Nuclear Localization of the ORF2 Protein Encoded by Porcine Circovirus Type 2

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Infectious porcine circovirus type 2 (PCV2) was generated following transfection of a porcine retina cell line (VIDO R1) with cloned circovirus DNA. Expression of open reading frame 2 (ORF2) was detected at 24 h postinfection and onwards increasingly throughout the infection by Western blot analysis using ORF2 specific polyclonal antibody. Moreover, the ORF2 protein was also detected in purified PCV2 virus, indicating that ORF2 is a structural component of PCV2 viral capsid. Nuclear localization of PCV2 ORF2 was demonstrated by immunofluorescence assay in PCV2-infected cells. An analysis of the subcellular localization of a series of truncation mutants of ORF2 fused with the green fluorescent protein indicated that the nuclear localization signal of ORF2 was conferred by the N-terminal 41 amino acids. This domain was further analyzed through site-directed mutagenesis, suggesting that the presence of basic amino acid residues at positions 12 to 18 and 34 to 41 are important for the strict nuclear targeting of PCV2 ORF2.

INTRODUCTION

As a member of the family Circoviridae, porcine circovirus (PCV) has a circular, single-stranded DNA genome with a nonenveloped, icosahedral virus shell (Lukert et al., 1995). PCV replicates in the cell nucleus and produces large intranuclear inclusion bodies (Murphy et al., 1999). Currently, there are two recognized types of PCV. PCV type 1 (PCV1) isolated as a persistent contaminant of the continuous porcine kidney cell line PK-15 (ATCC CCL31) does not cause detectable cytopathic effects in cell culture and fails to produce clinical disease in pigs after experimental infection (Allan et al., 1995; Tischer et al., 1982, 1986). In contrast, PCV type 2 (PCV2) is closely associated with postweaning multisystemic wasting syndrome (PMWS) in weanling pigs (Allen et al., 1998; Ellis et al., 1998; Morozov et al., 1998). Comparison of the nucleotide sequences of PCV1 (Mankertz et al., 1997; Meehan et al., 1997) and PCV2 (Hamel et al., 1998; Mankertz et al., 2000; Meehan et al., 1998) reveals <80% identity, although the genomic organization is similar, especially in the arrangement of the two largest open reading frames (ORFs) with a putative origin of DNA replication. The largest ORF, ORF1, of the two types of PCV shows only minor variation with a homology of 85% and has been demonstrated to be the Rep protein in PCV1 (Mankertz et al., 1998). A higher rate of variation displayed in the ORF2 sequences of PCV1 and PCV2 (homology ~65%) would suggest that the type-specific features of PCV might be determined by the respective ORF2 protein. In line with this speculation, several PCV type-specific epitopes have been mapped on ORF2 sequences by synthetic peptide scanning analysis (Mahe et al., 2000). In another recent study, PCV2 ORF2 has been identified as a major structural protein that can form viral capsid-like particles in insect cells infected with ORF2-expressing recombinant baculovirus (Nawagitgul et al., 2000).

Because of the potentially close relevance of PCV2 ORF2 to PCV2 pathogenicity, the characterization of PCV2 ORF2 has become one of our research goals. To this end, we have cloned PCV2 genome DNA isolated from a piglet with PMWS (Liu et al., 2000). We have also expressed the PCV2 ORF2 gene as a fusion protein in Escherichia coli and generated a polyclonal antibody in a rabbit (Liu et al., 2001). In this study we describe the generation of infectious PCV2 virus upon transfection of the cloned PCV2 genome DNA and the expression profile of the ORF2 gene in PCV2-infected cells. We also studied the intracellular localization of the PCV2 ORF2 protein. We found that PCV2 ORF2 encodes a nuclear protein directed by the N-terminal domain (i.e., amino acids 1 to 41). Site-directed mutagenesis experiments in this domain assigned an important role of the individual basic residues at positions 12 to 18 and 34 to 41 in the complete nuclear localization of PCV2 ORF2.

