Exploring Multimerization and the Cancer-Killing Potential of Human Torque Teno Virus VP3: An Apoptin Analog

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By

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Maria Arvena DeCicco

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James Scott Schiemer

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Approved:

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Professor Destin Heilman, Primary Advisor

Abstract

The Human Torque Teno Virus (TTV) is a small non-enveloped anellovirus with a single stranded circular DNA genome. Ninety percent of the world's human population is infected with this virus with no known pathogenicity or observed adverse affects. One of TTV's homologs, Chicken Anemia Virus (CAV), produces a similar protein known as Apoptin, which has been shown to multimerize and induce apoptosis selectively in transformed cells. In order to further examine the role of multimerization, using molecular cloning, we created two VP3 containing T-vector constructs, cloning intermediates, which can be used in future studies of TTV VP3 specifically in the area of multimerization. Also, we have shown that TTV VP3 has the ability to induce apoptosis in transformed cells via a caspase 3/7 dependent pathway, similar to that of CAV Apoptin. It is important to continue to compare TTV VP3 with CAV Apoptin to provide insight into new biochemical strategies for fighting cancer.

Acknowledgements

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Introduction

The Torque Teno Virus

The Torque Teno Virus (TTV) was first discovered in 1997 during a representational difference analysis of sera from post-transfusion hepatitis patients [1]. The patient in whom it was first discovered had the initials TT for which the virus is named. However it has since been renamed in 1999 Torque Teno Virus, where “Torque Teno” is Latin for “thin necklace” as it is a small circular single stranded DNA [2]. Due to its high concentrations in post-transfusion non-A to G hepatitis patients it was first introduced as a novel hepatitis agent. TTV is the first human DNA virus to be single stranded and circular. Additionally it is non-enveloped, and negatively stranded [2]. It is a relatively small virus of approximately 3.8kb, however the prototypic TA278 virus is known to have 3.852kb sequence [3].

Virus families with single stranded circular genomes, like that of TTV, are given the names of Microviridae, Geminiviriae, Nanoviridae, Inoviridae, or Circoviridae. The chicken anemia virus (CAV), a related virus, has a negatively stranded genome without a 9-nt structure common to Circoviruses, and is thus categorized as a Gyrovirus in the Circoviridae family. TTV has a similar genome, however it is slightly larger, and its open reading frames (ORF) are antisense rather than ambisense [3]. Thus TTV was assigned to a relatively new genus known as the Anellovirus [4].

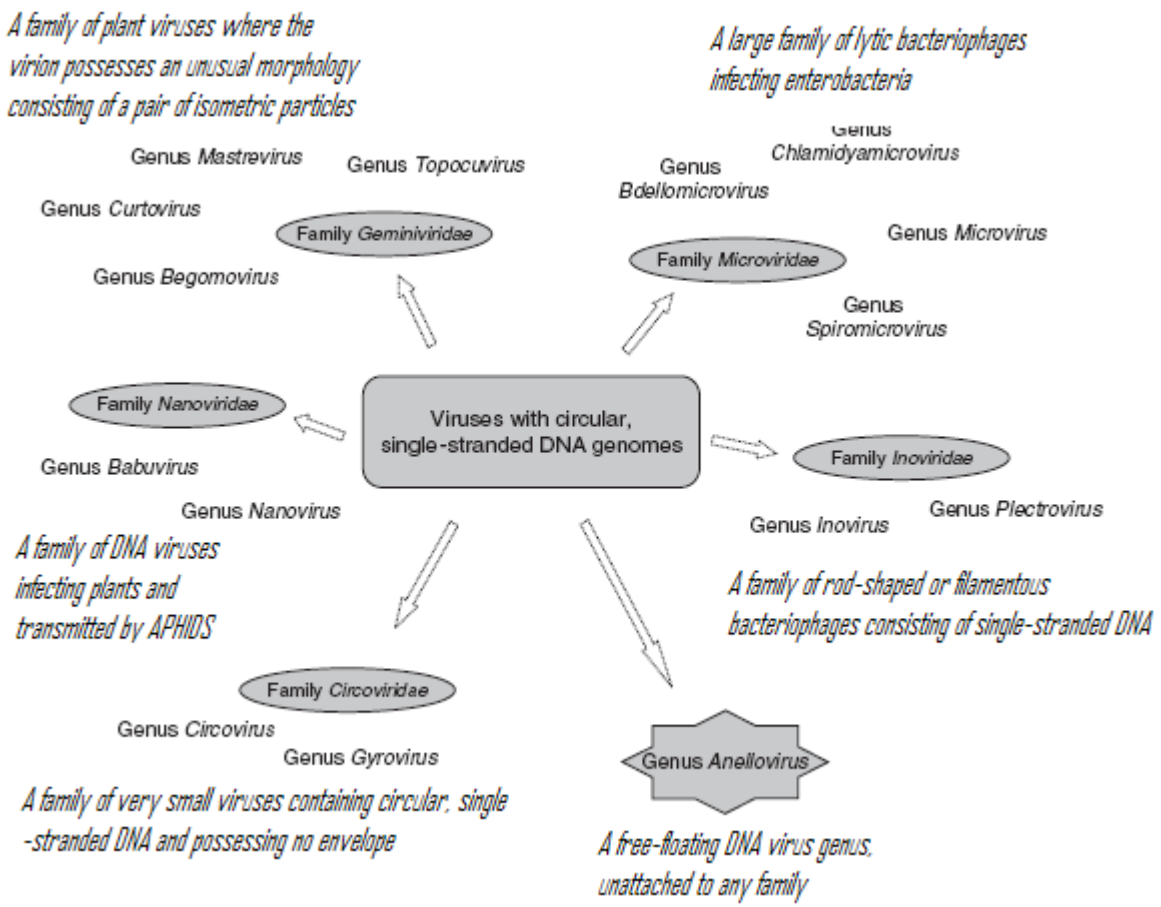

Figure 1 Official taxonomic description of viruses with circular, single-stranded DNA genomes [4]

Through epidemiological studies it was found that TTV has a large amount of genetic variability and is widespread. It was found to not be present in humans alone but in chickens, pigs, cows, sheep, chimpanzees, Japanese macaques, tamarinds, and owl monkeys [5, 6]. Additionally it was found to be present in over 90% of the human population. Though initially thought to be a novel hepatitis agent, there has been no evidence that can correlate it to human disease [5].

It has been found that the viral load of TTV in humans tends to be highest in the bone marrow, lungs, spleen, and liver tissues more than any other kind of tissue. Though TTVs

tropism is rather broad, it was found that most of its replication occurs in the liver [14]. It was also found that high concentrations of TTV are present in nasal secretions. Therefore it was proposed that entodermal epithelial cells may be another area of TTV replication. In addition to this, high concentrations of TTV are observed in pulmonary epithelium and may provide another area in which to replicate [23, 24].

