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Cloning and Expression of PCV1 ORF3

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Abstract

PCV1 is a non-enveloped, non-virulent, single-stranded DNA virus that infects pigs. Two related viruses, CAV and PCV2, encode VP3 proteins capable of selective induction of apoptosis in transformed cells, which is associated with cell-type specific localization. Our project is the first to examine the expression and localization of PCV1 VP3. Sequence analysis suggests NES and NLS motfis may exist in the protein. Expression of GFP-tagged PCV1 VP3 in H1299 cells conferred cytoplasmic localization to GFP, suggesting a functional NES may be present. This localization pattern is contrary to the nuclear localization pattern of apoptin but similar to the cytoplasmic localization pattern of PCV2 ORF3. Finally, the ability of PCV1 VP3 to induce apoptosis in transformed cells was assessed by monitoring changes in the confluency of H1299 cells following transfection with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 vector. The results of this assay suggest that PCV1 VP3 may be capable of inducing apoptosis in transformed cells.

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1 Background

1.1 The *Circovirus* Genus

The *Circoviridae* viral family encompasses an array of viruses that infect both plants and animals (31). A subset of the viruses within this family are grouped in the genus *Circovirus*. Circoviruses are icosahedral, non-enveloped, and infect vertebrates (32). Their monopartite genomes consist of a circular, single-stranded molecule of DNA (32).

One member of the *Circovirus* genus is *Porcine circovirus* type 1 (PCV1), which infects pigs and is one of the smallest animal viruses known (35). The virus was first discovered as a contaminant of the PK-15 porcine kidney cell line in 1974 (44). Since this discovery, many characteristics of the virus, such as its virulence and genomic organization, have been studied and described.

1.1 The Virulence of PCV1

PCV1 is unique among the circoviruses in that it is nonpathogenic (30, 45). However, interest in PCV1 piqued when *Porcine circovirus* type 2 (PCV2), a virus in which 76% of the genomic sequence is homologous to that of PCV1, began to be linked to a disease in pigs (10, 33). PCV2 was eventually identified as the causative agent of Post-weaning Multisystemic Wasting Syndrome (PMWS) (10, 33). Afflicting pigs 5-18 weeks in age, symptoms of the disease include weight loss, dyspnea, enlargement of the lymph nodes, and jaundice (11, 12).

During outbreaks of the disease, the mortality rate often peaks at about 10%, making the disease an economic concern for farmers worldwide (12).

Although PCV1 is not linked directly to PMWS, it is commonly found in the sera of healthy swine around the world (1, 9, 15, 45). Antibodies to PCV1 have also been found in the sera of mice, humans, and cattle, but the presence of the virus has not been linked to a specific pathology in any of these animals (43). Research has shown that the proteins Rep and Rep' of PCV1 are capable of initiating replication of the PCV2 genome (30). Given the widespread distribution of PCV1 among swine populations, it is possible that the virus could contribute indirectly to the virulence of PCV2 upon co-infection (30).

1.2 Organization of the PCV1 Genome

The full-length genome of PCV1 consists of 1759 nucleotides, as shown in Figure 1. Computational analysis revealed that the virus has an ambisense genome containing seven potential open reading frames (31). However, the only two reading frames known to encode proteins expressed in vivo are ORF1 and ORF2, which encode proteins necessary for viral replication and formation of the capsid. The two reading frames are oriented in opposite directions, and the promoters for each overlap the opposite ORF (28). Interestingly, it has been proposed that the transcript for the Cap protein encoded in ORF2 is processed in such a way as to possibly inhibit expression of a reading frame that overlaps ORF1 with a start codon in close proximity to the cap promoter (28). A possible identity for this hypothetically silenced ORF is ORF3 (see Figure 1).

ORF1 and ORF2 are separated by an intergenic region that contains a sequence of 111 nucleotides, which comprises the origin of replication (28, 29). The origin of replication is thought to form a stem-loop structure with the nonamer 5'-TAGTATTAC at the peak. Three repeats of the hexamer 5'-CGGCAG are found adjacent to the stem-loop structure along with a fourth repeat that differs in sequence at one nucleotide (29, 30). Although the function of these repeats is unclear, it has been proposed that they act as binding sites for proteins necessary for viral replicaton (29).

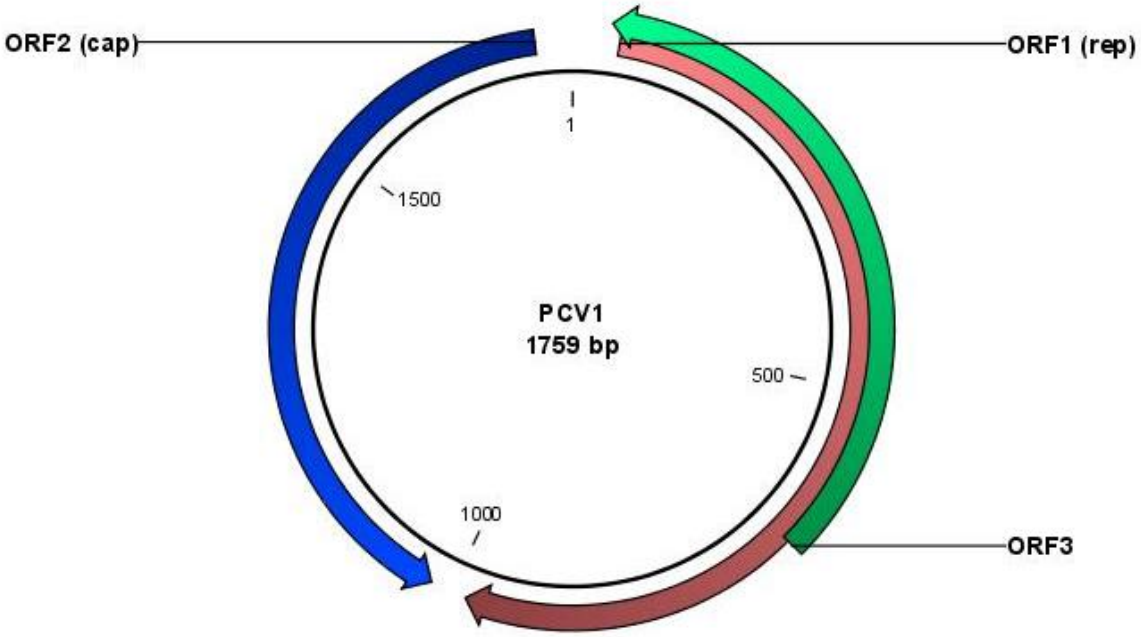

Figure 1-The PCV1 Genome. A circular monopartite single-stranded DNA molecule of 1759 nucleotides comprises the PCV1 genome. Out of seven potential ORFs, only ORF1 and ORF2 are known to encode viral proteins. ORF3 is 621 nucleotides in length and is oriented in the opposite direction of ORF1.

1.3 The Roles of Rep and Rep' in the Replication of PCV1

Following infection, the single-stranded PCV1 is thought to be converted into a double-stranded intermediate by cellular enzymes. Duplication of the genome then proceeds by rolling-

circle replication (RCR), a process which is well understood in geminiviruses but not in PCV1. In tomato yellow leaf curl virus, replication is mediated by the viral protein Rep and initiates with the introduction of a single-strand break in the origin of replication. The nick frees a 3' hydroxyl group that can subsequently function as a primer for DNA synthesis based on the sequence of the complementary strand. DNA synthesis is carried out by DNA polymerase III provided by the host cell. Regeneration of the of the origin signals Rep to cleave the strand again, terminating replication. Rejoining of the ends through a nucleotidyltransfer reaction catalyzed by Rep allows for the release of a new, circular, ssDNA viral genome (20, 40).