RESULTS

Generation of the PCV2 virus by DNA transfection

In order to obtain a pure PCV2 virus preparation, we transfected a molecularly cloned, double-stranded replicative form of PCV2 genome DNA (Liu et al., 2000) into a
fetal porcine retina cell line transformed by the human adenovirus-5 (HAV5) E1 gene (VIDO R1 cell line) (Reddy et al., 1999). A previous study has shown that PCV1 can replicate in several pig-derived cell cultures (Allan et al., 1994), so we assumed that VIDO R1 cells would be susceptible to PCV2 infection. Since the VIDO R1 cell line was transformed by HAV5 E1 that can induce the S phase of the cell cycle and transactivate transcription (Shenk, 1996), we thought that it might also enhance PCV2 replication. To test whether the VIDO R1 cell line could be used to generate PCV2 virus, 70% confluent cells grown in a six-well dish was transfected by 5 mg of cloned PCV2 DNA. Prior to transfection, plasmid containing full-length PCV2 genome was digested with Sac II and purified after agarose gel electrophoresis to remove the vector portion. When the cells were confluent, they were trypsinized and transferred to a T-25 flask. No CPE was observed for 12 days posttransfection. Cells were then harvested, frozen, thawed three times, and used to infect fresh VIDO R1 cells. Polymerase chain reaction (PCR) using DNA extracted from the infected cells as a template and PCV2-specific primers amplified a product of the expected size, while no DNA was amplified from the uninfected cells (Fig. 1A). Moreover, an immunohistochemical staining assay using anti-PCV2-ORF2 polyclonal antibody (Liu et al., 2001) showed a positive signal only in PCV2-infected cells, while no staining was observed in uninfected VIDO R1 cells (Fig. 1B). These data indicated that we had obtained the PCV2 virus. The virus was then expanded in cell culture and purified by ultracentrifugation. The immunostaining assay described above was also used to determine the virus titer by counting the positive-stained cells after serial dilutions of the pelleted virus. The virus titer of this preparation was calculated to be \(2 \times 10^7\) infectious units (IU)/ml (Fig. 1B).

Characterization of PCV2 ORF2 expression in infected cells

To analyze the expression of the ORF2 gene, VIDO R1 cells were infected with PCV2 virus at a multiplicity of infection (m.o.i.) of 1 and harvested at various hours postinfection (h.p.i.). Proteins in cell lysates were analyzed by Western blot with anti-ORF2 polyclonal antibody (Liu et al., 2001). While no protein was observed in uninfected cells, a 30-kDa protein band was detected in PCV2-infected cells as early as 24 h.p.i. and found to be accumulating to a greater amount at later time points during infection (Fig. 2). When the purified PCV2 virus was analyzed in Western blot with the same anti-ORF2 antibody, a similar band was also detected (Fig. 2). These results indicated that the PCV2 ORF2 gene was translated as a late protein that was incorporated into the virus particles.

PCV2 ORF2 localizes in the nucleus

To study the intracellular localization of PCV2 ORF2, VIDO R1 cells were seeded onto a two-well chamber slide and infected with PCV2. At 48 h.p.i., indirect immunofluorescence assay was carried out with anti-ORF2 antibody. While no staining was visible in uninfected VIDO R1 cells, nuclear fluorescence was observed in PCV2-infected cells (Fig. 3), demonstrating that PCV2 ORF2 was localized in the nucleus.
Intracellular localization of deletion mutants of PCV2-ORF2-GFP fusion proteins

The nuclear targeting of a protein in eukaryotes is frequently conferred by basic amino acid sequences. PCV2 ORF2 contains a basic N-terminal region that may have nuclear targeting activity (Fig. 4). To test this speculation, the wild-type and deletion mutants of PCV2 ORF2 were fused to the N-terminus of green fluorescent protein (GFP). Four C-terminal deletion mutants lacking 44, 139, 183, and 192 amino acids from the C-terminus of ORF2, respectively, and one N-terminal mutant lacking 41 amino acids from the N-terminus of ORF2 were constructed (Fig. 5A). To examine the expression of the proteins synthesized from the hybrid genes, Vero cells were transfected with each plasmid under the control of the CMV promoter. Equal aliquots of all lysates were analyzed by Western blotting with anti-GFP antibody. As shown in Fig. 5B, a fusion protein of the expected size was produced from each expression plasmid.