In the bone marrow it is postulated that TTV may prefer the erythroid series because the promoter activity of TTV was most significant in K562 cells of erythroid origin [25]. The same can be said for the megakaryocyte series, because thrombocytopenia has been reported in TTV infected patients [25]. When looking at HepG2 and Huh7 it was observed that the promoter was more active in the HepG2 cells in the case of the group 1 TTVs, while more active in Huh7 cells than Hepg2 when group 3 TTVs were used. This shows a variable tropism among different TTV groups.

TTV Pathogenicity

Since its discovery, very little has been determined about its biological nature [7]. A review published in 2000 by Springfeld et al. posed three of the most important questions for TTV research: 'What is the significance of TTV as a human pathogen?' 'What is the origin and molecular relatedness of the TT virus?' and 'What are the exact molecular mechanisms of viral replication?' [8]. Of these questions only the second has been answered with any certainty. The reasons for a lack of knowledge in answering the first and third question is most likely due to a number of obstacles outlined by Hino and Miyata in their recent review of TTV. They postulate that due to its near ubiquity when its 20 genotypes all combined it is difficult to understand its pathogenicity and biological function. Additionally, no confirmed culture system

has been developed for growing TTV in the laboratory [9]. Desai et al reported that the Chang liver cell line could be infected with TA278 and its supernatant could be transferred into culture. However there is no data to quantitatively show this. Their only proof is pictures that suggest induction of a clear cytopathic effect, and therefore is not convincing [10].

Though little is known of its biological function or pathogenicity the means by which it is transmitted has been evinced. It is un-enveloped, and therefore it is found in the feces and can be transmitted via a fecal-oral pathway [11]. Additionally, it can be transmitted parenterally, and is typically via transfusion in patients with hemophilia and those on hemodialysis as well as intravenous drug users [12]. However, unlike the almost free viral particle seen in feces, TTV is complexed with immunoglobulin G and can therefore be imaged with electron microscopy.

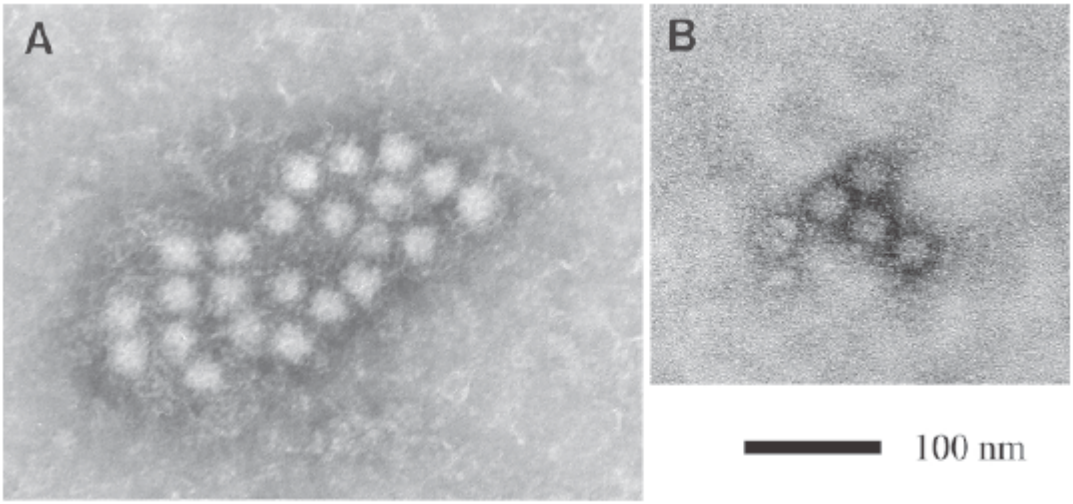

Figure 2: TTV aggregated in the blood with IgG [13]

Torque Teno Mini Virus

In 2000 Takashi et al discovered a single-stranded circular human DNA virus similar to TTV, and thus named it Torque Teno Mini Virus (TTMV) due to its smaller genome than TTV. It has a 2.9kb genome, and due to its similarity to TTV became known as an anellovirus as well.

Also like TTV, TTMV has an extremely diverse amount of genotypes, which are present in humans, often at the same time as TTV [26, 37]. Throughout its genome are nucleotide and amino acid sequences that are conserved in TTMV as well as TTV and CAV. Therefore many believe that this intermediate virus may unearth a great deal about the protein functionality and pathogenicity of both TTV and CAV [28].

Genetic Variability and Conservation Among TTV Genotypes

Despite the small size of the TT virus there are over 20 different genotypes of the TT virus and only 60% homology across all known genotypes [14]. This is a remarkable amount of diversity for such a small DNA virus, and is highly unusual. However phylogenetic analysis of full length or near full-length sequences reveals a division into 5 main groups. Group 1 is represented by the prototypical virus TA278, group 2 by US35, and group 3 by JA10, group 4 by TUS01, and group 5 by SANBAN [15, 16]. Most of the sequence variation occurs in the coding regions. It has been postulated that the high level of genetic variance is due to high levels of recombination, and may allow for a number of pathological differences among different groups [17].

However, despite such a large number of genotypes and groupings, the size of the viruses tends to be between 3.6kb and 3.8kb in length [16]. In addition, the UTR regions are highly conserved [18]. A key feature of this is a 36nt stretch that forms a stem-loop structure within an 113nt GC rich region. This allows for DNA replication to take place there [19]. Additionally the 3 ORFs coding for the putative proteins, as well as the mRNA strands derived from them, are conserved across all genotypes.

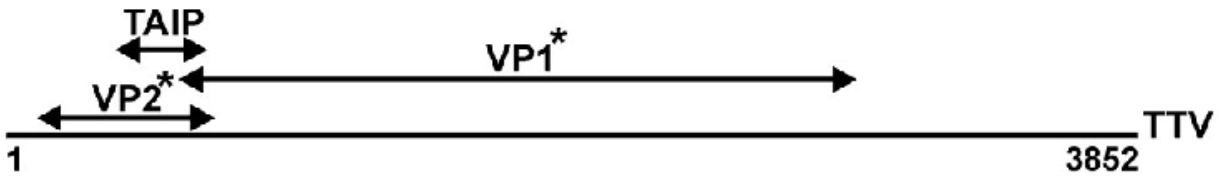

Figure 3: TTV proposed structure of TA278. Note the overlap of ORFs coding for VP2 and VP1 encoding TAIP [13]

It is known that the mRNA of the 3 open reading frames is approximately 3.0kb from ORF1, 1.2kb from ORF2, and 1.0kb in ORF3. It is the third mRNA strand coding for our protein of interest. It is known that each mRNA is polycistronic, and contains one ORF in each of the reading frames [9]. The ratio in which each mRNA strand is transcribed tends to follow the ratio of 65:5:35 for its first, second, and third reading frame respectively. It was originally thought to code for only one strand of mRNA, as it has only a single promoter, TATA box, and Poly-A signal [20, 21]. However Kamahora et al found the 3 separate mRNA strands, and discovered that the 3 ORFs overlapped but in different reading frames. It was found by Kamada et al that the region -154/-76 contains the critical element for the functioning of the TTV promoter [22]. The individual proteins expressed by these open reading frames are poorly characterized. However much of what is hypothesized about their function is based upon the well characterized proteins of the CAV, which shares much homology with TTV and will be discussed later.

