVFIFIMTLGAAGLAAATWVVWLYIVGGHDPEIDHV
AAAAYYVFFVFYQLFVVFGLGAFFHMMRHVGRAYAAVNTRVEVFPYRPRPTSPECAVE
EIELQEILPRGDNQDEEGPAGAAPGDQNGPAGAAPGDQDGPADGAPVHRDSEESVDEAA
GYKEAGEPTHNDGRDDNVEPTAVGCDCNNLGAERYRATYCGGYVGAQSGDGAYSVSC
HNKAGPSSLVDILPQGLPGGGYGSMGVIRKRSAVSSALMFH*

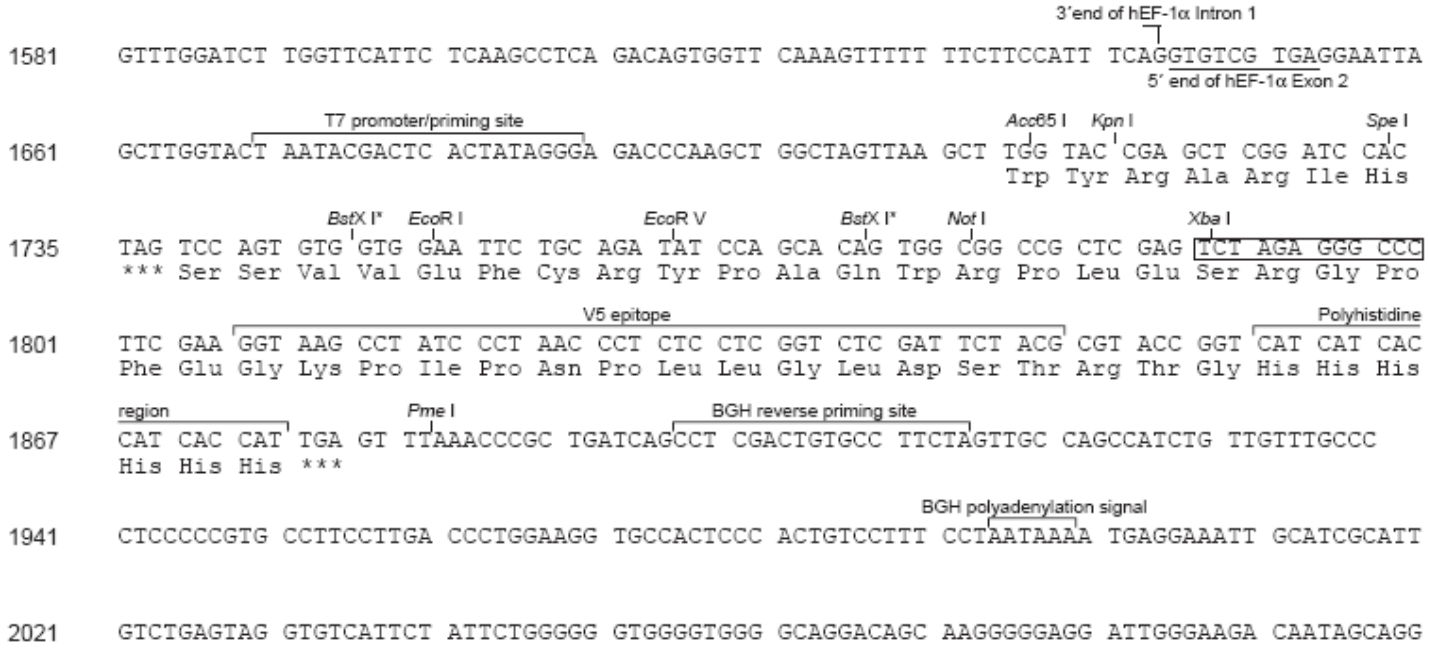
5.3 pTracer EF/V5-His A Vector Map:

Comments for pTracer™-EF/V5-His A
5933 nucleotides

EF-1α promoter: bases 476-1662
T7 promoter/priming site: bases 1670-1689
Multiple cloning site: bases 1715-1806
V5 epitope: bases 1807-1848
Polyhistidine region: bases 1858-1875
BGH reverse priming site: bases 1898-1915
BGH polyadenylation region: bases 1901-2128
CMV promoter: bases 2159-2777
EM7 promoter: bases 2781-2831
Cycle 3-GFP ORF: bases 2850-3554
Zeocin™ resistance gene: bases 3555-3926
SV40 polyadenylation signal: bases 3940-4070
pMB1 origin: bases 4256-4913 (complementary strand)
Ampicillin resistance gene: bases 5058-5918 (complementary strand)