Green fluorescence produced by the GFP portion in each fusion protein was then used to analyze the intracellular localization of these ORF2-GFP wild-type fusion and truncation mutants in transfected Vero cells 20 h after transfection. GFP expressed alone was distributed almost equally throughout the cell (b in Fig. 5C), whereas fusion of the wild-type ORF2 protein to GFP conferred nuclear localization (c in Fig. 5C). These results indicated that localization of the ORF2-GFP fusion protein was directed by ORF2, not by the GFP moiety. Moreover, nuclear localization of the ORF2-GFP fusion demonstrated that the ORF2 protein can localize to the nucleus without the assistance of other viral proteins.

The four mutants with progressive deletions from the C-terminus of ORF2 also exhibited nuclear accumulation (d–g in Fig. 5C), whereas the mutant with the N-terminal 41-amino-acid deletion localized almost exclusively in the cytoplasm (h in Fig. 5C). These results indicated that the nuclear targeting activity of PCV2 ORF2 was associated with the N-terminal 41 amino acid residues.

Intracellular localization of the mutants in the ORF2 N-terminal nuclear targeting domain

In order to further characterize the contribution of the basic amino acid residues in the N-terminal domain of PCV2 ORF2, eight mutants were constructed with the basic amino acid residues in this region substituted in groups of three or two (Table 1). The expression of each mutant PCV2 ORF2-GFP fusion protein in transfected Vero cells was demonstrated by Western blot analysis using anti-GFP antibody (Fig. 6A). The intracellular distribution of the eight mutants was determined after transfection into Vero cells (Fig. 6B and Table 1). The R5S-R6V-R7N, R9S-R10V-R11N, R24S-R25V-R26N, and H31S-R33G mutants (Fig. 6B) were exclusively localized in the nuclei of transfected cells, similar to the wild-type PCV2 ORF2-GFP (Fig. 5C), indicating that these basic residues are not required for the nuclear targeting of PCV2 ORF2. In contrast, the mutations of the basic residues in positions 12 to 14, 16 to 18, 34 to 37, and 39 to 41 in the N-terminal domain resulted in a diffuse distribution of both nuclear and cytoplasmic fluorescence (Fig. 6B), indicating that the basic residues at these positions are required for the complete nuclear localization of PCV2 ORF2.

DISCUSSION

The goal of this study was to investigate the expression and intracellular localization of the PCV2 ORF2 protein. First of all, we attempted to establish a pure PCV2 virus in cell culture. In two earlier published papers, PCV2 virus was obtained from PCV-free porcine kidney (PK-15) cells after transfection with a cloned PCV2 genome (Meehan et al., 1998; Mahe et al., 2000). In both cases the porcine kidney cells were treated with D-glucosamine, as it can stimulate entry to the S phase in the cell cycle in PK-15 cells (Tischer et al., 1987). It should

**FIG. 4.** Amino acid sequence of PCV2 ORF2. The single-letter amino acid code is used and the residues are shown in groups of ten. The sequence of the N-terminal domain is in bold letters with the residues important for the nuclear localization of PCV2 ORF2 underlined. Also shown are consensus patterns present in the PCV2 ORF2 sequence for potential posttranslational modifications indicated by symbols above the respective residues. □, N-myristoylation; ▽, protein kinase phosphorylation site; △, casein kinase phosphorylation site; ◊, N-glycosylation site.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1. pQBI25:</td>
<td>26.9 kDa</td>
<td>C+N</td>
</tr>
<tr>
<td>2. pORF2-GFP:</td>
<td>54.7 kDa</td>
<td>N</td>
</tr>
<tr>
<td>3. pORF2-Δ44(C)-GFP:</td>
<td>49.5 kDa</td>
<td>N</td>
</tr>
<tr>
<td>4. pORF2-Δ139(C)-GFP:</td>
<td>38.3 kDa</td>
<td>N</td>
</tr>
<tr>
<td>5. pORF2-Δ183(C)-GFP:</td>
<td>33.5 kDa</td>
<td>N</td>
</tr>
<tr>
<td>6. pORF2-Δ192(C)-GFP:</td>
<td>32.4 kDa</td>
<td>N</td>
</tr>
<tr>
<td>7. pORF2-Δ41(N)-GFP:</td>
<td>49.7 kDa</td>
<td>C</td>
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</tbody>
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be noted, however, that D-glucosamine is toxic for cell culture, so the treatment must be performed with caution and hence should be avoided whenever possible (Allan and Ellis, 2000). In this report, a fetal porcine retina cell line (VIDO R1) was transfected with a cloned PCV2 genome and infectious PCV2 virus was also generated. In contrast to the porcine kidney cell line, we found that D-glucosamine treatment of VIDO R1 cells was not necessary, presumably because the VIDO R1 cell line has been transformed by HAV5 E1, which can induce the S phase in the cell cycle.

