Replacement of the Replication Factors of Porcine Circovirus (PCV) Type 2 with Those of PCV Type 1 Greatly Enhances Viral Replication *In Vitro*[▽]

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Porcine circovirus type 1 (PCV1), originally isolated as a contaminant of PK-15 cells, is nonpathogenic, whereas porcine circovirus type 2 (PCV2) causes an economically important disease in pigs. To determine the factors affecting virus replication, we constructed chimeric viruses by swapping open reading frame 1 (ORF1) (*rep*) or the origin of replication (Ori) between PCV1 and PCV2 and compared the replication efficiencies of the chimeric viruses in PK-15 cells. The results showed that the replication factors of PCV1 and PCV2 are fully exchangeable and, most importantly, that both the Ori and *rep* of PCV1 enhance the virus replication efficiencies of the chimeric viruses with the PCV2 backbone.

Porcine circovirus (PCV) is a single-stranded DNA virus in the family *Circoviridae* (34). Type 1 PCV (PCV1) was discovered in 1974 as a contaminant of porcine kidney cell line PK-15 and is nonpathogenic in pigs (31–33). Type 2 PCV (PCV2) was discovered in piglets with postweaning multisystemic wasting syndrome (PMWS) in the mid-1990s and causes porcine circovirus-associated disease (PCVAD) (1, 9, 10, 25). PCV1 and PCV2 have similar genomic organizations, with two major ambisense open reading frames (ORFs) (16). ORF1 (*rep*) encodes two viral replication-associated proteins, Rep and Rep', by differential splicing (4, 6, 21, 22). The Rep and Rep' proteins bind to specific sequences within the origin of replication (Ori) located in the intergenic region, and both are responsible for viral replication (5, 7, 8, 21, 23, 28, 29). ORF2 (*cap*) encodes the immunogenic capsid protein (Cap) (26). PCV1 and PCV2 share approximately 80%, 82%, and 62% nucleotide sequence identity in the Ori, *rep*, and *cap*, respectively (19).

In vitro studies using a reporter gene-based assay system showed that the replication factors of PCV1 and PCV2 are functionally interchangeable (2–6, 22), although this finding has not yet been validated in a live infectious-virus system. We have previously shown that chimeras of PCV in which *cap* has been exchanged between PCV1 and PCV2 are infectious both *in vitro* and *in vivo* (15), and an inactivated vaccine based on the PCV1-PCV2 *cap* (PCV1-*cap*2) chimera is used in the vaccination program against PCVAD (13, 15, 18, 27).

PCV1 replicates more efficiently than PCV2 in PK-15 cells (14, 15); thus, we hypothesized that the Ori or *rep* is directly responsible for the differences in replication efficiencies. The objectives of this study were to demonstrate that the Ori and *rep* are interchangeable between PCV1 and PCV2 in a live-

virus system and to determine the effects of swapped heterologous replication factors on virus replication efficiency *in vitro*.

Construction of chimeric PCV infectious DNA clones. PCV1 and PCV2a infectious DNA clones (11, 12, 15) were used as the genomic backbone for the construction of 4 new chimeric infectious-virus clones, PCV1-PCV2 Ori (PCV1-Ori2), PCV1-PCV2 *rep* (PCV1-*rep*2), PCV2-PCV1 Ori (PCV2-Ori1), and PCV2-PCV1 *rep* (PCV2-*rep*1), by overlap extension PCR (Fig. 1A). The full-length chimeric genomes were assembled from two overlapping PCR fragments for chimera PCV1-Ori2 (Table 1, primers 3 plus 8 and 4 plus 7, PCV1 backbone) and chimera PCV2-Ori1 (primers 1 plus 6 and 2 plus 5, PCV2 backbone). Three fragments were used to assemble chimera PCV1-*rep*2 (primers 1 plus 15 and 2 plus 14, PCV2 backbone; primer 13 plus 16, PCV1 backbone) and chimera PCV2-*rep*1 (primers 3 plus 11 and 4 plus 10, PCV1 backbone; primer 9 plus 12, PCV2 backbone). Fusion PCR was used to assemble fragments of the chimeric DNA clones (primers 1 plus 2 or 3 plus 4). The chimeric fusion products were digested with KpnI or SacII and cloned into pCR2.1 or pBluescript II SK(+), respectively. Each chimeric clone was completely sequenced to confirm that no mutations had been introduced. Infectious-virus stocks of PCV1, PCV2, and the 4 chimeric viruses were generated by transfection of PK-15 cells with dimerized or concatamerized infectious clones (12, 15). PCV1 and PCV2 and all 4 chimeric infectious clones were found to produce infectious virus after transfection into PK-15 cells, and each infectious-virus stock was titrated by an immunofluorescence assay (12).