Chicken Anemia Virus (CAV)

The Chicken Anemia Virus (CAV) serves as an interesting comparison for TTV. Unlike the other circoviridae, which have ambisense genomes, CAV's genome is antisense like TTV's. CAV's highly conserved genome encodes for three proteins VP1, VP2, and VP3. Previous studies have established that VP1 is partially responsible for the assembly of the capsid and VP2 has dual

specificity phosphatase (DSP) activity and may act as a scaffold protein in virion assembly [20, 32]. The third protein, VP3, also known as Apoptin, is the most interesting with regards to our study of TTV. This protein has been shown, in previous studies, to induce apoptosis in both chicken and human tumor cells independent of the p53 pathway [32]. In a study by Noteborn and others, they explained that the CAV protein VP3 first exhibits a granular distribution in the nucleus. As the infection progresses, VP3 forms aggregates causing the cell to become apoptotic [22].

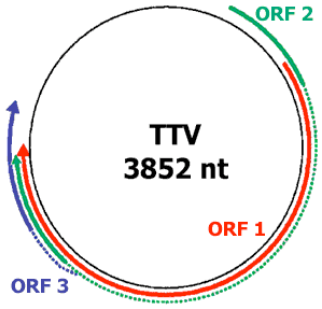

Figure 4: Map of TTV Genome showing the three overlapping Open Reading Frames. The ORF of interest in this study is ORF3.

Apoptin-CAV VP3

Apoptin is comprised of 121 amino acids containing two proline-rich regions and two basic regions [32]. It was also found using circular dichroism (CD) to lack alpha-helical structure and resembled proteins with unordered structure [29]. Apoptin is the protein of most interest in the study of torque teno virus as TTV encodes for a protein with similarity to Apoptin, VP3 also known as TAIP (TTV derived apoptosis inducing protein), which in preliminary studies by Noteborn et al in 2004 has shown the ability to induce apoptosis in cancerous cells [22]. As little is known about how TAIP functions, it is useful to consider Apoptin's established mechanism.

Cell Cycle and Regulation

Considering the like-like protein coded for by TTV is thought to selectively kill transformed but not primary cells, an understanding of what causes cells to become transformed is essential. Since very little is known of the means in which TTV induces apoptosis the following information should be kept in mind. The eukaryotic cell cycle is composed of 4 phases: G1, S, G2, and M. The G1 "gap" phase is the longest step in the cell cycle, typically consuming about 18 hours of a typical 24-hour cycle. The G1 phase is when a cell carries out its "normal" metabolic duties, such as energy metabolism and most protein synthesis. However terminally differentiated cells enter into phase known as G0, in which replication may never restart. Following the G1 phase, the S or "synthesis" phase is when DNA is replicated. Following this is the G2 phase, which is simply another gap phase. Following this is the Mitosis, or M phase, in which the cell undergoes chromosome separation, and finally cytokinesis into 2 cells.

Central to these phases are cyclins and cyclin-dependent kinases (Cdks). The cyclins are named as such because their concentration follows a cyclic pattern in each phase. Unique to each phase a cyclin will be present in large amounts and bind to Cdks to cause gene expressions unique to that phase. The cyclins then degrade as the phase comes to an end, and when a new phase begins additional cyclins unique to that phase are produced and degraded in the same way. In addition, Cyclin-dependent kinase inhibitors (CKIs) are present to halt the action of Cdks. These allow for the cell to inhibit cells from entering a new phase if it is dangerous for the cell to do so. These are negative controls that allow the cell to avoid entering a cancerous state.

Another means of protecting against uncontrolled growth is the p53/Mdm-2 cell cycle regulation. The p53 protein is an anti-tumor agent acting as a roadblock to many cancerous pathways. It is able to arrest the cell cycle in the case of pathway signals becoming dangerous

for the cell. If it finds the damage to the cell cannot be repaired it will induce intrinsic apoptosis. Often the cell is given a chance to repair itself during the arrested state. Mdm-2 on the other hand, is a p53 regulatory element, which ligates p53 with a tag that ultimately ends in the destruction of the protein.

Cell Death

In addition to understanding the role the cell cycle plays in cancer, it is also important to understanding how cells die and its role in the cell cycle. There are 2 ways in which a cell can die. The first is necrosis, in which the cell suffers a sort of trauma. This can be due to toxicity, passive degeneration, or some kind of mechanical stress in which the membrane is no longer functioning correctly. This is followed by the surrounding environment to flood in, destroy what is left of the membrane, and trigger lytic enzymes to degrade the cell.

The second way in which a cell dies is via apoptosis. However this can happen in one of two ways: intrinsic and extrinsic pathways. As the names suggest, intrinsic pathways occur when the cell takes it upon itself to induce apoptosis, whereas extrinsic pathways are caused by an outside cell that directs the target cell to commit to apoptosis through the interaction of membrane proteins. This causes a cascade of reactions ultimately leading to apoptosis. Apoptosis also differs from necrosis by the manner in which the cell dies. Apoptosis is characterized by cell shrinkage, condensation of chromatin, fragmentation of nuclei, and fragmentation of chromosomal DNA. After these events take place, the cell fragments form into membrane-enclosed bodies and are phagocytised by neighboring cells.

The first virus found to induce apoptosis directly was an adenovirus mutant (E1B-19K). It was found that this virus induces apoptosis in the infected cells. Since its discovery there have

been a number of additional viruses, both RNA and DNA, which can induce apoptosis in the cells it infects. However the mechanism by which apoptosis is induced differs among DNA and RNA viruses. DNA viruses will induce apoptosis if their genome acquires an unfavorable mutation and therefore does not want to have its progeny carry this on. RNA viruses simply induce apoptosis allowing for sufficient replication [33].

There are viruses that are able to selectively induce apoptosis in cancer cells while leaving most normal cells untouched. They also induce apoptosis independently of the p53 tumor-suppressor gene, which is important since this pathway is mutated in approximately half of all cancers [33]. The CAV is known to act in a p53 independent pathway as well. Therefore if TTV acts in a similar manner it may shed some light on these apoptotic mechanisms occurring selectively in cancer cells.

Apoptin's NES and NLS

Apoptin contains a nuclear localization sequence (NLS) located at the C-Terminus between residues 80-121. Heilman and others, through a point mutation study concluded that this bipartite NLS was functional [30]. In addition to the NLS, Apoptin also contains a leucine-rich nuclear export sequence (LR-NES) located at the N-Terminus between residues 33-46. Although several sites were thought to contain the NES of this protein, another point mutation study performed by Heilman and others established the location of this functional NES [33].