As a first report, we also developed a quantitative assay to titrate PCV2 virus based on immunohistochemical (IHC) staining of infected cells using anti-ORF2 antibody. Previous studies on PCV1 titration used an indirect immunofluorescence assay (IFA) and counted the number of fluorescing cells (Tischer et al., 1986, 1995). For a comparative purpose, the PCV2 virus preparation was also titrated by IFA and the result was the same as that obtained by IHC, indicating that the two assays have similar sensitivities. However, besides that the IHC staining lasts much longer than IFA, the stained monolayer dishes in IHC could be easily labeled and thus divided into small viewing areas under a light microscope, rendering the counting of the positive cells very easy to perform, as opposed to the IFA method.

The generated PCV2 virus was then used to study the expression of the ORF2 gene. In PCV2-infected cells, a 30-kDa protein was detected in Western blots using anti-ORF2 antibody, consistent with a recent study (Nawagitgul et al., 2000). In a previous report, we showed that in vitro translation of the PCV2 ORF2 gene also yielded a 30-kDa protein (Liu et al., 2001), suggesting that the ORF2 protein is not posttranslationally modified in virus-infected cells, although PCV2 ORF2 contains several consensus patterns (Fig. 4). The temporal regulation of PCV2 ORF2 gene expression in PCV2-infected cells indicated that ORF2 is probably a late protein because the expression was detected only after 24 h.p.i. and the amount increased at later time points in viral infection. Moreover, ORF2 was also detected in purified virus, suggesting that ORF2 probably takes part in the late events of the virus replication cycle, e.g., the assembly of the progeny viruses as a structural component.

Using anti-ORF2 antibody, PCV2 ORF2 was characterized to be a nuclear protein in virus-infected cells by immunofluorescence assay. Figure 3 is the nuclear fluorescence of ORF2 at 48 h.p.i. We also have data showing the nuclear fluorescence of PCV2 ORF2 at 24 and 72 h.p.i., suggesting that there is no change in the intracellular localization of ORF2 in the PCV2 replication cycle.

Many nuclear targeting sequences have been identified. Even though there does not exist a strict consensus nuclear localization signal (NLS), stretches of basic amino acids are the principal components of NLS sequences (Silver, 1991), which are generally classified as one of three types (reviewed in Boulakis, 1993; Hicks and Raikhel, 1995; Nakai and Kanehisa, 1992). The "pat4" motif consists of a continuous stretch of four basic amino