For growth characterization, PK-15 cells seeded in 24-well plates at 50% confluence were infected with a 0.5 multiplicity of infection of each virus, incubated for 1 h at 37°C, and washed three times with sterile Hanks' buffered salt solution. One ml of minimal essential medium (MEM) was then added to each well. Triplicate wells of infected cells for each virus were harvested every 12 h through 96 h postinfection (hpi), frozen and thawed three times, clarified at 2,500 × g at 4°C for 10 min, and stored at –80°C until titrated. At 12, 24, 36, 48, 60,

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FIG. 1. Schematic diagram of wild-type and chimeric PCVs and viral genomic DNA titer changes during *in vitro* replication. (A) Organization of wild-type and chimeric PCV infectious DNA clones. Genes encoding the viral capsid (*cap*) and replication-associated proteins (*rep*) are shown, along with the origin of replication (*Ori*). Genetic elements from wild-type PCV1 are shown in black, while those from wild-type PCV2 are white. Chimeric PCV clones were constructed by overlap extension PCR, using primers 1 to 16 in Table 1, and were cloned into plasmid vectors, using unique KpnI and SacII restriction enzyme sites located within *rep*. (B) Net changes of viral genomic DNA copy numbers during replication in PK-15 cells from 12 hpi to 96 hpi. The net change in viral genomic copies was calculated by subtracting the viral DNA genome copy numbers present at 12 hpi from those present at 96 hpi. The mean changes in log₁₀ viral genomic copies/ml ± the standard errors of the means (SEM) were compared among different viruses, using one-way ANOVA followed by Tukey's procedure for multiple comparisons, and are plotted for each virus. An asterisk (*) indicates a significant difference between the results for chimeras PCV1-Ori2 and PCV1-rep2 (*P* = 0.027).

72, 84, or 96 hpi, the infectivity titers of each virus were determined (12) and compared by exact Kruskal-Wallis one-way analysis of variance (ANOVA), followed by Dunn's procedure for multiple comparisons.

The genomic copy numbers of viral DNA at 12 and 96 hpi were quantified. Viral genomes containing the *rep* gene of PCV2 were quantified with a published quantitative PCR (Q-PCR) (24). A 25-μl Q-PCR mixture (200 nM each primer [PCV2-83F, 5'-AA AAGCAAATGGGCTGCTAA-3'; PCV2-83R, 5'-TGGTAACC ATCCCACCACTT-3']; 200 μM deoxynucleoside triphosphate [dNTP], 5 mM MgCl₂, and 1 μl DNA extract) was run in duplicate in a My iQ thermocycler. Viral genomes with the *rep* gene of PCV1 were quantified in duplicate in a 25-μl Q-PCR mixture (200 nM each primer [PCV1-qRepF, 5'-TGGAGAAGAAGTT GTTGT-3'; PCV1-qRepR, 5'-TCTACAGTCAATGGATACC-3'], 200 μM dNTP, 3 mM MgCl₂, and 1 μl DNA extract), using a similar program. Net genomic titer changes in log₁₀ genome

copy numbers from 12 hpi to 96 hpi were compared among different viruses (Fig. 1B).

PCV1 replicates more efficiently than PCV2 *in vitro*. The infectivity titer of PCV1 was found to be significantly greater (*P* ≤ 0.005) than that of PCV2 from 36 hpi to 96 hpi (Fig. 2A). This is consistent with our previous studies showing that PCV1 replicates to higher titers in PK-15 cells than PCV2 (14, 15). The enhanced replication ability of PCV1 in PK-15 cells is likely due to the fact that PCV1 is already adapted to grow in PK-15 cells, since it was first isolated as a persistent contaminant of the PK-15 cell line (33).

