An Analysis of the Unique Cytoplasmic Localization of Porcine Circovirus 1

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By

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Abstract:

PCV1-VP3 and its homologues in the *Circoviridae* family have the ability to selectively differentiate between normal and transformed cells. They induce apoptosis in cancerous cells through a possible CRM1-independent fashion. However, PCV1-VP3 is unique in the fact that it is localized primarily in the cytoplasm while its homologues, namely Apoptin, are not. In an attempt to shed light on this unique quality of PCV1-VP3, it will be forced into the nucleus via an SV40 NLS and its killing capacity will be compared to that of Apoptin under the same nuclear localized conditions. Possible explanations for the unique localization will then be provided and possible future experiments to test these methods will be provided in an attempt to greater understand PCV1-VP3, which could ultimately create an easier and more viable form of cancer treatment due to its p53 and CRM1 independent nature of apoptosis.

Acknowledgements:

I would like to thank the advisor of my project, Destin Heilman for all the effort he has put forth in shaping me into the scientist I am today. His knowledge in the field and cultivation of proper learning techniques has bolstered my love of science and convinced me that I have made a correct choice in pursuing a career in biochemistry.

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1. Background:

1.1 A Brief Overview of Cancer and its Mechanics

Cancer is classified as a group of diseases that involve abnormal cell growth and contain the ability to not only spread to other areas of the body, but to metastasize. Over one hundred different types of cancer affect humans, with many different factors affecting a person's possibility of developing any kind; genetic history, diet, cigarette use, and certain infections such as hepatitis B. Cancer is typically coupled with many types of genetic mutations that cause the cell cycle to continue indefinitely. This is followed by a change in the cellular and extra-cellular mechanics of the cells in question. Due to these changes cancer becomes, "a disease rooted in the dis-regulation of cellular signaling pathways that control cell proliferation and apoptosis," (1). In order for the dis-regulation of the cellular signaling pathways to occur, mutations in the genes that code for important tumor suppressors must occur. One of the most vital genes is that which codes for the p53 tumor suppressor. Inactivation of this gene is almost always indicative of a type of cancer developing.

1.2 Cancer Treatment and the p53 Tumor Suppressor

Due to the overwhelming prevalence of cancer amongst humans, treatment, and the research there of, is of vital importance. There are many types of treatments involved, depending on the type and severity of the cancer, namely: surgery, radiotherapy, chemotherapy, and immunotherapy, as a few examples. With the

advent of more advanced techniques for treating cancers, proper records must be maintained. To maintain proper records, “Minimum sets of patient and tumor characteristics are necessary for identification of the patient population under therapy,” (2). This ensures that reproducible treatments can be shared amongst different institutions.

When the p53 protein binds DNA, it can activate the transcription of genes and actively works to stop genomic rearrangement. The primary mutation that develops into cancer is one that involves this tumor suppressor with, “The majority of the mutations occur(ing) in the core domain which contains the sequence-specific DNA binding activity of the p53 protein (residues 102-292), and they result in loss of DNA binding,” (3). Due to missense mutations and/or deletions of p53 alleles, the tumor suppressor cannot perform its job of maintaining genome integrity. Not only does the p53 lose its function as a suppressor when it mutates, but many times it gains functions that help the tumors proliferate. This makes the current treatment, a type of chemotherapy that works off of the p53 tumor suppressor, much less effective. Addressing this problem means having a deeper understanding of the export method p53 is a part of, namely, the CRM1 pathway.

1.3 CRM1 (Exportin 1) Localization

Chromosomal maintenance 1 (CRM1), otherwise known as exportin 1, is a primary export protein that moves RNA and other such macromolecules across the nuclear membrane and into the cytoplasm. It was first observed in the yeast *Schizosaccharomyces pombe* and was found later to inhibit the nuclear export of Rev

snRNA's and command the localization of MAPK and cyclin B. "In mammalian cells, CRM1 was found to bind several nuclear pore proteins hence its role in nuclear-cytosolic transport was discovered" (8). In addition, CRM1 binds to Ran-GTP, which in turn causes the binding and subsequent nuclear export of many different suppressors and oncoproteins. One such suppressor is p53, making CRM1 an integral export protein in the elimination of tumor cells, and thus, in important protein for cancer research. In order to create a more viable form of cancer treatment that can work around these mutations, the method in question must be able to bypass the use of p53 and the CRM1 pathway. One such method could result from PCV1-VP3 and its homologues, which is theorized to engage in apoptosis of transformed cells in a CRM1 independent fashion.

1.4 A history of PCV/Apoptin and their Localization

Porcine circovirus, or PCV, is a member of the virus family known as Circoviridae. They are, "small-nondeveloped DNA viruses containing a unique single-stranded circular genome," (4). Through years of research into these viruses, two strains have been documented and observed: PCV1 and PCV2. PCV1 contains 1.759 kilobases of genetic material in its genome, while PCV2 has 1.766 kilobases. Once a pig is infected with type two, porcine circovirus associated disease and more recently, post weaning multisystemic wasting syndrome (PMWS) occurs. Type one on the other hand, readily infects, but does not cause disease in swine. Apoptin is the third viral protein (VP3) that is derived from chicken anemia virus (CAV) that specifically kills tumor cells while remaining inactive in non-cancerous cells. It is

comprised of 121 amino acids and contains both an NLS and an NES, which facilitates movement of the protein in and out of the nucleus depending on the type of cell it resides in. "In tumor cells, apoptin causes the nuclear accumulation of survival kinases including Akt and is phosphorylated by CDK2," (5). This causes the cell in question to undergo apoptosis instead of promoting cell survival.