Two proteins encoded by PCV1 designated Rep and Rep' are thought to function in a homologous manner to the Rep protein from tomato yellow leaf curl virus, especially since sequence analysis shows a high degree of homology between the Rep proteins (30). Rep and Rep' are essential for replication of PCV1 (27, 40). The hexamer repeats in the origin of replication serve as binding sites for Rep and Rep' and mediate the recruitment of these enzymes to the origin of replication. One study has shown that, in vitro, both Rep and Rep' are capable of nicking the viral strand between two specific nucleotides within the nonamer sequence found in the origin of replication. Rep and Rep' are also capable of ligating two ends of a strand that had previously been cut by one of the enzymes. Since site specific cleavage and ligation are necessary for RCR, these findings suggest that Rep and Rep' mediate viral replication through a mechanism similar to that of the homologous Rep protein from tomato yellow leaf curl virus (40).

ORF1 contains the rep gene, which encodes the two proteins Rep and Rep' (38). Two different transcripts are derived from the rep gene. The first transcript comprises the full length of the reading frame while the second is an alternatively spliced form of the first. The full length

transcript is translated into Rep, a protein 312 amino acids in length, while the spliced transcript is translated into Rep', which contains 168 amino acids (30).

1.4 The PCV1 Cap Protein

The cap gene is located within ORF2 and encodes a protein 234 amino acids in length which is thought to form the capsid of PCV1. An interesting feature of the cap genes from PCV1 and PCV2 is that they show the highest level of divergence in terms of sequence homology between the viruses (30). For this reason, the major difference in virulence between the two viruses has been attributed to differences in the Cap proteins of each (30). However, a study in which pigs were inoculated with chimeric clones of PCV1 where the cap gene had been replaced with that from PCV2 showed that the clone was insufficient to cause the development of PMWS. Pigs inoculated with a PCV2 clone did develop symptoms of the disease (11). Hence, the low homology between the cap genes from PCV1 and PCV2 does not correlate with a difference in virulence.

1.5 The Apoptotic Activity of Circovirus VP3 Proteins

Porcine circovirus type 2 (PCV2) and chicken anemia virus (CAV) are also members of the *Circoviridae* family, and thus are related to PCV1 (32, 35, 36). As mentioned previously, PCV2 is the agent responsible for the development of PMWS in swine. CAV infection in chickens causes the temporary development of severe anemia and immunodeficiency (47, 18) Each virus has been proven to induce apoptosis in infected host cells, which is thought to

contribute directly to pathogenesis in each case (34, 25). Additionally, for both PCV2 and CAV, apoptosis induction in host cells has been shown to be mediated by the protein encoded in ORF3, also referred to as the VP3 protein (34, 25).

Aside from its role in viral pathogenesis, the apoptosis-inducing activity of these proteins is of interest as a potential cancer therapy. Studies of CAV VP3 or "apoptin" have demonstrated that the protein selectively induces apoptosis in transformed cells whereas it persists in primary cells with no observed detrimental effects (7). Experiments conducted by previous lab members suggest that PCV2 VP3 is also capable of inducing apoptosis in transformed cells (4). These proteins, and those from other circoviruses, will continue to be studied in order to expand models for their activities that have already been elucidated.

1.6 The CAV VP3 (Apoptin) Mechanism of Apoptosis Induction

Apoptin is a 13.6 kDa protein consisting of 121 amino acids encoded by ORF3 of CAV. Unique structural features of the protein include its two proline-rich regions and its two basic regions. The protein is translated from a polycistronic mRNA molecule that also serves as the transcript for genes encoded in ORF1 and ORF2 (34). Apoptin was first demonstrated to be expressed in chicken lymphoblastoid T cells, in which it was simultaneously shown to be capable of inducing apoptosis (34). It was subsequently shown that apoptin induces apoptosis in transformed cells but not in normal cells (7, 48).

Our understanding of the mechanism by which apoptin induces apoptosis in transformed cells remains incomplete. It has been demonstrated that apoptin's activity is p53 independent and that the mechanism depends on nucleocytoplasmic shuttling and the interaction of apoptin

with the anaphase promoting complex (13, 14). To better understand this mechanism, we first describe the endogenous p53-regulated pathway to apoptosis and then compare it to the current model of apoptin-mediated apoptosis.

1.6.1 Endogenous Induction of Apoptosis via the p53-regulated Pathway

Apoptosis is a physiological process used by multicellular organisms to regulate the longevity of certain cells, remodel tissues during development or as part of a repair process, and to eliminate cells that have sustained extensive genetic damage. This so called "programmed cell death" is distinguished from necrosis, a form of cell death often induced by injury (16). The morphological and biochemical markers of apoptosis are also distinct from those of necrosis. The initial stages of apoptosis are marked by condensation and endonucleolytic cleavage of the genomic DNA, condensation and the formation of bulges or "blebs" in the nuclear and plasma membranes, detachment of the apoptotic cell from surrounding cells, and the loss of microvilli (16, 5). As the process continues, the cytoskeletal structure is disrupted, the cytoplasm condenses and compacts cellular organelles, and the cell finally shrinks and fragments into membrane bound vesicle-like structures called "apoptotic bodies". In vivo, these apoptotic bodies are rapidly cleared from the surrounding tissue by phagocytic cells such as macrophages (16, 5).

Many of the morphological and biochemical features of apoptosis are the result of caspase activity. The caspases are a family of proteases containing cysteine residues in their active sites that cleave specific sites following aspartic acid residues. The caspases are constituitively expressed as zymogens that only become activated following cytochrome c release into the cytoplasm during the initiation of apoptosis. Activation of initiator caspases in

this manner allows them to in turn activate effector caspases. The results of caspase activation include the degredation of the cytoskeleton, perturbation of the nuclear envelope, and activation of the nuclease responsible for DNA fragmentation (16).

The transcription factor p53 enhances the rate of transcription of a set of many genes, some of which promote apoptosis. The concentration of p53 is kept low in cells under normal conditions via protease-mediated degradation and possibly by sequestration of an inactive form of the protein (22). MDM2, encoded by one of the genes for which p53 enhances transcription, negatively regulates p53 by binding it such that the DNA binding domain is partially blocked and degradation is accelerated (16). The p53 concentration of a cell increases in response to damaging conditions such as hypoxia and DNA double strand breaks (22). When damage is detected, expression of the p53 gene is upregulated and the half-life of p53 in the cytosol increases dramatically due to phosphorylation, which enhances p53 activity and inhibits its binding to MDM2 (16).

The increased activity and upregulation of p53 leads to increased production of one of the proteins it regulates known as p21. The role of p21 is to bind to and inhibit cyclins and cyclin-dependent kinases, including cyclinD and CDK4 which normally complex and phosphorylate the retinoblastoma (Rb) protein, allowing the cell to progress from G_1 to S phase (16, 22). Once the cell is arrested in G_1 phase, the cell machinery has time to attempt to repair the damage that initiated p53 upregulation (16).

If the damage to the cell is extensive and irreparable, p53 functions to guide the cell down an apoptotic pathway. Members of the Bcl-2/Bax family of proteins are essential to this process. Bax is known to promote apoptosis while Bcl-2 inhibits it. Both proteins are found in cells: Bax resides in the cytoplasm while Bcl-2 is attached to the cytosolic surface of the outer

mitochondrial membrane, where it regulates ion transport and protects the integrity of the membrane (16). The relative levels of expression of these proteins are thought to be controlled by p53. When the ratio is shifted in favor of Bax production, Bax is thought to bind to Bcl-2 on the mitochondrial membrane, resulting in loss of selective ion permeability and the leaching of cytochrome c and apoptosis inducing factor (AIF) into the cytoplasm from the mitochondrial intermembrane space. Once in the cytoplasm, AIF and cytochrome c procede to initiate apoptosis by promoting chromatin condensation and caspase activation (16).