Nuclear Import and Export of Apoptin

Nuclear import and export is mediated by nuclear pore complexes (NPCs) and aided by karyopherins (kap). Kaps mediate both the import and export of Apoptin through these NPCs, as they are required for molecules larger than 50kDa. Importins (IMPs) and exportins are

responsible for the import and export respectively of molecules [31]. It has been shown that Apoptin can be imported into the nucleus via the IMP 1 protein through the Ran-GTP cycle. IMP 1 binds to Apoptin's NLS in the cytoplasm allowing VP3 to dock to the NPC. Apoptin is then translocated into the nucleus and Ran-GTP binds to IMP1 releasing Apoptin [31]. The exportin CRM1 utilizes this same Ran-GTP cycle to facilitate nuclear export. CRM1 binds to the NES of Apoptin causing it to dock once more to the NPC and be subsequently transported from the nucleus to the cytoplasm. The GTP is then hydrolyzed to GDP causing CRM1 to release Apoptin [31].

Apoptin Nuclear and Cytoplasmic Shuttling (NES and NLS)

In order for Apoptin to induce apoptosis, a shuttling mechanism is required. In normal cells, Apoptin is found mainly in the cytoplasm. However, in tumor cells, Apoptin is found in the nucleus prior to inducing apoptosis [30]. Having Apoptin present in the nucleus is not enough to induce apoptosis, however as Heilman, et al proved. When nuclear export was inhibited by leptomyosin B in tumor cells, Apoptin still localized in the nucleus, however its apoptotic ability was removed. The same was true when Apoptin was artificially localized in the nucleus of normal cells [30]. It is still unclear how this nucleo-cytoplasmic shuffling is regulated in tumor and normal cells, however one possibility is that multimerization is related as Apoptin's multimerization domain overlaps with its NES.

Multimerization of Apoptin

As discussed in the sections above, nuclear localization is important, but cannot induce apoptosis on its own. In addition to nuclear localization, the formation of soluble 30-40 subunit Apoptin multimers is thought to be required to induce apoptosis. Apoptin has a multimerization

domain between amino acids 33-46, which overlaps with its NES. In 2003, Leliveld et al. hypothesized that residues in the NES between Glu^{32} and Leu^{46} could fold as an anti-parallel beta sheet with Ala^{38} and Gly^{39} to form a beta-turn or a hairpin. This would result in the hydrophobic amino acids oriented on one side of the hairpin and hydrophilic residues on the other. This could result in Apoptin's ability to form multimers [29]. In primary cells it has been shown that Apoptin forms large insoluble aggregates localized within the cytoplasm, whereas in transformed cells smaller soluble 30-40 subunit multimers are seen localized in the nucleus. This difference in solubility of Apoptin multimers and its effect on localization within primary and transformed cells provide valuable clues as to its apoptotic pathway, as well as a valuable comparison for other viral homologs. The TTV VP3 has been shown to have markedly different distributions within transformed cells than Apoptin. At 24 hours the GFP tagged protein was shown to exist seemingly without preference in both the nucleus and the cytoplasm. At 48 hours, the GFP tagged protein was free of nuclear localization; appearing as punctate aggregates around the nuclear and cytoplasmic periphery [36]. This suggests that TTV VP3 may induce apoptosis independent of APC/C recruitment or that perhaps its nuclear concentration is insufficient. The important point of this is that TTV VP3 appears to have a functional NES, and its punctuate foci suggest it may form soluble multimers in transformed cells; two important aspects of Apoptin's apoptotic mechanism. In the study of TTV, if we examine its multimerization potential as well as solidify the location of its NES, we may be better able to understand this virus.

Materials and Methods

TTV VP3 Construct Design

Primers were designed based on those used in a previous study of TTV VP3 by M. Orme-Johnson and E. Evan-Browning. A few modifications were made to accommodate different restriction sites applicable to our studies. The primer design was based on the GenBank accession number (AB008394) and sequence published by Noteborn et al. for TTV VP3.

TTV VP3 Construct design **- For Cloning into pGBKT7 Vector**

TTV VP3 Sequence, 40bp Oligonucleotides	**Contains**
1. **5'** gc**G AAT TC**a ***atg*** atc aac act acc tta act ggc aat ggt **3'**(*39 nt*)	**1.EcoRI restriction site, 2.start codon**

TTV VP3 Reverse Compliment, 40bp Oligonucleotides	**Contains**
1. gc**GGATCC**gtct agcaggtctgcgtcttcgggtcctccag	**1.BamHI restriction site**

TTV VP3 Construct design **- For Cloning into pACT2 Vector**

TTV VP3 Sequence, 40bp Oligonucleotides	**Contains**
1. **5'** gc**G GAT CC**a ***atg*** atc aac act acc tta act ggc aat ggt **3'** (*39 nt*)	**1.EcoRI restriction site, 2.start codon**

TTV VP3 Reverse Compliment, 40bp Oligonucleotides	**Contains**
1. gc**CTCGAG**gtct agcaggtctgcgtcttcgggtcctccag	**1.XhoI Restriction Site**

Figure 5: Oligonucleotides used for PCR-directed gene synthesis, forward and reverse complement.

The following PCR protocol was used: |95°C, 4min (denature), 1x| [95°C, 30sec] [55°C, 30sec] [72°C, 45sec], 25x| 72°C, 4min, 1x| 10°C, ∞|. The PCR products were separated using agarose gel electrophoresis using TAE (0.04M Tris-HCl, 1 mM EDTA, 57.2µL glacial acetic acid) and 9% agarose gel. The products were purified from the agarose gel using a Promega Wizard PCR Preps DNA Purification System (cat. #A7170).

Molecular Cloning

The PCR products were cloned into a Promega pGEM-T Vector (cat. # A362A) following the standard protocol. Chemically competent DH5α E. coli were chemically transformed with the ligated T-vector. Cultures were grown on agar plates containing 0.1 mg/mL ampicillin plates with 40µg/mL x-gal, and 0.1mM IPTG overnight at 37°C. White colonies were selected and 3mL of media was inoculated with each selected culture. These were allowed to incubate overnight. Plasmid DNA was purified according to standard alkaline lysis protocols (0.2%N NaOH, 1% SDS). Chemically competent DH5α E. coli were transformed with the purified plasmid DNA and incubated in 100mL of LB media overnight with shaking. Plasmid DNA was purified using Promega Wizard Midipreps DNA Purification System (cat #A7640). The gene insert was cut out of the T-vector using restriction digest with BamHI and EcoRI for the pGBKT7 insert or BamHI and XhoI for the pACT2 insert. Restriction digests were performed on the Clonetech pGBKT7 vector (cat #K1612-1) and the pACT2 vector (cat #K1604-A) using BamHI and EcoRI for pGBKT7 vector, and BamHI and XhoI for pACT2 vector. The vectors were purified from 0.9% agarose gel using MP Biomedical's GeneClean Kit (cat. # 1001-200).