The first cases of virulent PCV2 were noted in North America and Europe around the early 1990s. Since then, the disease has been discovered in pigs in each of the major continents of the world. One of the main concerns around the discovery of this disease and any other type of livestock related disease is the possible transmission into the human population. Pigs in particular are of major importance to study as they harbor, "a wide range of viruses: arboviruses, circoviruses, flaviviruses, herpesviruses, nidoviruses, orthomyxoviruses, paramyxoviruses, and picornaviruses," (6) which causes many problems in the maintenance of the overall swine population.

Due to the devastation of the swine industry from this disease, analysis of the mechanisms involved in infection has been researched. PCV1 and PCV2 are theorized to share similar mechanisms due to both being localized in the cytoplasm of tumor cells. Contrary to this, apoptin is located in the cytoplasm of normal cells but is transported into the nucleus of tumor cells. This difference in localization is atypical when homologues are concerned. Due to this, many question whether apoptin kills in the nucleus or if it is simply localized there after performing its job in the cytoplasm. By using PCV as a medium, upregulation of p53 expression can occur, causing a halting of cell division and further resulting in virus induced

apoptosis. Apoptin on the other hand, can facilitate apoptosis even in the absence of p53, making it a vital protein of study.

With the advent of many tumors causing a mutation in the p53 suppressor, research into a CRM1 independent form of localized tumor suppression becomes vital. One-way to verify whether the specific localization is CRM1 dependent or not is to inhibit CRM1 using leptomycin B. PCV1-VP3 not only suppresses independent of p53, but through CRM1 inhibition, it is theorized that the third viral protein also engages in cytoplasmic localization for apoptosis in a CRM1 independent fashion.

1.5 Goal

This paper works to shed light to this issue of the PCV homologues and apoptin seemingly having different localizations in tumor cells. First, PCV1 will be forced into the nucleus by the addition of a nuclear import sequence (SV40) to a template of PCV1-GFP. Then the circovirus' function in the nucleus will be analyzed to observe whether it acts the same as its homologue, apoptin. By analyzing PCV1-GFP's specific killing capacity, an explanation for the method of CRM1-independent cytoplasmic localization will be surmised. By using PCV1 and apoptin as a medium for the treatment and elimination of cancer cells, the need for direct p53 facilitation is eliminated, and a much more simple and viable approach to the future of fighting cancer could be realized.

2. Materials and Methods:

2.1 Restriction Digests and PCR (primer sets 1-3)

A sample of wild type PCV1-GFP was first restriction digested using 1 uL of BamH1 for 1 hour at 37°C. Then an around the world PCR with complementary primers (figure 3-5) was used to insert an SV40 NLS to various sites around PCV1. The PCR began with an initial denaturation at 98°C for 10 seconds. Then, 25 cycles of the following were performed: a denaturation at 98°C for ten seconds, an annealing step at 50°C for 30 seconds, and an extension at 72°C for 6 minutes. To finish the PCR, a final extension was performed at 72°C for 2 minutes. The product was then restricted using 1uL of DPN1 for 1 hour at 37°C.

2.2 Double Restriction Digest (oligos fig. 6)

A double restriction was performed to cut a 20 base pair section of the DNA out between the Bgl2 and EcoR1 restriction sites. To do this a solution was made containing 5 uL Buffer D, 3 uL of PCV1-GFP wild type, 2 uL EcoR1, and 2uL of Bgl2. The solution was brought up to 50uL using ddH_20.

2.3 Agarose Gel Electrophoresis

To analyze the DNA after PCR or double restriction digest, gel electrophoresis was performed. To make the gel, 50 uL of 1X TAE was added to a flask containing .5g of Agarose. The solution was microwaved for 1 minute followed by 3 minutes of cooling. Finally, 3uL of ethydium bromide was added and it was poured into the electrophoresis apparatus. A comb was inserted into the gel and the

gel was subsequently allowed to solidify for 20 minutes before the combs removal. The apparatus was filled with 1X TAE and three different lanes of the gel were filled. The lanes used were 1,3, and 5 with lane 1 containing 10 uL of quick load purple 2-log DNA ladder. Lanes 3 and 5 contained 10 uL of the PCR-DPN1 product with 2 uL of loading dye and 10 uL of PCR or restriction sample. The gel was run for 55 minutes and then analyzed. The portion of gel containing the band related to the double restriction digest was carefully cut out for purification.