The relevance of the p53 pathway in cancer is that it is the means by which most therapies, including radiation and chemotherapy, bring about apoptosis of tumor cells (39). However, approximately 50% of all human tumors express a mutated p53 that renders these therapies ineffective (37). Therefore, apoptin-mediated apoptosis is a promising potential therapy for aggressive tumors because it does not rely on p53 (7.). Understanding the biochemical mechanism of apoptin-mediated apoptosis is essential to its development into a therapy.

1.6.2 Apoptin Associates with the Anaphase Promoting Complex/Cyclosome (APC/C) to Induce Apoptosis

The association of apoptin with the APC/C is vital to its mechanism of apoptosis induction (14). The APC/C is a multiprotein complex that functions as an E3 ligase that selectively ubiquitinates cyclins, targeting them for degredation by the proteasome so that the cell progresses through mitosis (2). Another protein thought to be targeted by the APC/C during the transition into anaphase is securin, which inhibits the enzyme separase from prematurely unbinding sister chromatids in metaphase, preventing their premature separation (2, 13).

Destruction of securin leads to the activation of separase and separation of the sister chromatids (17).

The APC/C must be bound to one of two coactivators, Cdh1 or Cdc20, in order for the complex to bind its substrates (2). It has traditionally been thought that the APC/C bound to Cdc20 was the complex active during early mitosis while the Cdh1-bound version of the complex was active in late mitosis and G1, but some evidence indicates that APC/C-Cdh-1 may be responsible for the destruction of securin in late metaphase (2). Phosphorylation of Cdh-1 is prevalent during early mitosis and prevents it from binding the APC/C while phosphorylation of the APC/C itself is necessary for Cdc20 binding (2, 23).

Once Cdc20 is bound to the APC/C, regulation of the complex is achieved by the spindle assembly chekpoint, a molecular system that inhibits the activity of APC/C-Cdc20 until all kinetochores are attached to microtubules (2). When all kinetochores are bound to microtubules, the APC/C-Cdc20 complex becomes activated, leading to cyclin B and securin degradation that results in progression into anaphase (2). Exactly how crucial Cdc20 is in this transition seems to vary by species: studies of Cdc20 knockout mouse embryos show the cells are arrested in metaphase while Cdc20 knockdown human somatic cells progress into anaphase normally (23, 3). The APC/C-Cdc20 targeted destruction of cyclin B activates phosphatases that act on Cdh1, allowing it to bind the APC/C. The resulting APC/C-Cdh1 complex targets Cdc20 for destruction, which facilitates the transition from APC/C-Cdc20 to APC/C-Cdh1 as the active complex in late mitosis (17).

The APC/C is also an important target for certain viral proteins (13). The interaction of these proteins with the APC/C often leads to cellular G_2/M arrest and apoptosis. Induction of G_2/M arrest seems advantageous to these viruses as it disrupts the nuclear envelope, allowing

viral components to freely access the nucleus to initiate replication and easing transit of viral genomes to sites of viral assembly in the cytoplasm following replication (13). Induction of apoptosis may also be advantageous in that it could facilitate viral egress from infected cells (13).

Apoptin is an example of a protein that induces G_2/M arrest and apoptosis exclusively in transformed cells (7, 41). In transformed cells, apoptin associates with APC1, the largest subunit of the APC/C complex that likely has an important structural role (41). This association leads to disruption of the APC/C complex and an accumulation of substrates normally targeted for destruction by the APC/C, which are also associated with G_2/M arrest and apoptosis (41). These effects have not been observed in nontransformed cells (41). The mechanism of apoptosis induction, and the reasons why apoptin-induced G_2/M arrest and apoptosis are cell-type specific, are not fully understood (41).

1.6.3 Nucleocytoplasmic Shuttling

From what is known of our limited understanding of the mechanism of apoptin-induced apoptosis, localization of apoptin to the nucleus of a transformed cell is required for association with the APC/C and induction of G_2/M arrest and apoptosis (14). Translocation of apoptin to the nucleus of transformed cells during interphase requires transiting the nuclear envelope, which is a highly regulated process for most proteins (42). Thus, the cellular machinery and mechanisms involved in nucleocytoplasmic shuttling are essential to apoptin's method of apoptosis induction in transformed cells.

All nucleocytoplasmic transport occurs through a unit known as the nuclear pore complex (NPC). The NPC is a discrete channel that spans the inner and outer membranes of the nuclear

envelope to connect the cytoplasm and nucleoplasm (8). The NPC can roughly be described as consisting of a scaffold that lines the channel and connects two rings, each of which lies on either the nuclear or cytoplasmic face of the channel. Each ring serves as the site of attachment for eight filaments, giving the complex its characteristic octuple symmetry (8). Unlike the cytoplasmic filaments, the ends of the nuclear filaments distal to the nuclear ring join together to form the nuclear basket (8).

The proteins that make up the nuclear pore complex are known as nucleoporins or nups (6, 8). Although a single NPC can consist of up to 1000 nups, only about 30 different nups exist in humans, which collectively exhibit a relatively small array of functional diversity (6, 8). One notable functional domain of certain nups consists of FG repeats that bind directly to transport receptors carrying substrates through the NPC (6).

In general, ions and molecules that are smaller than 40 kDa can passively diffuse through the NPC while larger molecules must be translocated in a process that involves interaction of the molecule's transporter with nups bearing FG domains (42). The exact mechanism by which association of transporter-cargo complexes bind to nups and translocate the NPC is unknown, but various models have been proposed in which transiting the NPC requires a transporter-cargo complex to overcome physical or energetic barriers (42).

Compared to current knowledge of the roles nups play in nucleocytoplasmic transport, the model that describes the roles of transporters in this process is much better understood (42). Most of these proteins are members of the karyopherin-β/importin-β family and can be classified as either importins or exportins based on their directionality of transport relative to the nucleus (42, 46). The movement of these transporters and their cargos is regulated and coordinated by Ran, a guanisine triphosphatase (42). Directionality of movement seems to be highly dependent

on the existence of a Ran·GTP gradient across the nuclear envelope, which is established by the activities of nucleoplasm-confined RCC1, a Ran guanine nucleotide exchange factor, and the cytoplasmic RanGAP, which promotes GTP hydrolysis by Ran (46).

The karyopherin-mediated process of import begins in the cytoplasm where an importin binds to its specific cargo, and the importin-cargo complex then enters the nucleus via the NPC. Inside the nucleus, the N-terminus of the importin is able to bind Ran·GTP, resulting in release of the importin's cargo. The importin-Ran·GTP complex then returns to the cytoplasm, where RanGAP stimulates Ran to hydrolyze its bound GTP, resulting in disassembly of the importin-Ran·GTP complex. The Ran importer NTF2 then binds to and returns Ran·GDP to the nucleus, where association with RCC1 causes the GDP to be replaced with GTP, allowing the cycle of import to begin again (46).

Alternatively, the newly regenerated Ran·GTP could associate with the N terminus of an exportin bound to its cargo in the nucleus to begin a cycle of export. Once the exportin-cargo-Ran·GTP complex enters the cytoplasm, hydrolysis of GTP induced by the activity of RanGAP disassembles the complex. The exportin and Ran are then transported back to the cytoplasm (46).

Binding of either an exportin or importin to its cargo requires the presence of a particular signal. An importin recognizes and binds to a protein bearing a nuclear localization signal (NLS) while an exportin recognizes and binds to a protein with a NES, or nuclear export signal (42). A NLS is generally comprised of an area of a protein enriched in basic amino acids and can be either monopartite, consisting of one cluster of basic residues, or bipartite, consisting of two

clusters of basic residues divided by a linker region of 10-12 amino acids of any type (21). Import of proteins containing a NLS often requires binding of an importin-α to recognize the NLS and binding of an importin-β to the importin-α to enable transport, although some proteins contain an NLS that can bind an importin-β directly (21). A NES generally consists of a single region in a protein enriched in hydrophobic amino acids (19). Many proteins bearing a NES are transported by the exportin Crm-1, and as such, sequence conservation of the NES among these proteins is fairly common (19). A study of various NES sequences led to the proposition that the majority of proteins transported by Crm-1 bear a NES that resembles one of three major consensus sequences (19).