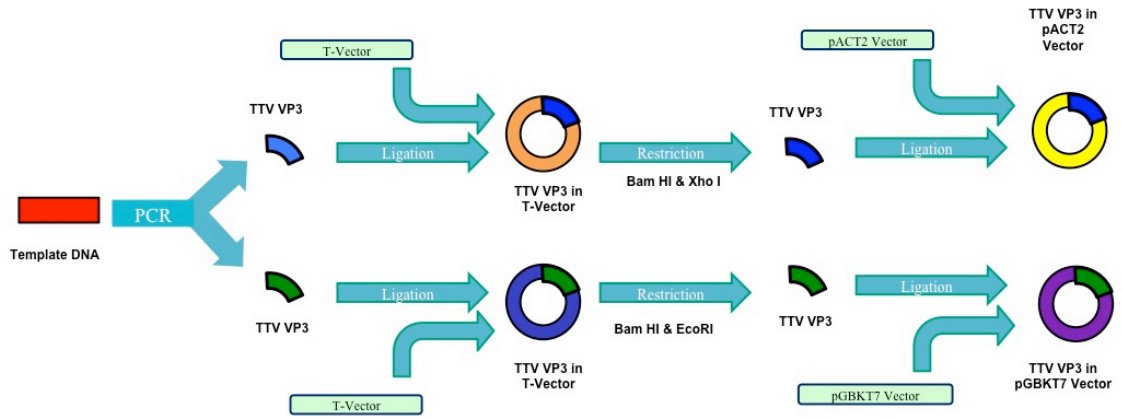

Figure 6: Overall molecular cloning strategy for creating a construct to assess multimerization. We have created two T-Vector constructs with our TTV VP3 insert. The steps following are for the creation of two final constructs for use in a yeast 2-hybrid system to continue multimerization analysis.

Cell Culture and Apoptosis Assay

H1299 cells were maintained in culture using DMEM + 10% FBS with 1x PSF. The cells were passed regularly before reaching complete confluency. For the apoptosis assay, H1299 lung carcinoma cells were passed into a 96 well plate at a concentration necessary to obtain 70% confluency within 1-2 days. The cells were then transfected with Apoptin as a positive control and Human TTV VP3, both in Flag vector constructs using the Effectene Transfection Reagent. The cultures were allowed to incubate for 24 hours at 37°C. After 24hours, the media was removed, the cells were washed with PBS, and 50µL of media was added back to the cells. An Apo-One Homogeneous Caspase-3/7 Assay kit was used to determine whether or not Apoptin and TTV VP3 had the ability to induce apoptosis along the caspase 3/7 pathway. 50µL of substrate was added to each well as prescribed by the assay. The cells were allowed to incubate for 18 hours at room temperature in the dark. Fluorescence readings were taken at λ_{ex} 499nm and λ_{em}521nm and verified that Apoptin and TTV VP3 induces apoptosis in H1299 cells.

Results

Sequence Analysis of TTV VP3 Compared to Apoptin

As TTV VP3 has not been fully characterized or studied extensively, a comparison to a better-known homolog was essential for this study. More work has been done to identify important sequences within the third open reading frame (ORF3) of CAV, which encodes for Apoptin.

The Chicken Anemia Virus protein Apoptin has been shown to selectively induce apoptosis in transformed cells but not primary cells. Two characteristics of the protein are thought to contribute to this selectivity. The first is the protein's localization within transformed cells compared to primary cells. In transformed cells Apoptin migrates into the nucleus, whereas in primary cells it is retained mainly within the cytoplasm. The second characteristic of the protein, which may play a role in Apoptin's cancer killing ability, is multimerization. It has been shown that Apoptin spontaneously forms 30 to 40 subunit multimers in vivo. The area responsible for multimerization overlaps with the Nuclear Export Sequence (NES) (aa 33-46), in which hydrophobic and hydrophilic residues alternate. Leliveld et al. (2003) determined the amino acids 32-46 may fold with the amino acids 38 and 39 as an anti-parallel beta sheet in hairpin orientation. In this case, the hydrophobic residues would be facing towards each other, while the hydrophilic residues would be protruding outwards in the opposite direction. This structural motif is hypothesized to account for Apoptin's ability to multimerize, perhaps by Ile and Leu residues interlocking between Apoptin monomers to form Apoptin multimers.

Similarly, TTV VP3 has significant cellular localization and multimerization properties that may be important in its cancer killing ability. Localization of TTV VP3-GFP in vivo has been

shown to be largely time dependent. After twenty-four hours in cancerous cells, the protein was found in both the nucleus and the cytoplasm. When examined at forty-eight hours the protein was found to be localized almost exclusively in the cytoplasm, largely in punctate foci at the nuclear and cytoplasmic periphery. Significantly, this shows that TTV VP3 may have a functional NLS and NES. Because the area responsible for multimerization in Apoptin overlaps with the NES, a comparative sequence analysis of with TTV VP3 was performed.

In order to perform this sequence analysis, the known, published sequences of Apoptin and TTV VP3 along with CLC workbench were used to perform the alignment. The data from this study can be found in Figure 7. The amino acids appear for each sequence and are color-coded based on hydrophobicity and charge. Black amino acids are hydrophobic while green are hydrophilic. Blue amino acids are hydrophilic and positively charged while red are hydrophilic and negatively charged. An exact amino acid match is indicated by the red regions below the amino acid residues. Interestingly, in aligning the two sequences and examining the NES and Multimerization domain in Apoptin's sequence, it was observed that the residues (AA 33-46) alternate between hydrophobic and hydrophilic residues while in TTV VP3's (aa 24-39) they are all hydrophobic. This could suggest possible structural and functional differences between the two proteins. Despite this difference, it is important to note that all the proline residues in the two sequences were aligned suggesting possible structural similarity as proline produces a distinctive kink in a polypeptide chain. Leliveld et al. predicted that it was Apoptin's canonical sequence that gave it the necessary conformation to form multimers. TTV VP3's difference in structure could have several implications in its cancer killing mechanism and evolutionary divergence from Apoptin.