2.4 Purification of the PCR/ double restriction product

After the PCR was restricted using DPN1 or directly after the double restriction digest, the product was purified. First membrane binding solution was added to the products based on the volume of PCR product or the mass of gel slice recovered from the Agarose gel (double digest gel). Then this solution was transferred into an SV minicolumn in a collection tube and was purified through multiple rounds of centrifugation after incubating at room temperature for 1 minute. First the minicolumn was centrifuged at 16,000x g for 1 minute and the liquid in the collection tube was then discarded. Then the column was washed using 700 uL of membrane solution 95% ethanol and the column was once again centrifuged at 16,000x g for one minute and emptied as before. The wash was repeated with 500 uL of the same membrane wash solution. The column was then centrifuged again, this time for 5 minutes at 16,000x g. The collection tube was emptied and the column went through another centrifugation at 16,000x g for 1 minute. The minicolumn was transferred to a clean 1.5 uL microcentrifuge tube and

50 uL of nuclease free water was added directly to the center of the column. The tube was incubated at room temperature for 1 minute and a final centrifugation was performed for 1 minute at 16,000x g.

2.5 Gibson Assembly (primer sets 1-3)

Gibson Assembly was then performed directly on the purified PCR product. To perform the Gibson Assembly, on ice, 3 uL of the purification product was added to 10 uL of Gibson Assembly Master Mix and 7 uL of ddH_20. This was incubated at 50°C for 45 minutes.

2.6 Oligo Annealing and Ligation into Restricted PCV1 GFP

The oligos were annealed prior to ligation in order to ensure greater success in binding to the Bgl2 and EcoR1 sites. To do this, 5 uL of each oligo was placed into a 1.5 mL tube and heated at 95°C for 5 minutes on a heating block. Then the block was removed and allowed to naturally cool to 37°C. Following this step, 1 uL of polynucleotide kinase was added to a tube containing 20 uL of the annealed primers and 5 uL of reaction buffer in order to re-phosphorylate the 5' end of the oligo. The reaction was brought up to 50 uL and incubated at 37°C for 30 minutes. To ligate the now purified gel slice using the newly annealed oligos, a ligase solution was prepared containing 2 uL ligase buffer, 1 uL of the purified gel, 1 uL ligase (added last) and a varying amount of annealed oligos, ranging from 1 uL to 7 uL. The solution was raised to 20 uL and sat at room temperature for 25 minutes.

2.7 Transformation of the Gibson/Ligation Product into JM109

First, 1uL of the product was added to a tube containing JM109 chemically competent E. coli. The cells were then placed on ice for 30 minutes and then heat treated for exactly 45 seconds in a 37°C water bath. Directly after the head treatment, the cells were placed again on ice for 2 minutes. Four hundred fifty uL of LB media at room temperature was added to the transformation reaction. This was then incubated in a shaker for 1 hour at 37°C. Finally, 150 uL and 200uL of media were plated on kanamycin plates and incubated overnight at 37°C.

3. Results:

3.1 Overview

PCV1-VP3's unique cytoplasmic localization makes it a prime target of study amongst the *Circoviridae* family. The selective apoptosis in transformed cells that it engages in, coupled with the fact that it may do so in a CRM1 and p53 independent manner, only increases the need to study PCV1-VP3 as a possible form of cancer treatment. It would be able to avoid the mutation of the p53 tumor suppressor that is extremely prevalent in today's cancers. FOR PCV1-VP3 to be used as a medium for treatment however, its particular mechanism for selective apoptosis and its possible influences on healthy cells must be understood. In an attempt to shed light on this possible CRM1 independent method of nuclear transport, PCV1-GFP was to be

forced into the nucleus by use of an SV40 NLS to analyze if its selective apoptosis could still occur.

Each of the methods to be explained below were done in an attempt to successfully clone the PCV1-GFP with the SV40 NLS into JM109 competent E. coli. The subsequent primers and the oligo set were the result of a need for redesign after failed transformation. With proper verification after each step, the issues seemed to arise from either the Gibson Assembly or the final transformation. Even minute details such as a small change in volume added to plates, a change in volume of restriction enzyme added, or various dilutions of annealed oligos were attempted in order to achieve transformation.

In order to properly assess the killing capacity of PCV1-VP3 when forced into the nucleus, two different approaches were taken to ensure that the SV40 NLS was carried over into JM109 competent E. Coli. The first method utilized around the world PCR using three different primer sets that could work properly in the transformation. Following PCR, Gibson Assembly was used to assemble the DNA fragments. In the second method Linkers were utilized along with ligation in an attempt to remove a portion of the PCV1-GFP DNA and replace it with a section of around the same length containing the NLS. For each of these methods different trial of gel electrophoresis were performed in order to assess whether or not the desired digests occurred within the PCV1-GFP.