Both NLS and NES sequence motifs have been identified in apoptin (14). The NES lies in the N-terminus of the protein, spanning residues 37 to 46, while the NLS lies in the C-terminus, spanning residues 70 to 121 (14). The NLS and NES are functional in both transformed and nontransformed cells, and export of apoptin is Crm-1 dependent (14). Thus, nucleocytoplasmic shuttling is responsible for the cell-type specific localization of apoptin.

More importantly, it is this shuttling process that allows apoptin to induce apoptosis in a cell-type specific manner. Loss of a functional NLS renders apoptin incapable of entering the nuclei of transformed cells and interacting with the APC1 to induce apoptosis (14). Interestingly, loss of a functional NES also renders apoptin incapable of inducing apoptosis in transformed cells, perhaps by eliminating a region involved in multimerization, which could be critical to the process of apoptosis induction (14). Nucleocytoplasmic transport is therefore a critical component of the mechanism of apoptosis induction employed by apoptin.

Careful study of nucleocytoplasmic shuttling of apoptin has provided tremendous insight into its mechanism of selective induction of apoptosis. It seems reasonable to expect that

examination of the nucleocytoplasmic transport of related proteins with similar functions could also provide evidence of processes and interactions critical to their mechanisms of apoptosis induction. To this end, we will continue with an examination of the sucellular localization pattern of PCV2 VP3 and a putative mechanism by which it induces apoptosis in transformed cells.

1.7 Apoptosis Induction by PCV2 VP3

In terms of genomic organization, PCV2 is similar to PCV1. The genome of PCV2 consists of a circular, single-stranded DNA molecule with multiple overlapping, ambisense ORFs (30). As is typical of a circovirus, the largest reading frames, ORF1 and ORF2, are oriented in opposite directions about an intergenic region of the genome (30, 4). Like PCV1, PCV2 ORF1 encodes the Rep protein while PCV2 ORF2 encodes the Cap protein (25). Replication of the viral genome is thought to proceed by a RCR mechanism similar to that of PCV1 (30).

Unlike PCV1, the expression of ORF3 of PCV2 has been studied. The apoptotic activity of PCV2 VP3 is known to be a direct cause of viral pathogenesis in vivo (25). In addition, PCV2 VP3 induces apoptosis in H1299 cells but not in nontransformed cells, implying that like apoptin, it is capable of inducing cell-type specific apoptosis (4). Induction of apoptosis in these cells was associated with a cytoplasmic localization of PCV2 VP3, although cytoplasmic localization of PCV2 VP3 was also observed in primary cells (4). Putative NES and NLS motifs were identified in PCV2 VP3, but in concordance with the observed localization pattern, the putative NES was a better match to the consensus than was the NLS (4). The difference in

localization of PCV2 VP3 relative to apoptin is therefore an indication that the proteins may induce apoptosis in transformed cells by different mechanisms.

Indeed, a unique mechanism for the apoptotic activity of PCV2 VP3 has been proposed (26). In this mechanism, PCV2 VP3 binds to a porcine E3 ubiquitin ligase known as pPirh2 in the cytosol of transformed cells. This interaction leads to destabilization of pPirh2 and a decrease of the protein in the cell that is associated with a rise in p53, triggering the onset of apoptosis (26). Although evidence in support of this mechanism has been documented, the implication that PCV2 VP3 induces apoptosis in a p53 dependent manner conflicts with findings that PCV2 VP3 can induce apoptosis in H1299 cells, which are derived from a p53-negative non-small cell lung carcinoma cell line (4, 26). The mechanism for apoptosis induction by PCV2 VP3 needs to be investigated further to resolve this discrepancy.

1.8 Investigation of PCV1 for Proteins that Selectively Induce Apoptosis

Studies of CAV VP3 and PCV2 VP3 indicate that these two viral proteins localize to separate, distinct regions within transformed cells, but somehow both are able to bring about apoptosis of these cells (7, 4, 14, 26). It is possible that the CAV and PCV2 VP3 proteins evolved different strategies of apoptosis induction that are advantageous to each virus (13). Alternatively, the shared phylogeny of these two viruses, albeit limited, suggests that their mechanisms of apoptosis induction may have more in common than is currently realized (35, 36). One means of distinguishing between these two possibilities to better understand the implications of viral induced apoptosis in transformed cells is to examine other members of the

Circovirus genus and evaluate the apoptosis-inducing potential of their proteins in transformed cells.

In this regard, PCV1 is one such candidate for further study. The categorical relationship of this virus with other *Circovirus* members is not superficial, as is evident from the aforementioned 76% sequence conservation in the PCV1 and PCV2 genomes and the results of phylogenetic analysis of the two viruses (35, 33). A comparison of the ORF3 regions in PCV1 and PCV2 demonstrates that 61.5% of the expected amino acid sequence is conserved, suggesting that PCV2 VP3 and the putative PCV1 VP3 may share some functional domains (24). As far as we are aware, the apoptotic activity of PCV1 VP3, and expression of the protein in general, have never been studied. Thus, our interests focus on examining the expression of PCV1 VP3 in transformed cells to determine its subcellular localization pattern and apoptotic potential.

2 Materials and Methods

2.1 Sequence Analysis

Sequence alignments were used to compare PCV1 ORF3, PCV2 ORF3 and CAV ORF3 as well as the amino acids encoded by these three reading frames. Alignments were performed on CLC Free Workbench 4.0.2, developed by CLC bio A/s, using the Clustal alignment feature. Clustal alignments were also used to compare PCV1 genomes to determine a consensus for the sequence of PCV1 ORF3. Comparison of the sequence of our PCV1 ORF3 isolate with published PCV1 ORF3 sequences was also performed in this manner.

2.2 Isolation and Sequencing of PCV1 ORF3

PCV1 ORF3 was isolated and amplified by PCR using centrifuged PK-15 cells as a source of template DNA. Primers were designed based on the consensus sequence for PCV1 ORF3 determined by Clustal alignment. The forward primer had an EcoRI site and a kozak sequence. The sequence for the forward primer is: 5' GCGAATTCTACACCATGGCTCACTTTCAAAAG 3'. The sequence of the reverse primer is: 5' CCGGATCCTCAGTGAAAATGCCAAG 3'. The reverse primer had a BamHI site and also overlapped the stop codon of PCV1 VP3. The melting temperature of the forward primer was 66.62 and 64.77 for the reverse primer, as calculated in CLC Free Workbench 4.0.2.

The PCR reaction mixture consisted of PCR buffer (50 mM KCl; 10 mM Tris-HCl (pH 8.3); 15 mM $MgCl_2$), 200 µM of each dNTP, 0.4 µM of each primer, 1 Unit/20 µL Taq DNA polymerase, and PK-15 cells. The PCR cycle consisted of an initial denaturing phase at 95° for 4 minutes followed by 30 amplification cycles (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute). The reactions were held at 72°C until retrieved from the PCR machine.

Following completion of PCR, the reaction components were separated by gel electrophoresis on a 1% agarose gel containing 0.20 µg/mL ethidium bromide. A 0.600 kb amplicon from the PCV1 ORF3 reaction was identified, excised, and gel purified using the GeneClean kit from MP Biomedicals, LLC. The purified amplicon was inserted into a T-vector using the Promega pGEM-T Vector kit. DH5α competent *E. coli* were transformed with the products of the T-vector ligation and grown overnight at 37°C on agar plates containing 0.100 mg/mL ampicillin, 0.1 mM IPTG, and 40 µg/mL Xgal. The T-vector is designed to have a β-

galactosidase gene span the point of insertion, so white colonies were selected. Plasmid DNA was isolated by alkaline lysis minipreparation, digested with BamHI and EcoRI, and separated by electrophoresis on a 1% agarose gel containing 0.20 µg/mL ethidium bromide. A colony carrying a plasmid with restriction digest fragments similar in size to the T-vector and PCV1 ORF3 insert was grown overnight at 37°C in 100 mL LB media with 0.100 mg/mL ampicillin. The plasmid DNA was then harvested by midipreparation using the Promega Wizard® Plus Midipreps DNA Purification System and sent to be sequenced.