PCR and Molecular Cloning

As multimerization was thought to be an important part of Apoptin's mechanism for selectively inducing apoptosis in transformed cells, further examination as to whether or not TTV VP3 also had the capability to multimerize was performed. One way of determining if VP3 can multimerize is to use a yeast 2-hybrid system. This experiment utilizes the internal mechanisms of a yeast genome, in that if the Gal4 upstream activating sequence is turned on by a particular transcription factor it will activate the LacZ gene. This gene encodes the enzyme Beta Galactosidase, which can metabolize X-Gal, a substance introduced experimentally. This will produce a blue dye, which can allow for phenotypic selection of colonies of yeast that have the transcription factor for Gal4. To test for multimerization, the transcription factor is divided into two parts; the Gal4 binding domain contained on our pGBKT7 vector, and a Gal4 activating domain contained in the pACT2 vector. Should the TTV VP3 protein be present in both these vectors, expressed within the cells, and multimerize with each other they will bring the transcription factors back into close proximity with each other and activate the LacZ gene. Before this can be performed, constructs must be made of the VP3 and inserted into the appropriate vectors. As two different terminal vectors are used in this experiment and contain different restriction sites, two separate constructs had to be designed and synthesized. In order to do this, two sets of primers that included the appropriate restriction sites for each vector were constructed. After PCR was performed using the VP3 template that was synthesized by E. Evan-Browning and M. Orme-Johnson last year, the purified products were run on an agarose gel to confirm the product. This gel is shown in figure 8A. Lane 1 contains the molecular weight

marker. In lanes 2 and 3, the two purified PCR products can easily be seen as bright bands near the bottom of the gel. Lane 2 contains the purified product for the pACT2 vector. Lane 3 contains the purified product for the pGBKT7 vector. With the two PCR products purified and confirmed, the next step was to ligate them into T-vectors. The T-vectors serve as a useful stable cloning intermediate that can be transformed into E. coli to increase insert copy number. Once the insert is ligated into a T-vector, it must be restricted out and purified, then it can be ligated into the terminal pACT2 and pGBKT7 vectors for the yeast 2-hybrid assay. Performing the restriction digest and running the products on a gel is a useful way to confirm that the insert was successfully ligated into the T-vector. Figures 8B and 8C shows the gels that were run of the restriction digests to verify that the insert had been ligated into the vector. Lane 1 in each is a molecular weight marker while lane two contains the samples. The upper band on both gels shows the empty restricted vector (pACT2 in B and pGBKT7 in C) while the lower faint band is that of the restricted insert that has been successfully cleaved from the vector. To additionally confirm that the constructs contained the appropriate inserts, samples of each were sent out for sequence analysis and results are pending. Once confirmed by sequence analysis, ligation of the inserts into the pACT2 and pGBKT7 vectors can be performed along with the multimerization analysis via a yeast 2-hybrid assay.

Apoptosis Assay

In parallel with our study, apoptosis experiments to examine TTV VP3's ability to selectively induce apoptosis in transformed cells were performed. In order to perform these experiments, Flag constructs of TTV VP3 and native Apoptin were transfected into H1299 cells in a 96 well plate. The Apoptin served as a positive control. Cells and media that were taken

through the transfection process without DNA served as a negative control. Apo-One Homogeneous Caspase-3/7 Assay kit was used to determine whether or not TTV VP3 had the ability to induce apoptosis along the caspase 3/7 pathway. This kit contains a substrate, which is cleaved and fluoresces if the caspase 3/7 pathway is activated. After an 18-hour incubation at room temperature in the dark, fluorescence readings were taken of each of the samples. A λ_{ex} 499nm and λ_{em}521nm were used to scan the samples over a range of 510-547nm. Figure 9A is a graph of the curves generated by each of the samples. The x-axis shows the nm range the samples were scanned along while the y-axis is a measure of relative fluorescence units (rFU). The negative controls showed only baseline levels of apoptosis and the curves for each are clustered on the lower portion of the graph and show no peak at 521nm. Visually, when observed under a microscope, the cells remained healthy with very low levels of cell death. The cells transfected with Apoptin and TTV VP3, when inspected visually showed very high levels of apoptosis with cell debris comprising the majority of each well. In Figure 9A, it can be observed that the curves for Apoptin and TTV VP3 are interspersed in a fairly tight range. Each curve exhibits a peak at 521nm, which is expected for this assay if apoptosis was induced. The rFU values for Apoptin and TTV VP3 were much higher than that of the negative controls as well. This confirmed that both Apoptin and TTV VP3 induced apoptosis in the H1299 cells through a caspase 3/7 dependent pathway. Interestingly, not only did TTV VP3 also induce apoptosis in the H1299 cells, but the levels of apoptosis were similar to that caused by the positive control Apoptin. In Figure 9B, the averages of the three samples for each the negative control, positive control (Apoptin), and TTV VP3 were taken and plotted in a bar graph. In this graphical

representation, it is easy to see that Apoptin and TTV VP3 induced apoptosis at similar levels significantly higher than those seen by the baseline shown by the negative control.

Discussion and Future Experiments

Sequence analysis of TTV and Apoptin

TTV VP3 is considered a homolog to the Chicken Anemia Virus Apoptin; a protein capable of inducing apoptosis selectively in cancerous and transformed cells. Though the mechanism by which it accomplishes this is largely unknown, its nuclear localization [34] as well as its ability to multimerize with itself has been shown to be essential in its apoptotic capability. By performing a sequence analysis of TTV VP3 and Apoptin we have found that the mechanism of multimerization, and possibly apoptosis, may be significantly different.

Apoptin's nuclear localization was originally thought to be required because it is localized in the nucleus in all cancer cells and is thought to target subunit 1 of the anaphase promoting complex/cyclosome (APC/C) while it is in the nucleus [35]. The APC is a master regulator of the cell cycle and is also responsible for several important checkpoints during mitosis. One of its most important activities is the transition from metaphase to anaphase, as its name suggests. It performs this action by separation of sister chromatids once all of the mitotic spindles have attached to the kinetichores. If even one spindle is not connected this pathway will shut down. Such a sensitive and important pathway is therefore a very attractive target for a viral protein. The only problem with this theory is that Apoptin's entry into the nucleus causes cell cycle arrest at G2/M phase, followed by apoptosis; yet, there is no nucleus at this point of the cell cycle. Therefore it may be possible that the localization sequence is not necessary to gain access to the nucleus, however studies have shown that its presence is required for apoptosis.

When the sequence analysis of Apoptin is compared with that of TTV VP3 the alternating hydrophobic-hydrophilic residue sequence of Apoptin's multimerization domain is not seen in TTV. This is significant because the way in which Apoptin multimerizes has been shown to affect its ability to induce apoptosis. This suggests that perhaps TTV VP3 A) does not multimerize, or B) multimerizes in a manner different than Apoptin. Should the former be true, it may support the idea that Apoptin multimerizes as an alpha helix, with its hydrophilic residues protruding outside and its hydrophobic residues inside. This would mean that because of TTV VP3's only hydrophobic residues at Apoptin's area of multimerization TTV VP3 does not undergo multimerization. However, if TTV VP3 did undergo multimerization its possible that it does in fact induce apoptosis independent of nuclear APC/C, and therefore may not need to be in the nucleus to induce apoptosis. In either situation, the incongruity of amino acid sequence may provide valuable insight into the apoptotic pathways for both of these viral proteins, as well as the role multimerization and nuclear proteins play in TTV VP3.

Interestingly, when the sequence for nuclear localization in Apoptin is compared to that of TTV VP3 they again differ. The canonical bipartite nuclear localization sequence, typically made of leucine and arginine amino acids, are found in Apoptin is made of KKR (amino acids 86-88) and KRR (amino acids 115-117) which are polar hydrophilic amino acids. When compared to the TTV VP3 sequence the only sequence matched up is EHH (amino acids 86-88), which are hydrophilic and polar but do not fit the bipartite sequence expected of nuclear localization. This evidence favors the theory that nuclear localization is not a necessary part of inducing apoptosis in. Instead, this begs the question as to what part of Apoptin's NLS are playing a part in its apoptotic ability.