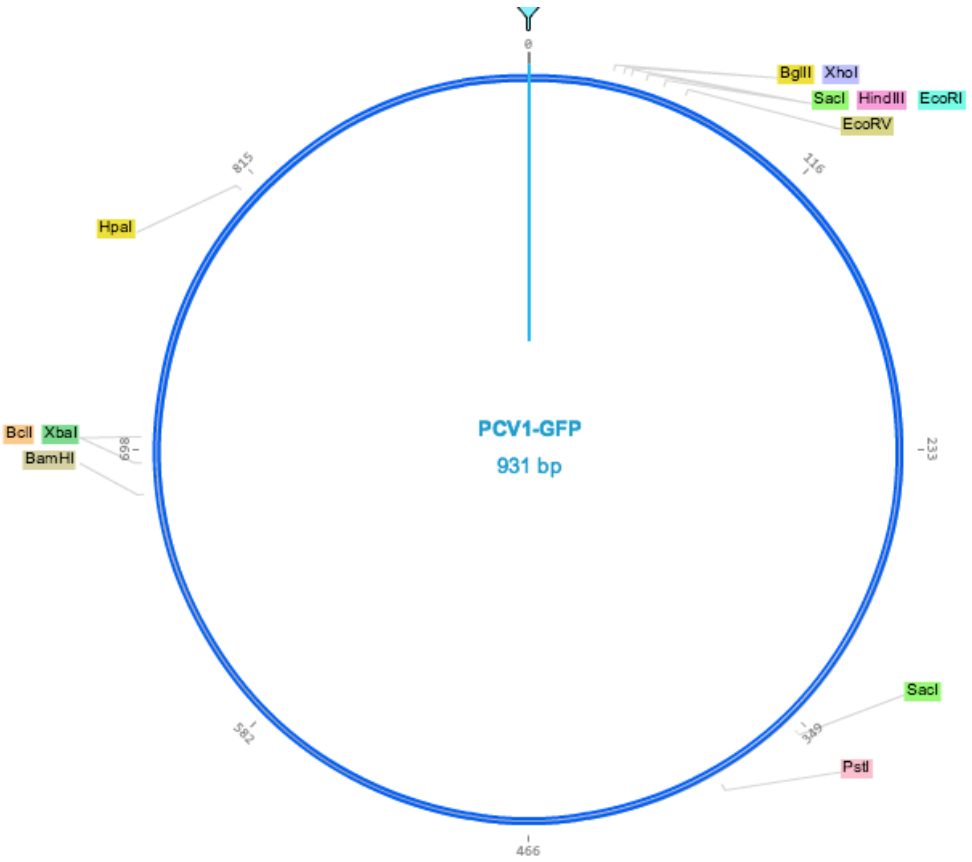

Figure 1: A diagram of the PCV1-GFP being used in the following experiments. The figure contains important restriction sites in the target as well as their general location.

For the first primer set (figure 3), both the forward and reverse primers were situated around the BamH1 site. This was done to ensure that a stop codon was present in both primers so extraneous overhangs could be omitted during the process. For the second primer set (figure 4), a different approach was taken. The forward primer was to be situated at the EcoR1 site while the reverse primer was to be situated adjacent to a stop codon by the BamH1 site. The third primer set (figure 5), adopted another different approach with the forward primer starting near the BamH1 site from the previous primer set. The reverse primer was situated at the

very beginning of PCV1 in the construct. Both were flanked by stop codons to eliminate overhangs.

3.2 PCR and Gibson Assembly

For the first three sets of primers, the Gibson Assembly was the primary method used in an attempt to achieve fully assembled DNA from different sized fragments. Each of the forward and reverse primers were situated by a stop codon in order to eliminate extraneous overhangs during the around the world PCR. Once the PCR was completed, restriction digests (BamH1 followed by DPN1 to remove methylated DNA) were performed on each of these first three primer sets. Finally, Gibson Assembly was used to assemble the purified fragments into fully assembled DNA. These were then ready to be transformed into JM109 competent E. Coli. For the PCR's the starting sites of each strand were chosen based on the above image (figure 1).

3.3 Choice of Linkers

In case of failure in transformation of the first three primer sets, a set of oligos using two unique linkers was created. When choosing the two sites, it was vital for the eliminated portion of DNA to be around the same length in base pairs as the SV40 NLS, so as to stay in frame. In this effort, EcoR1 and Bgl2 were chosen as the two linkers as the base pair removal was fairly close to the 27 base pair addition

that the linker plus NLS was to enact (figure 6). A depiction of the finished product with the SV40 NLS added is shown below (figure 2).