2.3 PCV1 ORF3 Sub-cloning into the pEGFP and p3XFLAG-*myc*-CMV-26 Vectors

After confirming that PCV1 ORF3 had been isolated by sequencing, PCV1 ORF3 was excised from the T-vector clone by restriction digest with BamHI and EcoRI. The excised fragment was isolated by electrophoresis on a 1% agarose gel containing 0.20 µg/mL ethidium bromide and purified using the GeneClean kit from MP Biomedicals, LLC. The pEGFP and p3XFLAG-*myc*-CMV-26 vectors were each isolated similarly following digestion with BamHI and EcoRI.

Ligation reactions using T4 DNA ligase were then performed to incorporate PCV1 ORF3 into each of the vectors. Each reaction mixture was used to transform DH5α *E. coli* following heat shock at 42°C for 1 minute. *E. coli* transformed with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 reaction mixture were grown overnight at 37°C on agar plates with 0.100 mg/mL ampicillin and those transformed with the pEGFP-PCV1 ORF3 reaction mixture were grown overnight at 37°C on agar plates with 0.100 mg/mL kanamycin. Colonies were selected from each plate and were further screened by alkaline lysis minipreparation of the plasmid DNA

followed by restriction digestion with EcoRI and BamHI and separation of the resulting fragments by electrophoresis on a 1% agarose gel containing 0.20 µg/mL ethidium bromide. Plasmids consisting of fragments similar in size to PCV1 ORF3 and the respective vector were further cloned and purified by midipreparation and sent away for sequencing as previously described.

2.4 Transfection of H1299 cells

Cells from the H1299 non-small cell lung carcinoma cell line were grown in 6 well plates in DMEM/High glucose with 10% FBS and PSF (100 units/mL Pen G sodium; 100 mg/mL streptomycin sulfate; 0.25 mg/mL amphotericin B) overnight. The cells were then transfected with either the pEGFP-PCV1ORF3 or p3XFLAG-*myc*-CMV-26-PCV1ORF3 vector using the Qiagen Effectene Transfection Reagent kit. Mock transfected cells served as negative controls for the cells transfected with the p3XFLAG-*myc*-CMV-26-PCV1ORF3 vector and cells transfected with the pEGFP vector alone were negative controls for cells transfected with the pEGFP-PCV1ORF3 vector. At both 24 hours and 48 hours post-transfection, pEGFP-PCV1ORF3 transfected cells were fixed with 4% paraformaldehyde in PBS, stained with DAPI (4',6'-diamidino-2-phenylindole), mounted on slides with mounting media (50% glycerol; 100 mM Tris (pH 7.5); 2% DABCO), and imaged by epifluorescence microscopy. At 72 hours post-transfection, p3XFLAG-*myc*-CMV-26-PCV1ORF3 transfected cells were fixed with 4% paraformaldehyde in PBS and stained with crystal violet.

3 Results

3.1 The ORF3 Sequences of PCV1, PCV2, and CAV are Homologous and May Encode Proteins with Similar Functions

Preexisting data showed that the proteins encoded by ORF3 of CAV and PCV2 normally induce apoptosis in host cells during an infection (25, 34). Both of these viruses are members of the *Circoviridae* family, and in both, ORF3 overlaps a larger reading frame that encodes a protein necessary for viral replication (24, 34). The *Circoviridae* family member PCV1 also contains an ORF3 oriented in this manner (see Figure 1). The similar taxonomy of these viruses and the similar orientations of their ORF3 regions suggest that this portion of the genome could have been evolutionarily conserved. If true, this would in turn imply that PCV1 ORF3 could encode a protein similar in function and structure to the VP3 proteins of CAV and PCV2.

To investigate the possibility that ORF3 has been evolutionarily conserved among these viruses, Clustal alignments of the PCV1, PCV2, and CAV genomes were implemented in CLC Free Workbench 4.0.2. These alignments established ORF3 of PCV1 as a region homologous to PCV2 ORF3 and CAV ORF3. Approximately 81% of the sequence in PCV2 ORF3 was conserved in PCV1 ORF3, while only 39% of the sequence in CAV ORF3 was conserved in PCV1 ORF3. A unique feature of PCV1 ORF3 is that it is almost double the length of either CAV ORF3 or PCV2 ORF3. The end of PCV2 ORF3 is defined by the stop codon UAA, while the homologous codon in PCV1 ORF3 is UAU, which encodes a tyrosine residue. The result of this alteration is that PCV1 ORF3 consists of 621 nucleotides while PCV2 ORF3 and CAV ORF3 are 315 and 366 nucleotides in length, respectively.

Clustal alignments of the amino acid sequences determined from the ORF3 sequences of the three viruses were also performed to identify regions of the putative PCV1 VP3 involved in nucleocytoplasmic transport. The reported sequence of the putative NES identified in PCV2 VP3 is VYIG/SLPITLL while in apoptin the sequence of the putative NES is IAGITITLSL (4, 14). We identified the region from amino acids 38 to 49 as the putative NES of PCV1 VP3, which has the sequence VYSCLPITLLHL as shown in Figure 2. In apoptin, the putative NLS was reported to be bipartite, consisting of two sequence elements enriched in basic amino acids located in the C terminus. Both sequence elements of apoptin's NLS contain three consecutive basic residues (14). Visual inspection of the C terminal region of PCV1 VP3 demonstrates that regions enriched in basic residues exist, but these residues do not cluster together as is seen in apoptin's NLS. Although this does not rule out the existence of a NLS in PCV1 VP3, it precludes a definitive identification of a NLS based on amino acid alignment data.

3.2 Isolation of PCV1 ORF3

To study the expression of PCV1 ORF3, this portion of the PCV1 genome was isolated and amplified by PCR using PCV1-infected PK-15 cells as a source of template. As shown in Figure 3, an amplicon of approximately 600 nucleotides was subsequently detected by gel electrophoresis and inserted into a T-vector as described in the Materials and Methods section. Sequencing of this clone confirmed that PCV1 ORF3 had been inserted in its entirety. A comparison of the ORF3 sequence from this clone to PCV1 sequences archived in the NCBI database demonstrated that the isolate was 100% identical to ORF3 of PCV1 strain SC-11.

Next, PCV1 ORF3 was excised from the T-vector clone and inserted into pEGFP-C1 and p3XFLAG-*myc*-CMV-26 vectors using engineered BamHI and EcoRI restriction sites (see Figure 3). Sequencing of positive screened clones showed that no mutations had been introduced into PCV1 ORF3 during the cloning process. Moreover, PCV1 ORF3 was in frame with the genes for GFP and 3X FLAG in each of the respective vectors.

3.3 Expression of PCV1 ORF3 in H1299 Cells Localized to the Cytoplasm and was Associated with Lowered Confluency

To determine the subcellular localization pattern of PCV1 VP3 in transformed cells, H1299 cells were transfected with the pEGFP-C1-PCV1 ORF3 construct. As is shown in Figure 4, GFP-PCV1 VP3 expressed in cells fixed and observed by epifluorescence microscopy 24 hours after transfection demonstrated a distinct cytoplasmic localization compared to GFP controls. The same localization pattern was seen in cells expressing the GFP-PCV1 VP3 fusion product 48 hours after transfection (see the lower and upper rows of panels in Figure 4).