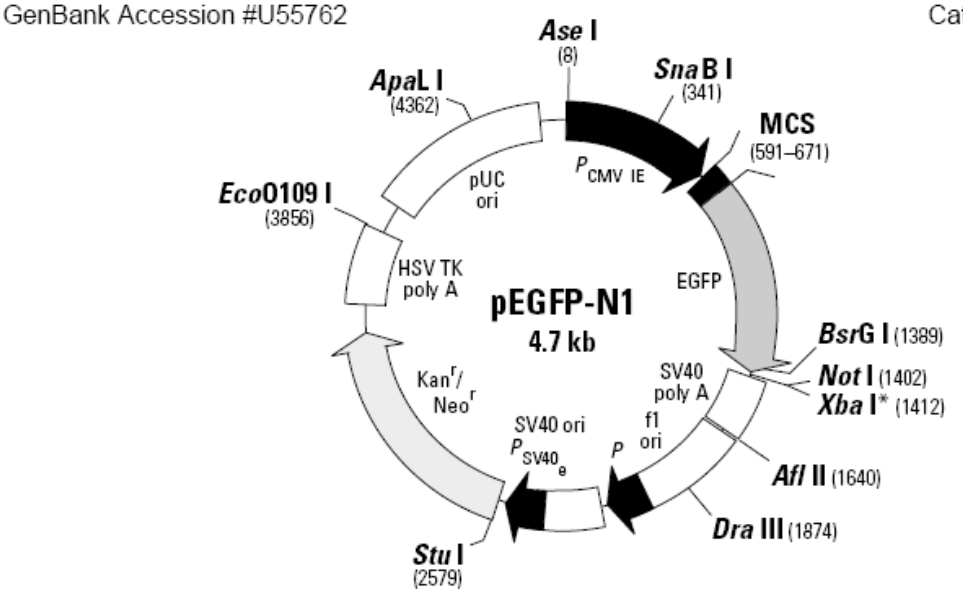
5.4 pTracer EF/V5-His A Multiple Cloning Site:

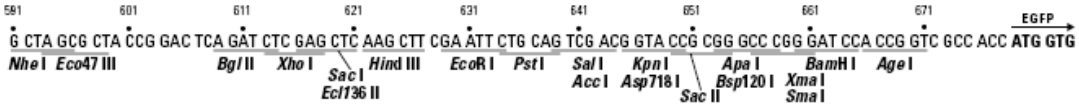


5.5 pEGFP-N1 Vector Map:

pEGFP-N1 Vector Information PT3027-5

GenBank Accession #U55762 Catalog #6085-1





5.6 PCR Primers:

M1: AA ACT AGT GCC ACC ATG AGC AGT TTT CAA AAG AAA TG
SpeI Kozak

M2: AA GAA TTC CAG TAT TTT TAA TGC ATT TTT GCT C
EcoRI

M3: GAG CAA CTG ATT GTT TTT ATT TTC ATT ATG AC

M4: GAA AAT AAA AAC AAT CAG TTG CTC TAG AAC AGG CGA GC

M5: TG CAA AGT TGG ATT CAG TGT TTA AAA TCA TCT CAT TG

M6: AA ACA CTG AAT CCA ACT TTG CAA GCA TGT ATG G

M7: G GGT CTG GAA GCT AAC TGG ATA GTT TTA TGT G

M8: T CCA GTT AGC TTC CAG ACC CTC CTG GTA AG

M9: GT GCG TTT TTC TTT GAG TTT AAG AGC TTA TGT AG

M10: CTT AAA CTC AAA GAA AAA CGC ACC CAA CCC AA

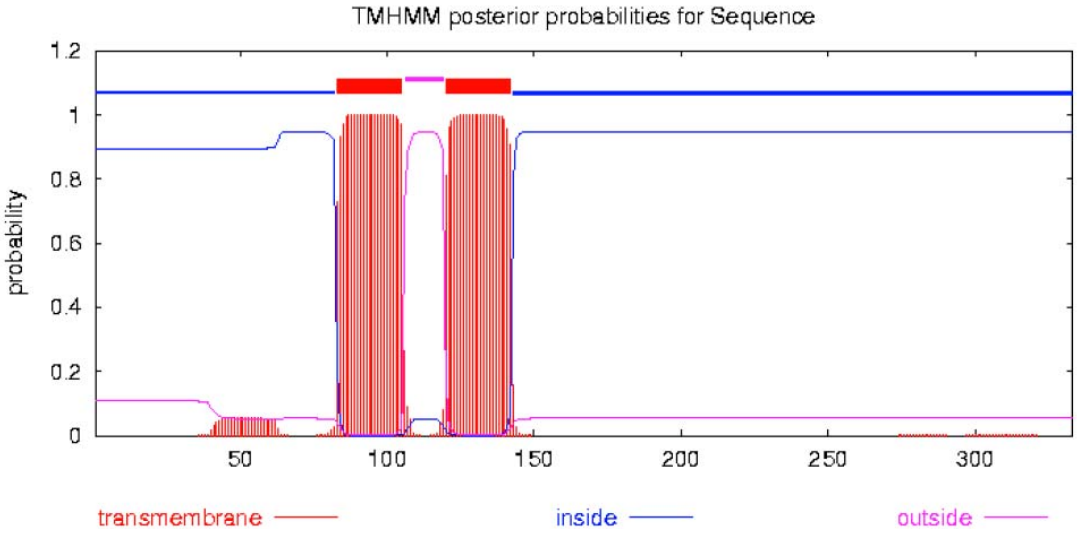
M11: AAA AGA TCT GCC ACC ATG AGC AGT TTT CAA AAG AAA TG
BglII Kozak

M12: A GGA TCC CCC AGT ATT TTT AAT GCA TTT TTG CTC
BamHI

M13: AA GAA TTC GCC ACC ATG AGC AGT TTT CAA AAG AAA TG
EcoRI Kozak

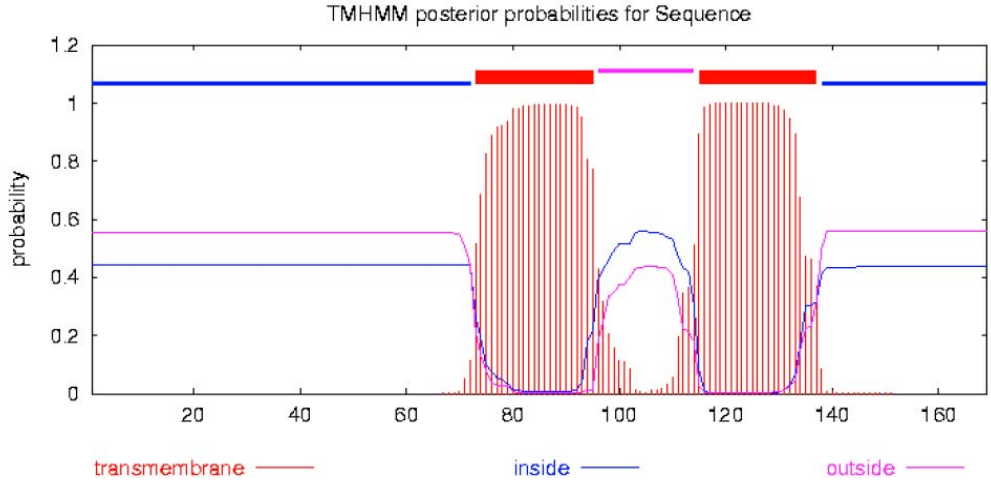
5.7 K3 Structure Prediction:

```
# Sequence Length: 333
# Sequence Number of predicted TMHs:  2
# Sequence Exp number of AAs in TMHs: 47.12182
# Sequence Exp number, first 60 AAs:  1.04266
# Sequence Total prob of N-in:        0.89327
Sequence        TMHMM2.0        inside       1    82
Sequence        TMHMM2.0        TMhelix     83   105
Sequence        TMHMM2.0        outside    106   119
Sequence        TMHMM2.0        TMhelix    120   142
Sequence        TMHMM2.0        inside     143   333
```



5.8 Orf12 Structure Prediction:

```
# Sequence Length: 169
# Sequence Number of predicted TMHs:  2
# Sequence Exp number of AAs in TMHs: 45.04891
# Sequence Exp number, first 60 AAs:  0.0002
# Sequence Total prob of N-in:        0.44402
Sequence        TMHMM2.0        inside       1    72
Sequence        TMHMM2.0        TMhelix     73    95
Sequence        TMHMM2.0        outside     96   114
Sequence        TMHMM2.0        TMhelix    115   137
Sequence        TMHMM2.0        inside     138   169
```



6 Works Cited

[1] R. Means, S. Lang, Y. Chung, J. Jung,(2002) *Kaposi's Sarcoma-Associated Herpesvirus Immune Evasion Strategies*, Frontiers in Bioscience 7, pgs. 185-203.