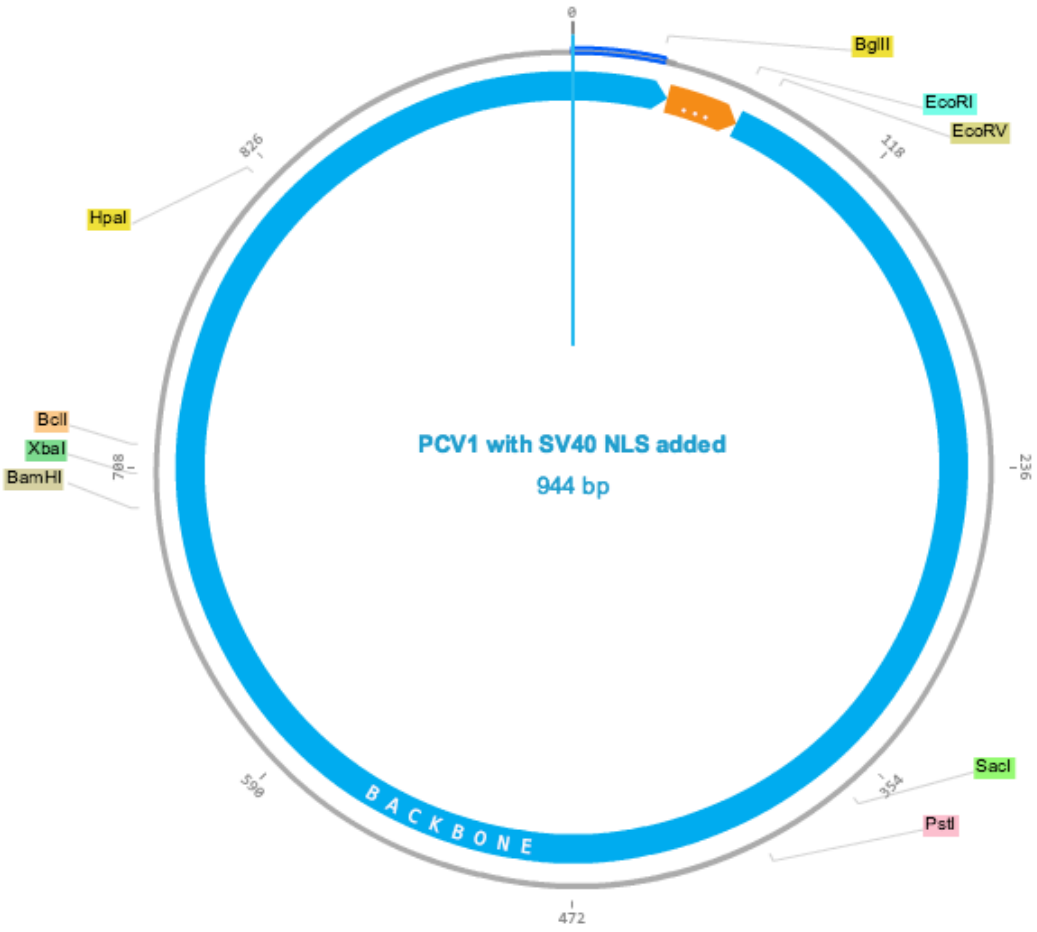

Figure 2: A diagram of the proposed PCV1-GFP with an SV40 NLS added (in orange). This is to be achieved through a set of oligos created using Bgl2 and EcoR1 as restriction sites. The two sites were chosen based on the SV40 NLS being a similar size to what would be removed by the double restriction digest.

3.4 Gel Electrophoresis

Over the course of these experiments, gel electrophoresis was used for a multitude of applications. When the primer sets (figures 3-5) were restricted using DPN1, gels were constructed to show that the restriction was occurring properly. Figure 7 below denotes this with a gel consisting of a pseudo PCR (contains all elements for PCR but not run) and a sample that went through PCR and was DPN1 treated to remove any excess template. The next utilization of gel electrophoresis was to ensure that the template PCV1-GFP was cutting correctly at various restriction sites. To determine this, a gel consisting of a number of samples of wild type PCV1-GFP cut by various restriction enzymes was constructed (figure 8). Furthermore, the annealed oligos (figure 6) were added to the far lane to ensure that the annealing was successful. The final use of gel electrophoresis in these trials was used on a previously purified gel slice using the oligos (figure 6) as a base. This gel (figure 9) was done as verification as to whether or not DNA remained following excision and purification of a previous gel slice.

4. Discussion and Localization Analysis:

Porcine circoviruses is notable due to its killing capacity when in reference to tumor cells. The mission of this study was to better understand the mechanisms of PCV1 in order possibly use it as a type of non-p53 dependent therapy to fight cancer. In order to better understand the mechanism behind porcine circovirus' CRM1 independent cytoplasmic localization, forcibly transporting it to the nucleus

was required. Attempts to analyze PCV1 and its killing capacity after nuclear localization are currently ongoing. The main issue that must be resolved moving forward is the method of cytoplasmic localization utilized by PCV1-VP3 if it is indeed CRM1 independent. In order to test further for CRM1 independent localization, the use of leptomycin B should be utilized to inhibit the protein. If localization still occurs independently, then a few different options for the overall mechanism present themselves.

4.1 Possible Manipulation of the NPC

One possible way in which PCV1-VP3 can engage in localization independent of CRM1 is by directly manipulating the nuclear pore complex. The main component to this method would be direct interactions between PCV1-VP3 and nucleoporins, thus bypassing the need for CRM1. One way that further research could check for this method is to determine whether or not ran-GTP is utilized during translocation of PCV1-VP3. Then in order to determine if manipulation of nucleoporins is occurring, an assay could be run to examine if localization occurs in PCv1-VP3 while nucleoporins are blocked. If so, then the localization could be fairly similar to the existing beta catenin where, "the occlusion of nucleoporins with wheat germ agglutinin (WGA) blocks the nuclear import of these signal transducers," (7). One of the peculiar aspects of beta catenin that makes it such an important protein of study is that it does not have an NLS typical to nuclear localized proteins. It was determined that importin beta and beta catenin use the same method of nuclear transport and furthermore engage in competitive inhibition with one another. Due

to these vital proteins accessing the nucleus due to direct nucleoporin binding, it is feasible that PCV1-VP3 could engage in the same transport. One way to verify this is to engage PCV1-VP3 through its entire cell cycle and analyze the nuclear pore complex for phosphorylation and glycosylation of its nucleoporins. Once the specific nucleoporins of transport have been identified (if any), it is possible to block them and determine if PCV1-VP3 transport is inhibited.