In addition to being distinctly cytoplasmic, the observed localization of GFP-PCV1 ORF3 in transfected cells was also perinuclear. As seen in the top row of panels in Figure 4, the GFP expressed in transfected control cells was distributed in a diffuse pattern throughout the cell, and cytoplasmic extensions, such as pseudopodia, were visible at the edges of the cells. In the cells that expressed GFP-PCV1 VP3, less detail was visible at the edges of the cell by green fluorescence (see the middle and lower rows of panels in Figure 4). Overall, cells that expressed GFP-PCV1 VP3 displayed a more elongated morphology than those that expressed GFP alone (compare the top row of panels in Figure 4 to the middle and lower rows).

Cells transfected with the pEGFP-C1-PCV1 ORF3 construct also presented a more punctate pattern of GFP-PCV1 ORF3 expression compared to control cells that expressed GFP. Inspection of the middle and lower rows of panels in Figure 4 demonstrates the existence of foci of GFP-PCV1 VP3 expression that clustered in the center of cells close to the nucleus. Comparison of these cells with controls that expressed GFP (shown in the top row of panels in Figure 4) indicated that these foci were markedly absent in control cells.

Aside from determining the subcellular localization of GFP-PCV1 ORF3, transfections were also conducted with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 vector to determine if PCV1 VP3 is capable of inducing apoptosis in transformed cells. After 72 hours, wells on a 6 well plate that had been transfected with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 vector displayed lower confluencies of H1299 cells than a mock-transfected control (see Figure 5). Furthermore, the confluencies of both wells transfected with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 vector were comparable.

4 Discussion

Apoptin has been extensively studied due to its ability to specifically induce apoptosis in transformed cells. Recent study of the expression of PCV2 VP3 demonstrated that this protein is also capable of inducing apoptosis in transformed cells (4). Similar structural elements have also been identified in both viral proteins (4). Taken together, these findings could indicate that apoptin and PCV2 VP3 represent of a class of homologs conserved within the *Circoviridae* family, the majority of which have yet to be identified.

The taxonomical relationship of CAV and PCV2 with PCV1, in addition to similarities in the orientation of ORF3 within each viral genome, implied that PCV1 could potentially encode a VP3 protein with functions similar to apoptin and PCV2 VP3. A comparison of the genomes of these three viruses supported this hypothesis by demonstrating that 81% of the sequence in PCV1 ORF3 is identical to that in PCV2 ORF3. Additional support was provided by a comparison of the amino acid alignments of all three proteins, from which it was evident that the NES of PCV1 is very similar to that of PCV2, and to a lesser extent, that of apoptin (see Figure 2).

Clustal alignments of the amino acid sequences of PCV1 VP3, PCV2 VP3, and apoptin revealed disparate characteristics as well. Although conservation is evident in the N-termini of these proteins, their C-termini are far more divergent. In particular, the C-terminus of PCV1 VP3 is almost twice as long as the C-terminus of either apoptin or PCV2 VP3 (see Figure 3). The NLS, which is situated in the C-terminus of PCV2 VP3 and apoptin, is indiscernible by Clustal alignment. These unique structural features could confer unique functional features to PCV1 VP3. The additional amino acids in its C-terminus could indicate that PCV1 VP3 has a functional domain that is not present in apoptin or PCV2 VP3. Additionally, the lack of a distinct NLS could mean that PCV1 VP3 is incapable of inducing apoptosis, as this element is essential to apoptosis-induction by apoptin (14).

An important means of studying the functionality of structural elements of PCV1 VP3 further was to determine the subcellular localization of pattern of GFP-PCV1 VP3 in transformed cells. Nucleocytoplasmic shuttling of apoptin, specifically its import into the nucleus, has been shown to be a prerequisite for the induction of apoptosis (14). This would imply that the subcellular distribution of GFP-PCV1 VP3 may play a role in determining its

ability to induce apoptosis in transformed cells. Finally, ascertaining the subcellular localization pattern of GFP-PCV1 VP3 was also of interest because, to our knowledge, PCV1 VP3 had never before been characterized.

GFP-PCV1 VP3 was predominantly distributed in the cytosol of transfected H1299 cells, which was clearly distinct from the diffuse distribution of GFP in controls (see Figure 4). An immediate implication of this difference was that the PCV1 VP3 portion of our construct had been expressed. Furthermore, this localization pattern suggests that the identified putative NES could be functional. However, it is not possible to determine from these results alone if GFP-PCV1 VP3 is even able to gain access to the nucleus. If PCV1 VP3 does not have a functional NLS, this property, coupled with the size of GFP-PCV1 VP3, could preclude its entry into the nucleus altogether, resulting in the observed localization pattern. On the other hand, the cytoplasmic distribution of GFP-PCV1 VP3 also does not exclude the possibility of the existence of a functional NLS, as a cytoplasmic distribution could still be favored in the event that both a NLS and a NES are present and functional.

The observed cytoplasmic localization of GFP-PCV1 VP3 in H1299 cells is similar to that of GFP-PCV2 VP3 but contrasts the nuclear localization displayed by GFP-apoptin expressed in transformed cells (4, 7). Despite these differences, both apoptin and PCV2 VP3 have been shown to be capable of inducing apoptosis in these cells (4, 7). Thus, examination of the subcellular distribution of GFP-PCV1 VP3 alone would have allowed nothing to be determined concerning the ability of PCV1 VP3 to selectively induce apoptosis. Instead, we monitored changes in the confluency of H1299 cells following transfection with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 vector. It was evident from our observations of the transfected cells through a light microscope that more dead cells were present in the p3XFLAG-*myc*-CMV-26-

PCV1 ORF3-transfected wells compared to the mock transfected control. Indeed, crystal violet staining of these wells 72 hours post-transfection does demonstrate that control cells are more confluent than those that presumably expressed 3X FLAG-PCV1 VP3 (see Figure 5). However, the observed differences in confluencies are slight and confirmation of our results using a more sensitive and specific assay is necessary before we can conclusively determine that PCV1 VP3 is capable of inducing apoptosis in transformed cells.

In spite of this shortcoming, hypotheses can still be made about the putative apoptosis-inducing activity of PCV1 VP3. Based on the significance of nucleocytoplasmic transport to the mechanism of apoptosis-induction employed by apoptin, an obvious hypothesis is that the exclusion of PCV1 VP3 and PCV2 VP3 indicates that they induce apoptosis by a mechanism distinct from that of apoptin. Indeed, some researchers have already presented evidence in support of this hypothesis (26). Alternatively, these proteins may employ the same mechanism of apoptosis-induction but differ in terms of when they are able to access the nucleus. For example, PCV1 VP3 and PCV2 VP3 could access the APC/C during mitotsis following breakdown of the nuclear envelope. The latter hypothesis could even apply to apoptin, meaning that nucleocytoplasmic shuttling per se would not be necessary to its ability to kill transformed cells. Further study of structural elements of PCV1 VP3, notably the NES and NLS, may help distinguish between these hypotheses. Further study of structural elements of PCV1 VP3, notably the NES and NLS, may help distinguish between these hypotheses.

The most straightforward means of evaluating the functionality of the NES and NLS in PCV1 VP3 would be to uncouple these elements. Primers could be designed to amplify separate segments of PCV1 ORF3 containing part or all of the sequence encoding one element and none of the sequence encoding the other. Each of these truncation mutants could be ligated into the

pEGFP-C1 vector so the localization of each mutant can be followed separately. Localization patterns of these mutants would be investigated in both H1299 cells and primary cells to account for cell-specific differences in localization patterns. We would expect that the truncated form of PCV1 ORF3 encoding the NES would localize to the cytoplasm when expressed in H1299 cells. Nuclear localization of PCV1 ORF3 truncation mutants encoding C terminal areas of the protein would demonstrate the existence of a functional NLS in PCV1 VP3 and would simultaneously indicate in which regions of the protein the NLS lies. An observation of diffuse or cytoplasmic localization patterns for all truncated versions of PCV1 VP3 expressed in both cell types would indicate that the protein is incapable of accessing the nucleus in a karyopherin-dependent manner. These experiments would also need to be repeated in primary cells to determine if localization patterns and nucleocytoplasmic shuttling is cell-type specific.