[2] S.Ishido, J-K. Choi, B-S. Lee, C. Wang, M. DeMaria, R.P. Johnson, G. Cohen, J. Jung, (2000) *Inhibition of Natural Killer Cell-Mediated Cytotoxicity by Kaposi's Sarcoma-Associated Herpesvirus K5 Protein*, Immunity, vol. 13, pgs. 365-374.

[3] L. Coscoy, D. Ganem, (2000) *Kaposi's Sarcoma-Associated Herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis*, PNAS, vol. 97, pgs. 8051-8056.

[4] E. Bartee, M. Mansouri, B. Hovey Nerenberg, K. Gouveia, K. Frueh, (2004) *Downregulation of Major Histocompatibility Complex Class I by Human Ubiquitin Ligases Related to Viral Immune Evasion Proteins*, J. of Virology, vol. 78, no. 3, pgs. 1109-1120.

[5] A. Ensser, M. Thurau, S. Wittmann, H. Fickenscher, (2003) *The genome of herpesvirus Saimiri C488 which is capable of transforming human T cells*, Virology, vol. 314, pgs. 471-487.

[6] E. Hewitt, L. Duncan, D. Mufti, J. Baker, P. Stevenson, P. Lehner, (2002) *Ubiquitination of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation*, EMBO, vol. 21, no. 10, pgs. 2418-2429.

[7] M. Haque, J. Chen, K. Ueda, Y. Mori, K. Nakano, Y. Hirata, S. Kanamori, Y. Uchiyama, R. Inagi, T. Okuno, K. Yamanishi, (2000) *Identification and Analysis of the K5 Gene of Kaposi's Sarcoma-Associated Herpesvirus*, J. of Virology, vol. 74, no. 6, pgs. 2867-2875.

[8] M. Lorenzo, J. Jung, H. Ploegh, (2002) *Kaposi's Sarcoma-Associated Herpesvirus K3 Utilizes the Ubiquitin-Proteasome System in Routing Class I Major Histocompatibility Complexes to Late Endocytic Compartments*, J. of Virology, vol. 76, no. 11, pgs. 5522-5531.

[9] R. Means, S. Ishido, X. Alvarez, J. Jung, (2002) *Multiple endocytic trafficking pathways of MHC class I molecules induced by a Herpesvirus protein*, EMBO, vol. 21, no. 7, pgs. 1638-1649.

[10] D. Sanchez, L. Coscoy, D. Ganem, (2002) *Functional Organization of MIR2, a Novel Viral Regulator of Selective Endocytosis*, J. of Biological Chemistry, vol. 277, no. 8, pgs. 6124-6130.

[11] N. Zheng, P. Wang, P. Jeffrey, N. Pavletich, (2000) *Structure of a c-Cbl-UbcH7 Complex: RING Domain Function in Ubiquitin-Protein Ligases*, Cell, vol. 102, pgs. 533-539.

[12] L. Coscoy, D. Ganem, (2001) *A viral protein that selectively downregulates ICAM-I and B7-2 and modulates T cell costimulation*, J. of Clinical Investigation, vol. 107, no. 12, pgs. 1599-1606.

[13] D. Corda, C. Hidalgo carcedo, M. Bonazzi, A. Luini, S. Spano`, (2002) *Molecular aspects of membrane fission in the secretory pathway*, CMLS, vol. 59, pgs. 1819-1832.

[14] V. Chau, J. Tobias, A. Bachmair, D. Marriott, D. Ecker, D. Gonda, A. Varshavsky, (1989) *A Multiubiquitin Chain is Confined to Specific Lysine in a Targeted Short-Lived Protein*, Science, New Series, vol. 243, issue 4898, pgs. 1576-1583.

[15] K. Cadwell, L. Coscoy, (2005) *Ubiquitin on Nonlysine Residues by a Viral E3 Ubiquitin Ligase*, Science, vol. 309, pgs. 127-130.