4.2 Microtubule Transport and Cytoskeletal Facilitation

This particular method has been known to occur in viruses and in proteins that regulate cancer, so it is feasible that this could be the substitute for CRM1 in PCV1-VP3's localization into the cytoplasm. Viruses such as HIV engage in interactions with microtubules, "As a regulatory mechanism to retain virus or viral gene products in the cytoplasm, but many more exploit the microtubular network for efficient nuclear delivery" (9). One of the driving forces in this method has been theorized to be the protein dynein. It has been known to facilitate the movement of particular proteins such as Rb and PTHrP along the microtubules by increasing the already present NLS's ability to perform its job. Rb and PTHrP interact with what is known as dynein light chains and these light chains then increase the overall efficiency of transport by interacting directly with the present NLS. Conversely, if one wishes to decrease nuclear import of these specific proteins, treatment with nocodozole; a depolymerizing agent for microtubules, has been known to achieve this result. The microtubules break down, and therefore cannot adequately transport the proteins across the nuclear membrane. This makes the overall process

fairly easy to control, allowing for accurate influence over import and export to and from the nucleus by using dynein and nocodozole.

In the case of cytoskeletal facilitation, when the actin cytoskeleton is in the presence of NF-KB, actin stress fibres form and aid in the importins task of nuclear transport by working directly with importins (primarily beta), which recognizes NF-KB. When previous studies tried to depolymerize and stabilize the actin using cytochalasin D and jasplakinolide respectively, there was no change on the accumulation of NF-KB in the nucleus. The main issue with these particular methods is that they mostly act to enhance traditional nuclear import and export through importins, exportins, and other NLS. For this method to be feasible, the actin-cytoskeleton or microtubule transport would need to facilitate enhanced movement to the nucleus, and then another method, such as manipulation of the NPC as stated above, would need to occur in order to bypass the need for importins and exportins. Furthermore, as will be stated below, there are transporters such as calmodulin that engage in competitive inhibition with Imp. beta, so it is possible to utilize transporters that are similar to this in order to achieve CRM1 independent transport.

4.3 Utilizing Transporters other than Importins

Besides importins, there exist other transporters that can provide the same job of nuclear shuttling, namely: calmodulin and calreticulin. Utilizing both of these is dependent on calcium concentrations within the cell. Calmodulin works as a nuclear export tool when high intracellular calcium is present and has its transport

dubbed the calmodulin dependent pathway. The calmodulin will work in tandem with sex- determining region Y (SRY) protein after a conformational change due to the high intracellular calcium levels. This conformational change allows the binding of calmodulin that beats out Imp B, thus allowing for the desired import method by transport through nuclear pores. The exact method of calmodulin transport is still under debate, but could be resolved with the discovery of a calmodulin-responsive effector protein to drive the transport. Although, facilitated diffusion appears to allow calmodulin access to the nucleus according to previous literature. To test for this method, future experiments should involve differing levels of calcium and an analysis of whether nuclear import occurs.

Calreticulin on the other hand, acts as the nuclear export agent in this method and it does this by binding to glucocorticold receptor (GR). Previous work has shown that GR starts in the cytoplasm, is imported to the nucleus, and then is exported again in a CRM1 independent fashion, as this occurred even with the addition of leptomycin B. As with the previous import with calmodulin, calreticulin export requires high amounts of intracellular calcium to occur. This allows calreticulin to bind to nuclear export substrates resulting in a, "Conformational change allowing for the interaction between calreticulin and the GR," (9). Interestingly enough, GR export is facilitated by release of calreticulin from the endoplasmic reticulum, so this makes the binding under correct conditions and export fairly easy to control with various intracellular calcium concentrations. Future tests should be careful when isolating calreticulin mediated export however, as some interactions, such as its interaction with TRα, works in tandem with CRM1 to initiate the export. Although, with proper calcium

regulation, discerning if this is indeed the method through which PCV1-VP3 performs localization and transport is highly feasible.

5. Figures:

Forward

5’ [CCGAAAAAGAAACGTAAAGTA]TGAACCGGATCTAGATAACTGAT 3’

Reverse

5’ ACT[TACTTTACGTTTCTTTTTCGG]GTGAAAATGCCAAGCAAGAA 3’

Figure 3: The forward and reverse primers used to add the SV40 NLS (in brackets) to the PCV1-GFP construct. The annealing sites for the primers are situated around the BamH1 site.

Forward with EcoR1

5’ GAATTCAATGATATCCATCC[CCCGAAAAAGAAACGTAAAGTA]TGA 3’

Reverse adjacent to stop codon

5’ [TACTTTACGTTTCTTTTTCGG]TCAGTGAAAATGCCAAGCAAGAA 3’

Figure 4: A second set of primers used to add the SV40 NLS to the PCV1-GFP construct. The forward primer is based around the EcoR1 site while the reverse primer was situated adjacent to a stop codon near the BamH1 site.

Forward

5’ TTCTTGCTTGGCATTTTCAC[CCGAAAAAGAAACGTAAAGTA]TGA3’

Reverse

5’AGTGGTGGGATGGATATCAT[TACTTTACGTTTCTTTTTCGG]TCA3’

Figure 5: These primers were created with the forward being situated at the stop codon adjacent to BamH1 while the reverse primer was situated at the N-terminus of PCV1.