Another feature of the localization pattern of GFP-PCV1 VP3 that should be investigated further is the presence of foci, which is similar to the punctate expression pattern of GFP-apoptin in H1299 cells (4). This pattern suggests that both proteins could be multimerizing when expressed in H1299 cells. To test this hypothesis for PCV1 VP3, yeast two-hybrid assays could be performed. Transfection of primary cells with our GFP-PCV1 VP3 construct should also be conducted to determine if the observed aggregation is cell-type specific. These experiments could also be expanded to investigate the possibility that PCV1 VP3 aggregates with proteins endogenous to the host cells.

A final inquiry worthy of investigation concerns the expression of PCV1 VP3 during viral infection of porcine cells. If a difference in the effectiveness of apoptosis induction exists between PCV1 VP3 and PCV2 VP3 in primary cells, it could explain why a PMWS phenotype develops in swine only as a result of PCV2 infection. Alternatively, if the proteins are equally

effective in inducing apoptosis of primary cells, the difference in virulence between the viruses could be the result of silencing expression of ORF3 in PCV1 by an unknown mechanism. Both hypotheses demonstrate the importance of studying PCV1 VP3 to better understand the pathology of PMWS.

We have shown that PCV1 VP3 is a homolog of apoptin and PCV2 VP3, both of which are known to induce apoptosis when expressed in transformed cells (4, 7). This activity makes the proteins of great interest as possible therapeutic agents. Moreover, PCV2 VP3 is of interest due to its suggested role in the development of PMWS in swine. Although PCV1 VP3 is not associated with a pathology and has not been conclusively shown to be capable of mediating apoptosis in transformed cells, understanding the structural differences between this protein and its homologs in PCV2 and CAV may explain its functional difference. Studying the structure and function of PCV1 VP3 is crucial to understanding structural elements in PCV2 VP3 and CAV VP3 critical to their roles in inducing apoptosis.

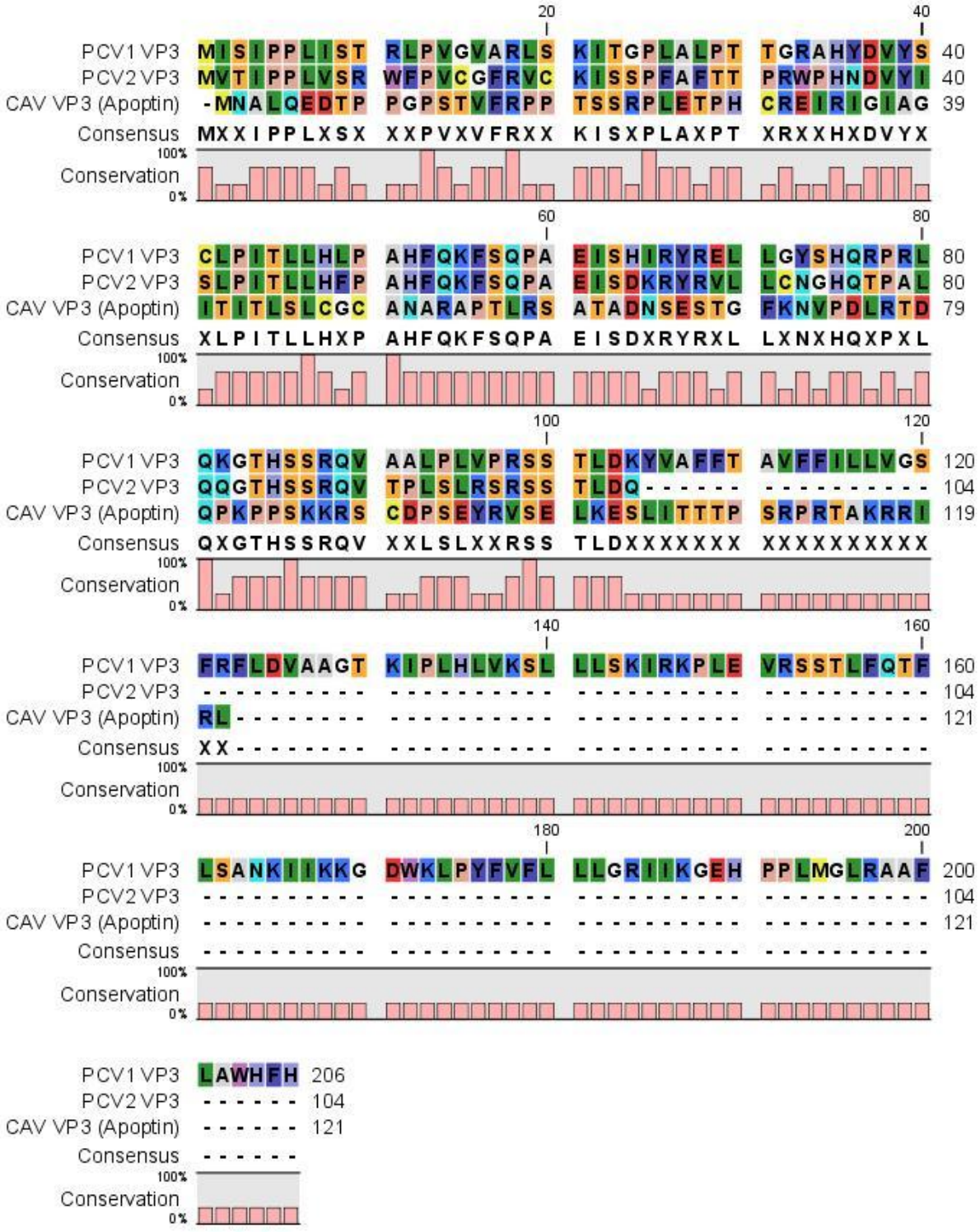

Figure 2-Amino acid sequence alignment of PCV1 VP3, PCV2 VP3, and CAV VP3 (apoptin). Residues 37 to 46 of apoptin comprise its NES while core basic residues of each element of the bipartite NLS are seen from residues 86 to 88 and 116 to 118. The putative NES of PCV1VP3 incorporates residues 38 to 49.