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**TABLE 1**

Overview of Site-Directed Mutagenesis Experiments

<table>
<thead>
<tr>
<th>PCV2 ORF2-GFP genotype</th>
<th>Amino acid sequence (positions 1 to 41)</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>MTYPRRRYRRRRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N</td>
</tr>
<tr>
<td>R5S-R6V-R7N</td>
<td>MTYPSVNRRRRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N</td>
</tr>
<tr>
<td>R9S-R10V-R11N</td>
<td>MTYPRRRSYVRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N</td>
</tr>
<tr>
<td>R12S-H13V-R14N</td>
<td>MTYPRRRYRRRRHRPSALQILRRPWLHPRHRYWRRK</td>
<td>N + C</td>
</tr>
<tr>
<td>R16V-R18A</td>
<td>MTYPRRRYRRRRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N + C</td>
</tr>
<tr>
<td>R24S-R25V-R26N</td>
<td>MTYPRRRYRRRRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N + C</td>
</tr>
<tr>
<td>H31S-R33G</td>
<td>MTYPRRRYRRRRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N + C</td>
</tr>
<tr>
<td>H34V-R35N-R37V</td>
<td>MTYPRRRYRRRRHRPRSHLQILRRPWLHPRHRYWRRK</td>
<td>N + C</td>
</tr>
<tr>
<td>R39S-R40V-K41N</td>
<td>MTYPRRRYRRRRHRPRSHLQILRRPWLHPRHRYWRSVN</td>
<td>N + C</td>
</tr>
</tbody>
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*Note. The intracellular localizations of the resulting PCV2 ORF2-GFP fusion products are indicated (N, nuclear; C, cytosolic).

* A The eight double or triple amino acid mutations underlined were carried out on the N-terminal domain of PCV2 ORF2.
acids or three basic amino acids with histidine or proline. The pat7 motif begins with proline and is followed within three residues by a segment containing three basic residues of four. The third type of NLS, the "bipartite" motif, consists of two basic amino acids, a 10-amino-acid spacer and a 5-amino-acid segment containing at least three basic residues. The actual functioning of a particular NLS sequence in nuclear localization of a protein requires several additional factors, such as whether the motif is exposed on the surface of the molecule.

In search of the NLS within PCV2 ORF2, we have identified two amino acid stretches that represent the nuclear targeting sequence. This experiment began with the evaluation of the intracellular localization of ORF2-GFP constructs expressing truncations of the ORF2 gene. When the first 41 amino acids of the PCV2 ORF2 were deleted, the resulting mutant PCV2 ORF2-GFP fusion protein became cytoplasmic, in contrast to the specific nuclear localization of the wild-type PCV2 ORF2-GFP. We also demonstrated that this 41-amino-acid domain was sufficient to target GFP to the nucleus when linked to the 5'-end of the GFP gene. The site-directed mutagenesis studies performed in the nuclear targeting domain revealed that the mutant proteins with replacement of the arginine and histidine residues in two stretches consisting of seven and eight amino acids (\[11\text{R-R-H-R-14}\] and \[34\text{H-R-Y-R-W-R-R-K-41}\]), respectively, lost the strict nuclear localization, as observed in the case of the wild-type PCV2 ORF2-GFP fusion protein. The first nuclear targeting stretch overlaps a pat4 motif (\[11\text{R-R-H-R-14}\]) and also represents part of the N-terminal portion of a bipartite motif (\[11\text{R-R-H-R-P-S-H-18}\] and \[34\text{H-R-Y-R-W-R-R-K-41}\]), while the second stretch comprises the C-terminal portion of another bipartite sequence (\[25\text{R-R-P-W-L-V-H-P-R-H-R-Y-R-W-R-R-K-41}\]). In contrast, mutations of the basic amino acids within the other two pat4 motifs and
one pat7 sequence present in the PCV2 ORF2 did not affect nuclear signaling.

In conclusion, we have shown that PCV2 virus could be generated by DNA transfection of VIDO R1 cells. We have also described the expression of PCV2 ORF2 as a late protein and a structural component of the virus particles. Finally, we have shown that the N-terminal 41 amino acids of PCV2 ORF2 were necessary and sufficient for its specific nuclear localization. Detailed analysis of the N-terminal domain by site-directed mutagenesis revealed two amino acid stretches \textit{\textsuperscript{12}R-H-R-P-R-S-H} and \textit{\textsuperscript{34}H-R-Y-R-W-R-R-K}, as being essential for the strict nuclear targeting of PCV2 ORF2.

**MATERIALS AND METHODS**

**Cell culture**

Fetal porcine retina cell line (VIDO R1) (Reddy et al., 1999) and Vero cells were maintained at 37°C with 5% CO\textsubscript{2} in Eagles-based MEM media supplemented with 10 or 5% heat-inactivated fetal bovine serum (FBS), respectively.