Forward

5’ GATCT[CCGAAAAAGAAACGTAAAGTA]G3’

Reverse

5’ AATTC[TACTTTACGTTTCTTTTTCGG]A 3’

Figure 6: A set of oligos were created using linkers situated at the Bgl2 site (forward) and the EcoR1 site (reverse). The 27 base pair addition in between the two sites are a suitable addition in place of the base pairs that are to be removed from the construct by restriction.

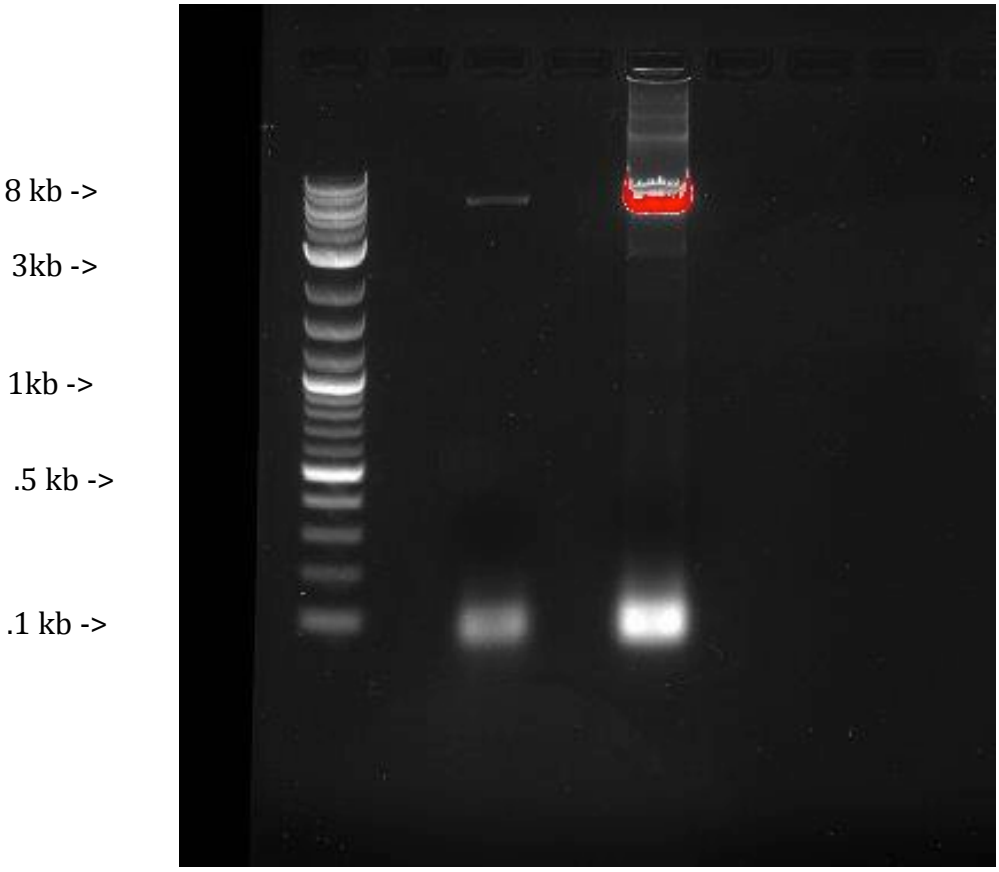

Figure 7: Gel Electrophoresis of a restriction digest involving PCV1-GFP using the primers from figure 3. Lane 1 denotes the ladder while lane 3 denotes a pseudo PCR (a PCR that was set up but not run). Lane 5 denotes a single digest involving DPN1. Lane 3 exhibits bands around 8kb and .1kb while lane 5 exhibits these same bands but at a higher intensity.