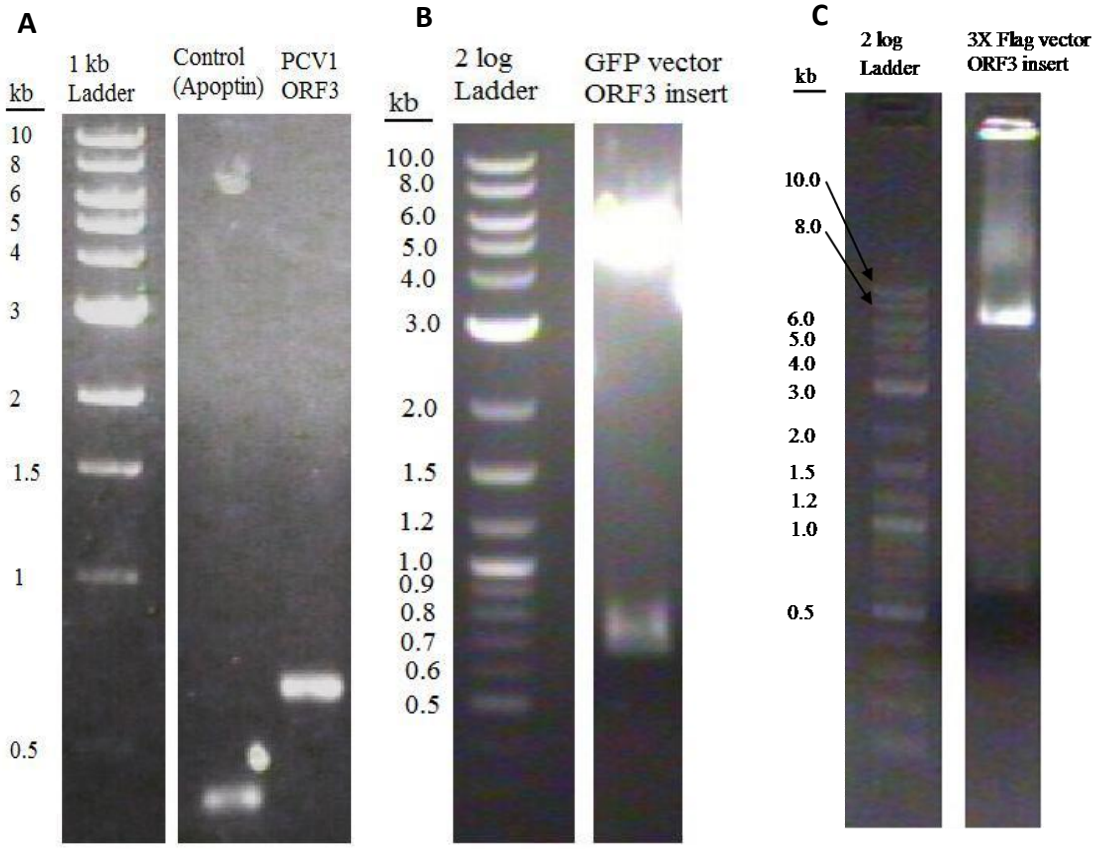

Figure 3-PCR and Cloning into pEGFP-C1 Vector. (A): PCR using PCV1-infected PK-15 cells as a source of template resulted in isolation of an amplicon of approximately 0.6 kb. Apoptin serves as a positive control. (B): Restriction of plasmid DNA from a Kan+ colony using BamHI and EcoRI yielded a 4.7 kb fragment and a 0.7 kb fragment, representing the pEGFP-C1 vector and PCV1 ORF3 insert, respectively. (C): Restriction of plasmid DNA from a Amp+ colony using BamHI and EcoRI gave a 6.3 kb fragment and a 0.6 kb fragment, representing the p3XFLAG-*myc*-CMV-26 vector and PCV1 ORF3 insert, respectively.

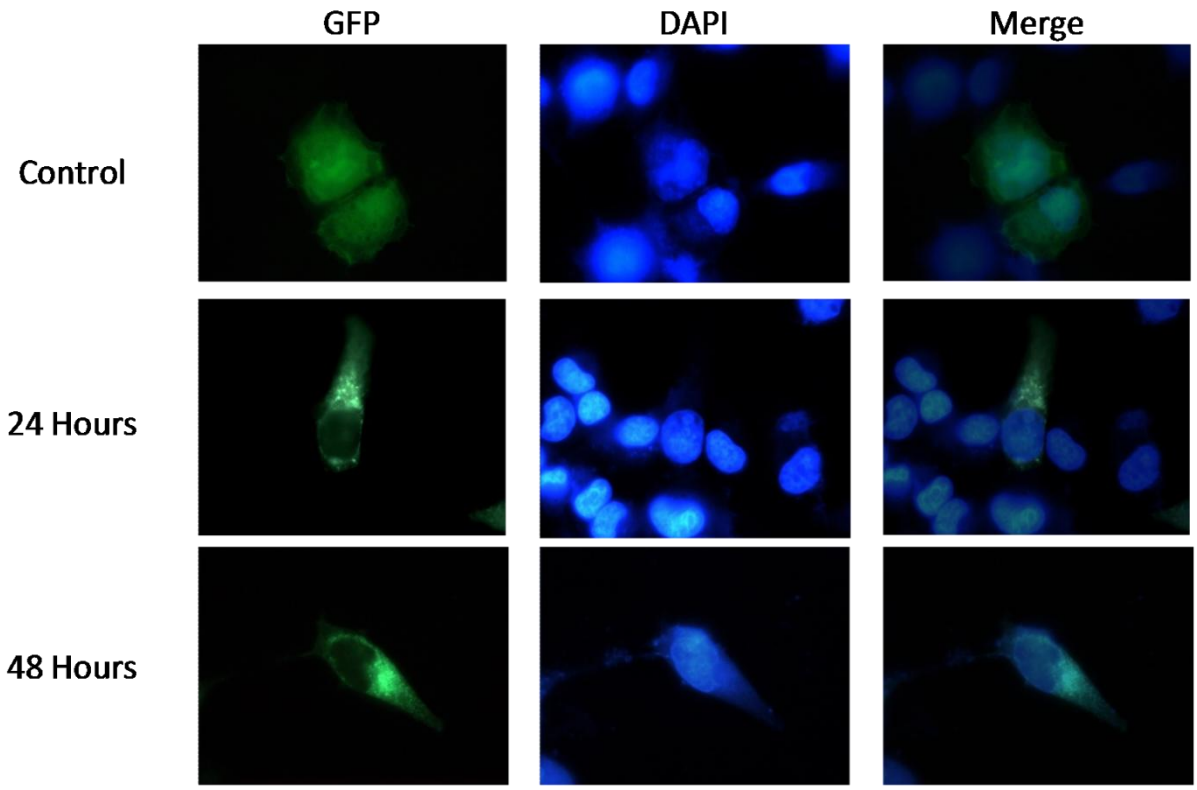

Figure 4-Subcellular localization of GFP-PCV1 VP3 construct in H1299 cells. The GFP-PCV1 VP3 fusion product displayed a distinct cytoplasmic localization compared to the GFP control both 24 hours and 48 hours after transfection. Foci are clearly visible in transfected cells, which could represent points of GFP-PCV1 VP3 aggregation. The lack of GFP-PCV1 VP3 in the nucleus suggests that PCV1 VP3 could have a functional NES.

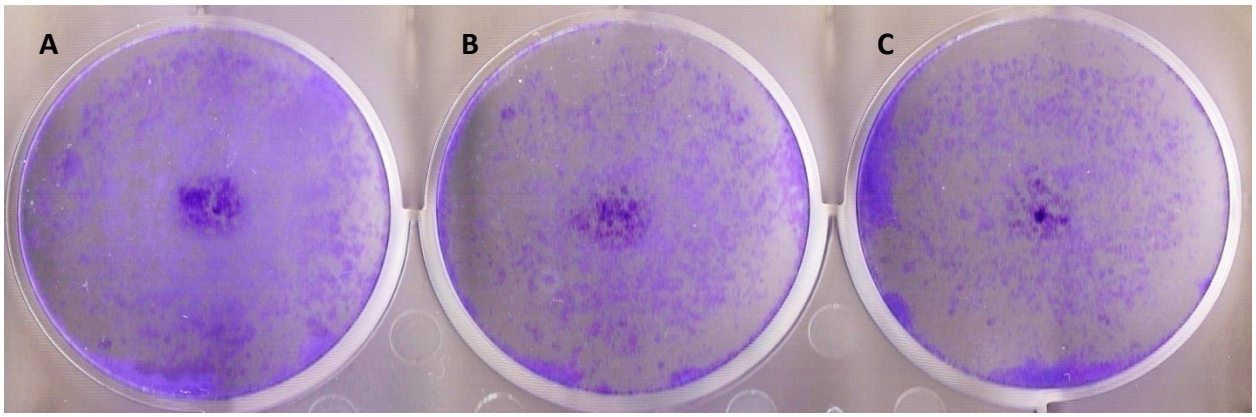

Figure 5-Confluency of H1299 cells 72 hours after transfection with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3**.** H1299 cells in a 6 well plate were fixed and stained with crystal violet 72 hours post-transfection. The well shown in (A) contains mock-transfected controls while (B) and (C) are duplicate wells transfected with the p3XFLAG-*myc*-CMV-26-PCV1 ORF3 vector.

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