**Transfection and infection**

Monolayers of VIDO R1 cells grown in a six-well dish were transfected with cloned PCV2 DNA using Lipofectin according to the manufacturer’s recommendations (GIBCO BRL). Prior to transfection, the PCV2 full-length genome was released from the plasmid by digestion with SacII (Liu et al., 2000). For infection, the transfected VIDO R1 cells were subjected to three cycles of freezing (−70°C) and thawing (37°C). The lysate was then clarified by centrifugation and used to infect fresh VIDO R1 cells.

For transient expression, analysis of GFP-containing fusion proteins, Vero cells in six-well dishes, or chamber slides were transfected by Lipofectamine and Plus reagent (GIBCO BRL). The intracellular localization of each fusion protein was observed 20 h after transfection with the aid of a fluorescein isothiocyanate (FITC) filter-equipped fluorescence microscope (Olympus AH-2).

**Virus purification and titration after immunohistochemical staining**

For purification of the PCV2 virus, PCV2-infected VIDO R1 cells were incubated with 0.5% Triton X-114 in phosphate-buffered saline (PBS) at 37°C for 45 min followed by Freon 113 (1,1,2-trichlorotrifluoroethane) extraction. The cell debris and membranes were clarified by centrifugation at 2000 g for 15 min. The viruses in the supernatant were pelleted at 35000 g for 3 h through a 20% sucrose cushion. The virus pellet was suspended in PBS and stored at −70°C. Virus titers were determined as infectious units by quantitative ORF2 protein immunoperoxidase staining. For this purpose, the cell monolayers in 12-well dishes were infected with serial dilutions of virus. After adsorption of virus for 1 h at 37°C, the cells were washed and overlaid with MEM containing 2% FBS and 0.7% agarose. On day 3 postinfection, the agarose overlay was removed and the cells were fixed and permeabilized with methanol/acetone (1:1 in volume) for 20 min at −20°C. After blocking with 1% bovine serum albumin for 1 h at room temperature, the cells were incubated with rabbit anti-ORF2 serum (Liu et al., 2001). After 2 h incubation, the plates were washed with PBS and then processed using the VECTASTAIN Elite ABC kit (Vector Laboratories). The reaction was developed with 3,3’-diaminobenzidine (DAB) tetrahydrochloride and observed under a microscope. By counting the positively stained cells, the virus titer was expressed as IU, where 1 IU was defined as one positively stained cell/focus at 3 days p.i.

**Viral DNA extraction and characterization**

Viral DNA was extracted from PCV2-infected VIDO R1 cell monolayers by the method of Hirt (1987). The viral DNA was then analyzed by PCR as described earlier (Liu et al., 2000).

**Plasmid constructions**

Molecular cloning was performed by standard techniques (Sambrook et al., 1989).

Construction of the full-length PCV2 genome clone. The cloning of the PCV2 genome was described previously (Liu et al., 2000). The double-stranded replicative form of the full-length PCV2 genome DNA can be released from the resulting plasmid upon SacII digestion.

Construction of PCV2-ORF2-GFP wild-type fusion and deletion mutants. Plasmid constructs are schematically shown in Fig. 5A. The vectors used in the construction were pQBI25 [containing a GFP coding sequence under the control of a cytomegalovirus (CMV) immediate early promoter; Quantum Biotechnologies] and pTargetT (containing a CMV immediate early promoter; Promega). The \textit{NheI-PstI} (753 bp) fragment containing the GFP gene from pQBI25 was ligated to a \textit{NheI-PstI}-digested PCR product (template, PCV2 full-length clone; primers, 5’-AAT TCT GCA GAT GAA TAA TAA AAA CCA TTA CGA A-3’ and 5’-AAC TCT GCA GAA TTC GCT AGC GGG TTC AAG TGG GGG GTC-3’). The ORF2-GFP fusion gene was then amplified by PCR from the resulting plasmid using primers 5’-GGG GGG GGA TCC CTT TTT CAG CTA TGA CGT ATC-3’ and 5’-TCC CAG CGT TAG AAG CTC TC-3’, and the \textit{BamHI}-digested fragment (1440 bp) was inserted into pTargetT vector, creating pORF2-GFP (Fig. 5A). Expression plasmids encoding the fusion of GFP with a series of deletion mutants of PCV2 ORF2 were generated from the pORF2-GFP plasmid by PCR primer-mediated mutagenesis. For this purpose, the coding sequences for
the N-terminal 41, 50, 94, and 189 as well as the C-terminal 192 amino acid residues were amplified by PCR and substituted for the full-length ORF2 in pORF2-GFP via appropriate restriction enzymes. The PCR fragments were confirmed by DNA sequencing.