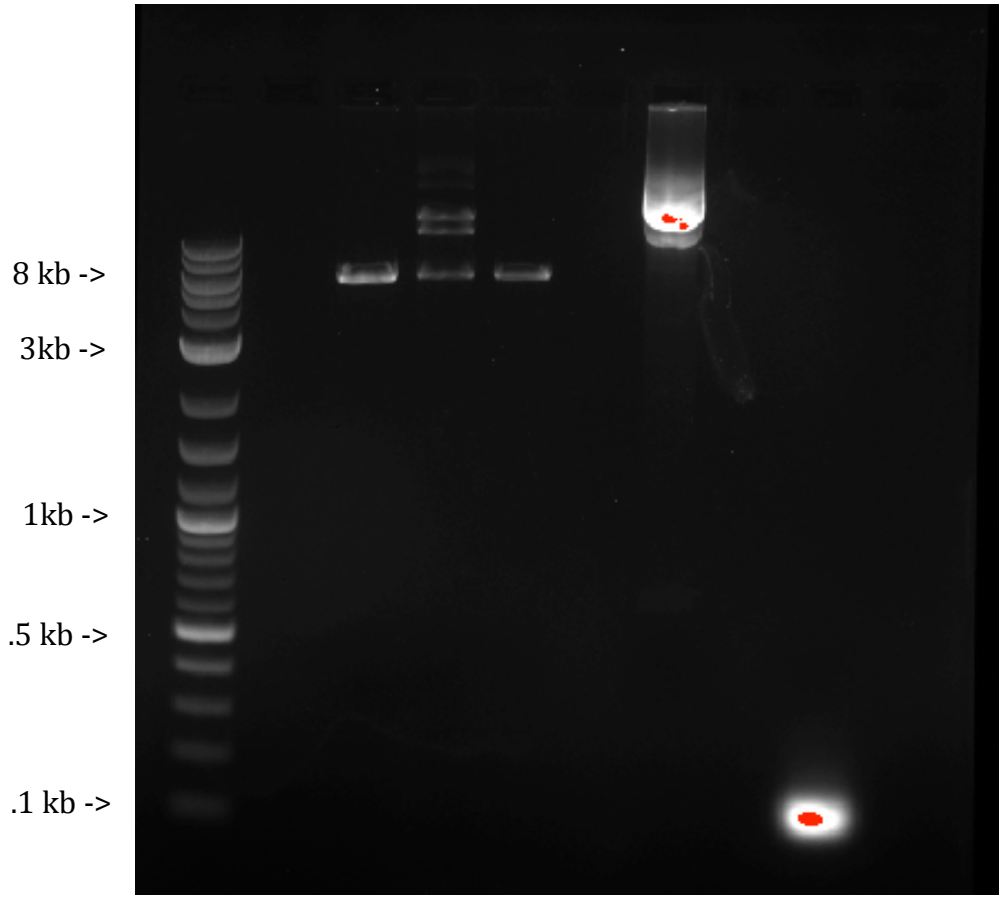

Figure 8: Gel Electrophoresis of varying restriction digests performed on PCV1-GFP wild type. Lane 1 denotes the ladder. Lanes 3, 4, and 5 are restrictions involving Bgl2, EcoR1, and then Bgl2 x EcoR1 respectively. Lane 7 denotes uncut PCV1-GFP wild type while lane 9 denotes the annealed oligos from Fig. 4. Lanes 3, 4, and 5 all show bands around 8kb while lane 4 shows additional bands around 10-11 kb. Lane 7 shows a high intensity band around 11kb, possibly hinting at concatenation being present in the WT. Lane 9 shows a high intensity band around .1 kb.

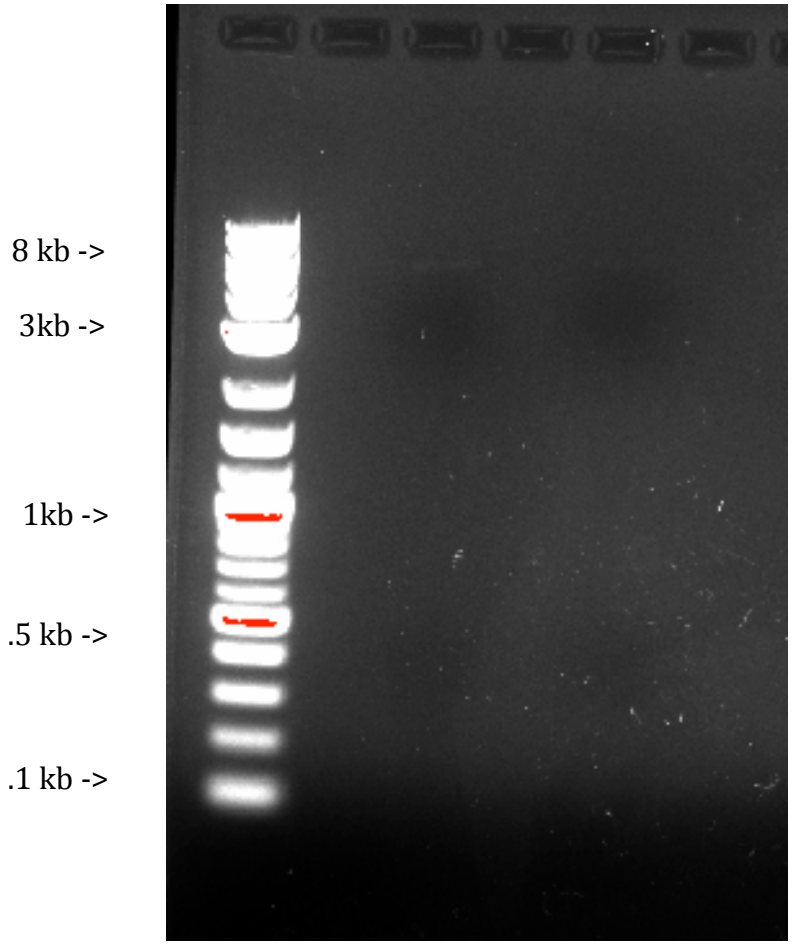

Figure 9: Gel electrophoresis of two different restriction digests utilizing purified gel slices from Fig. 6. Lane 1 denotes the ladder while lane 3 denotes a control Bgl2 restriction. Lane 5 denotes the restriction of interest; the Bgl2 x EcoR1 restriction. Both lane 3 and lane 5 show low intensity bands around 8kb, showing that there is DNA present after gel purification.

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