Despite the sequential differences seen in many of Apoptin's well-characterized sequence, there are some similarities. The most striking of these is the overlapping proline residues in five disparate areas corresponding to Apoptin's amino acids 20, 58, 84, 96, and 116. Proline has a distinct cyclic structure, which gives it a very strong rigidity that forms into a kink on a peptide chain. Because these groups are highly conserved in both viruses suggests that they still may share a similar 3-D orientation and functionality. Much effort has been put into examining Apoptin's nuclear import and export sequences as well as localization within the cell, but without a nucleus during the phase in which Apoptin arrests the cell it is difficult to know the exact importance these have on inducing apoptosis. Therefore finding conserved residues between the two homologous viruses may aid in future studies.

Apoptosis Study

In parallel with the comparative sequence analysis, the apoptotic capability of TTV VP3 was determined. Prior to our experiments only one experiment, performed by Noteborn et al, showed putative cancer killing ability. The shortcomings of this experiment were that the data was qualitative and showed no discernable pathway or mechanism by which TTV VP3 was inducing apoptosis. Therefore it was critical that we provide a concrete quantitative experiment that followed a specific mechanism. The apoptosis study performed in H1299 (human lung carcinoma) cells showed that TTV can induce apoptosis in H1299 cells through a specific caspase 3/7 dependent pathway. Visually, noticeable levels of apoptosis were observed in the TTV and Apoptin (+CTRL) samples 24hrs after the transfections. When the fluorescence readings were taken at 521nm after an additional 18-hour incubation, TTV VP3 and Apoptin were shown to induce apoptosis at similar levels. Both of these samples were significantly

higher than the negative control that was only cells in media. In the two trials performed, we used six samples for each. Results were reproducible and consistent within the trial and within the two that were performed as well showing low variability. We attribute the variability of this assay due to the unknown transformation efficiencies within the individual wells as well as the rate of substrate cleavage. This study can be used as a starting point for further experiments to explore TTV VP3's apoptosis inducing ability more in depth. To continue this study, we recommend repeating this assay as a time course over 18 hours, taking fluorescence readings every hour for 18 hours. In addition, we recommend performing this same experiment using primary cells to ensure that TTV VP3 is selectively inducing apoptosis in transformed cells only.

Yeast 2-Hybrid Multimerization Experiments

Multimerization has clearly been a topic of great interest among those studying Apoptin and its homologs. Whether or not TTV VP3 in fact does undergo multimerization has yet to be definitively determined. What is important is that both TTV VP3 and Apoptin are capable of inducing apoptosis via a caspase 3/7 pathway. Therefore the molecular cloning performed should be continued into further its final stage; the Yeast 2-hybrid assay. Using the T-vector constructs, the TTV VP3 inserts should be restricted out and ligated into the pACT2 and pGBKT7 vectors. A Yeast 2-Hybrid assay can then be performed to determine whether VP3 has the ability to multimerize with itself. If the result of this assay confirms the ability for multimerization, a few experiments should be done to further explore its importance. First, the multimerization domain should be identified and a knockout mutant of the multimerization domain should be created. Using this knockout mutant, the apoptosis experiment performed in

this study should be repeated to determine multimerization's role in inducing apoptosis in transformed cells.

If it is found that TTV VP3 does not have the ability to multimerize with itself, then the question becomes what is the cause of the selective induction of apoptosis in transformed cells but not primary cells. If multimerization is not a part of VP3's apoptosis inducing pathway, there must be some other factor such as another protein specific to transformed cells it is interacting with. Multimerization is thought to be a regulatory factor in Apoptin in that in primary cells it is seen as insoluble aggregates that cannot induce apoptosis while in transformed cells the multimers are smaller and soluble allowing for apoptosis induction. Without this mechanism of regulation in TTV VP3, another protein could be acting to inhibit and inactivate it from inducing apoptosis. Another possibility is that the differences in primary and transformed cell environments can contribute to the activation or inactivation of TTV VP3. Although these two scenarios are possible, it is unlikely that TTV VP3 does not multimerize as it exhibits similar punctate cellular localization patterns to that of Apoptin, which has been shown to multimerize.

Localization Experiments

An additional experiment that should be conducted is further analysis into the localization of TTV VP3 in transformed and primary cells. Preliminary data has shown that in transformed cells TTV VP3 will localize primarily in the cytoplasm and appear in a punctate pattern along the nuclear periphery. By performing localization experiments in primary cells, meaningful data can be collected as to its preferred location in transformed cells as opposed to primary cells, as well as the area in which it is present during apoptosis. Additionally, it may be

interesting to locate the Nuclear Localization and Nuclear Export Sequences. Once this is done, knockout mutants of each of these can be created for further analysis of nuclear-cytoplasmic shuttling.

Structural Analysis

In order to fully understand how the multimerization domain, NLS, and NES interact, the crystal structure of TTV VP3 must be discovered. Before attempting to crystallize VP3, it may be useful to perform Circular Dichroism to analyze the secondary and tertiary structures of the protein. This can provide valuable information about the flexibility of the protein that can aid in developing a crystallization strategy. Once the crystal structure is obtained, experiments can be devised to explore the interaction of VP3's domains with other proteins and itself. This can help to provide information about the pathways involved in VP3's apoptosis inducing ability. In addition, knowledge of the crystal structure can help with determining how VP3 changes in primary cell versus transformed cell environments. This may also help to provide insight into VP3's mechanism of action.

Conclusion

The human Torque Teno Virus is a largely unstudied virus. TTV is thought to be homologous to the Chicken Anemia Virus, which is a very well characterized virus, and therefore meaningful comparisons can be drawn from analysis of the two. As mentioned in the discussion of multimerization, the mechanism by which Apoptin and TTV VP3 induce apoptosis may be different. The sequence analysis showed that TTV VP3 may multimerize differently than Apoptin, if at all, and preliminary localization studies show that at 48 hours TTV VP3 does not even appear in the nucleus of a transformed cell, whereas Apoptin has been shown to recruit nuclear proteins in order to induce apoptosis. An interesting result of this apoptosis assay is that both Apoptin and TTV VP3 use a very specific caspase 3/7 dependent pathway in order to induce apoptosis in H1299 cancerous cells. Therefore the apoptotic result is the same, yet the mechanism by which apoptosis induces the caspase pathway is still to be determined in future experiments. The implications of these studies of Apoptin and TTV VP3 to cancer research as a whole is very exciting. If the specific pathways involved in targeting cancer cells and selectively inducing apoptosis can be discovered and characterized, potential therapeutics can be designed to mimic this mechanism.