Site-directed mutagenesis of the basic amino acid residues in the N-terminal domain. Eight double or triple amino acid substitutions in the N-terminal region of PCV2 ORF2 gene were constructed either by primer-directed PCR fragment mutagenesis or through insertion of pairs of complementary oligonucleotides between two restriction sites (Table 1). Synthetic oligonucleotides used were designed to mutate all the basic amino acid residues in the N-terminal domain as follows: for the R5S-R6V-R7N mutation, forward primer 5'-CAAGC TTACCA CCAAG ACAAC-3' and backward primer 5'-GCCGG TGTCT TCTCT TGGG TAGTT AACAG-3'; for the R9S-R10V-R11N mutation, forward primer 5'-CAAGG TTACA CGGCT AACAG ACAC-3' and backward primer 5'-GGGAC TGTCT GTTAA CTACC GCAGA AGAAG-3'; for the R24S-R25V-R26N mutation, forward primer 5'-GGGGG GGTTG CACG TATGG AGAAG GAAAA-3' and backward primer 5'-CAAGG AGGCG TTACA GCGTT AACAG ACAC-3'; for the R39S-R40V-K41N mutation, forward primer 5'-GGGGG GCCCG GGGCT GACGA GCCAG GCAC CGCTA CCGTT GGAGA A-3' and backward primer 5'-CAAGG AGGCG TTACA GCGTT AACAG ACAC-3'; for the H34V-R35N-R37V mutation, forward primer 5'-GGGGG GCCCG GGGCT GACGA GCCAG GCAC CGCTA CCGTT GGAGA A-3' and backward primer 5'-CAAGG AGGCG TTACA GCGTT AACAG ACAC-3'; for the R12S-H13V-R14N mutation, forward primer 5'-GGGGG GCCCG GGGCT GACGA GCCAG GCAC CGCTA CCGTT GGAGA A-3' and backward primer 5'-CAAGG AGGCG TTACA GCGTT AACAG ACAC-3'; for the R16V-H18A mutation, forward primer 5'-GGGGG GTACG TATGG AGAAG GAAAA-3' and backward primer 5'-CAAGG AGGCG TTACA GCGTT AACAG ACAC-3'.

Western blot analysis

Proteins in cell lysates were separated by SDS–PAGE in 10% gels using a discontinuous buffer system (Laemmli, 1970). Western blotting was carried out using anti-ORF2 polyclonal (1:200) (Liu et al., 2001) or anti-GFP monoclonal (1:500, Clontech) antibodies and peroxidase-labeled anti-species antibodies (1:5000, Kirkegaard & Perry Laboratories). The blots were developed with 4-chloro-1-naphthol (Bio-Rad).

Indirect immunofluorescence assay

Cells were grown in a two-well chamber slide and infected with PCV2 at a multiplicity of infection of 1. At 48 h.p.i., cells were washed with PBS before they were fixed and permeabilized with methanol/acetone (1:1 in volume) for 20 min at −20°C. The cells were blocked for nonspecific reactivity by incubation with 1% bovine serum albumin in PBS for 1 h. The cells were incubated with rabbit anti-ORF2 antibody (1:500 in PBS) for 2 h followed by a 1-h reaction with FITC-conjugated anti-rabbit IgG (1:500 in PBS, Jackson ImmunoResearch Laboratories Inc.). Cells were observed and photographed with a fluorescence microscope at a 400X magnification.

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