Figures:

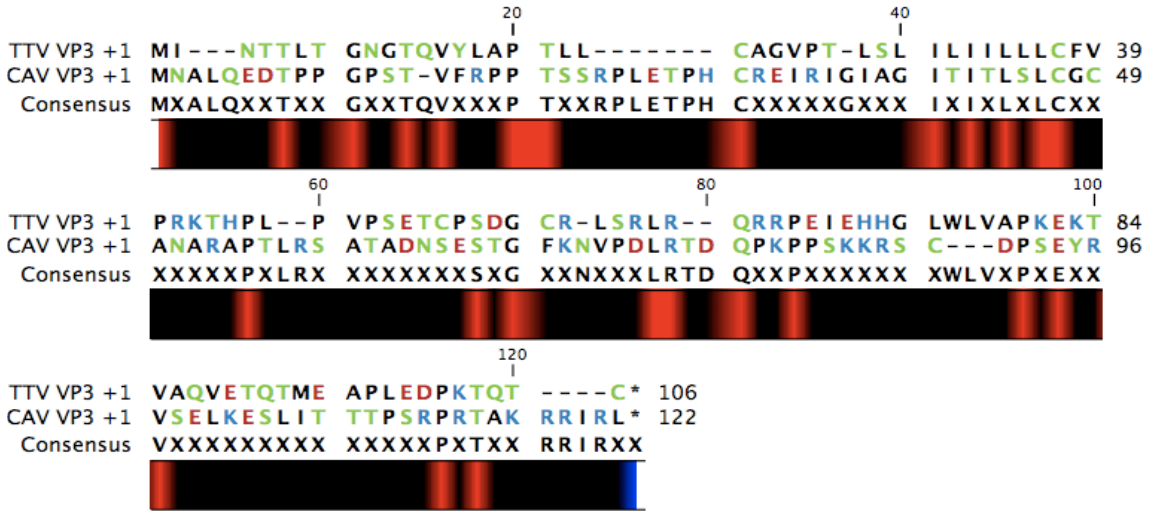

Figure 7: Sequence Analysis of TTV VP3 and CAV VP3. Black amino acids are hydrophobic. Green amino acids are hydrophilic. Blue amino acids are hydrophilic and + charged. Red amino acids are hydrophilic and – charged. An exact amino acid match is shown by the red regions below the amino acid residues.

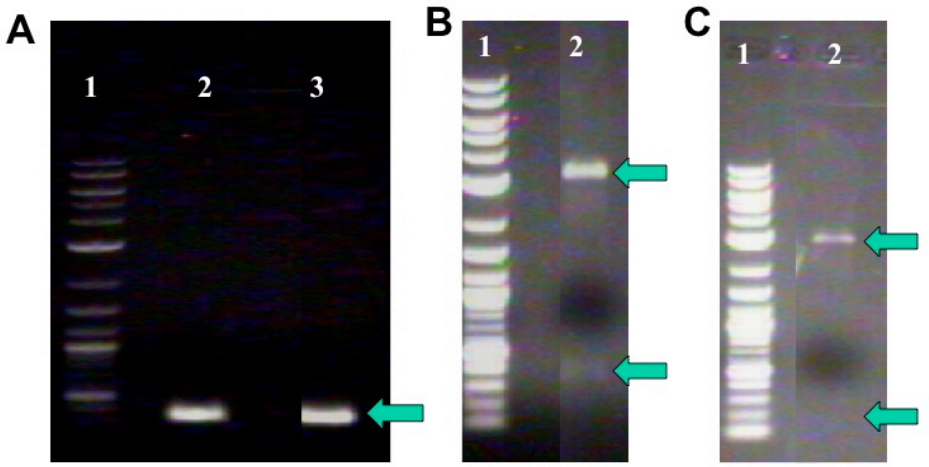

Figure 8: (A) Agarose gel of PCR products. Lane 1 contains the Molecular Weight Marker. Lane 2 contains the TTV VP3 product from the primers designed for the pACT2 vector. Lane 3 contains the TTV VP3 product from the primers designed for the pGBKT7 vector. (B) Agarose gel of restricted T-Vector with TTV VP3 Dropouts. Lane 1 contains a Molecular Weight Marker. Lane 2 shows the Bam HI and XhoI restricted T-vector in the top band and the TTV VP3 pACT2 insert in the bottom band. (C) Agarose gel of restricted T-Vector with TTV VP3 Dropout. Lane 1 contains a Molecular Weight Marker. Lane 2 shows the BamHI and EcoRI restricted T-vector in the top band and the TTV VP3 pGBKT7 insert in the bottom band.

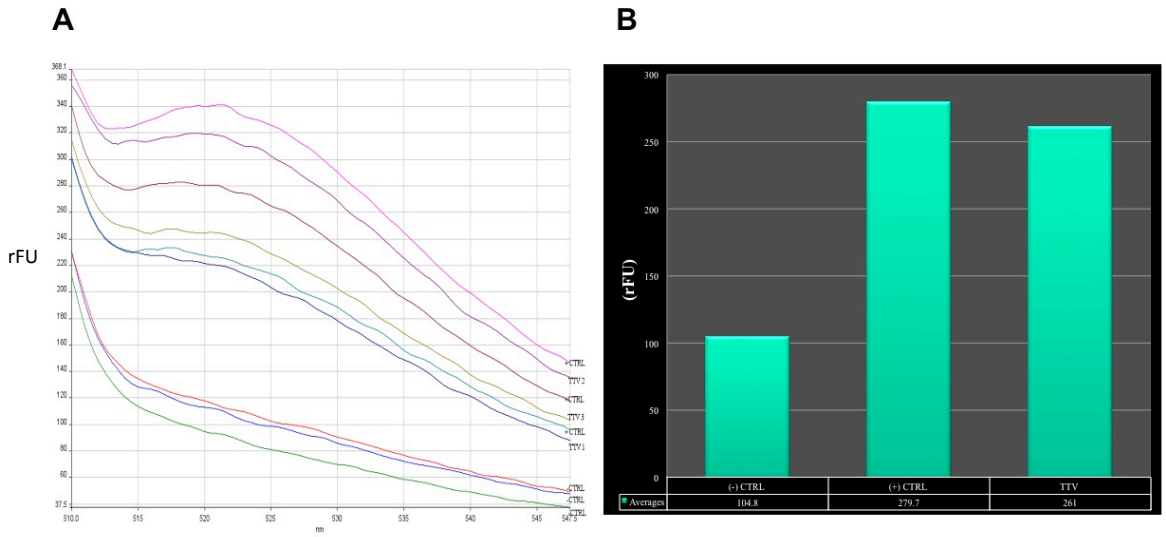

Figure 9: (A) Apoptosis Assay Curves for + Control, -Control, and TTV samples. Apoptosis is the result of the activation of caspase 3/7, which results in a cleavage of the fluorescent substrate. Fluorescence readings were taken at 521nm. (B) Quantified averaged data from Apoptosis Assay of the samples. Negative CTRL (cells and media only) showed baseline apoptosis. Positive CTRL (Apoptin) set the bar for apoptosis level. TTV samples were all in the range (or higher than + CTRL)

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