Chimeric PCV2 viruses containing replication factors of PCV1 have enhanced replication *in vitro*. Chimeric PCV2 viruses containing *rep* and the *Ori* of PCV1 were found to have increased replication efficiencies (Fig. 2B). The infectious titers of chimera PCV2-*rep*1 virus were significantly higher (*P* ≤ 0.047) at 72 and 96 hpi than those of PCV2 (Fig. 2B). By 96

TABLE 1. Oligonucleotide primers used in the construction of chimeric PCV infectious DNA clones	
Primer	Sequence (5'→3')
1.....	TTTCCGCGGGCTGGCTGAACTTTTGAAG
2.....	AGCCGCGGAAATTCTGACAAACGTTAC
3.....	GGAGGTACCCGAAGGCCGATTTGAAGCAG
4.....	TCGGGTACCTCCGTGGATTGTTCTCCAGC
5.....	AGTATTACCAGCGCACTTCGGCAGCGGCAGCACCTCGGCAGCGTCAGTGA AAAATGCCAAGCAAGAA
6.....	TGCCGCTGCCGAAGTGCGCTGGTAATACTACAGCAGCGCACTTCTTTCACTTTTATAGGATGACGT
7.....	TTACCAGCGCACTTCGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCAGCAAGAA
8.....	TGCTGCCGCTGCCGAAGTGCGCTGGTAATACTACAGCGCACTTCTTTCTGTTTTTCAGCTATGACGT
9.....	TCCCATATAAAATAAATTACTGAGTCTTTTTTATCACTTCGTAA
10.....	GGCAGCACCTCAGCAGCAACATGCCAAGCAAGAAAAGCGGCCCG
11.....	TTACGAAGTGATAAAAAAGACTCAGTAATTTATTTTATATGGGA
12.....	CGGGCCGCTTTTCTTGCTTGGCATGTTGCTGCTGAGGTGCTGCC
13.....	TTCCATATGAAATAAATTACTGAGTCTTTTTTGTATCACATC
14.....	CCTCGGAGCGTCAGTGA AAAATGCCAGCAAGAAAGAAATGGAAG
15.....	GATGTGATAACAAAAAGACTCAGTAATTTATTTTCATATGGAA
16.....	CTTCCATTCTTCTTGCTGGGCATTTTCACTGACGCTGCCGAGG

FIG. 2. One-step growth curve of wild-type and chimeric PCVs in PK-15 cells. PK-15 cells were infected with PCV1 (●), PCV2 (○), chimera PCV1-Ori2 (■), chimera PCV2-Ori1 (□), chimera PCV1-*rep*2 (▲), or chimera PCV2-*rep*1 (△) at a multiplicity of infection of 0.5. Cells in triplicate wells were harvested every 12 h through 96 hpi. The median 50% tissue culture infectious dose (TCID₅₀) for each virus was plotted, with bars showing the range of the data. (A) The growth kinetics of wild-type PCV1 and PCV2 were compared; an asterisk (*) indicates a significant difference. PCV1 replicates more efficiently than PCV2. (B) Comparison of the growth characteristics of wild-type PCV2 and two chimeric PCV2 viruses containing the Ori (□) or Rep (△) of PCV1. The TCID₅₀ for each virus was plotted, with bars showing the range of the data and asterisks indicating significant differences. Chimeric PCV2 viruses containing PCV1 Ori or Rep replicate more efficiently than wild-type PCV2. (C) Comparison of the growth characteristics of wild-type PCV1 and two chimeric PCV1 viruses containing the Rep (▲) or Ori (■) of PCV2. The two chimeric PCV1 viruses containing the Rep or Ori of PCV2 had decreased replication efficiencies compared to that of wild-type PCV1, although the difference was not statistically significant.

hpi, cells infected with chimera PCV2-*rep*1 produced at least 10-fold-more infectious virus than cells infected with PCV2 (Fig. 2B). The chimera PCV2-Ori1 infectivity titers were also increased compared to those of PCV2 prior to 72 hpi (Fig. 2B). Conversely, chimeric PCV1 viruses containing the Ori or *rep* of PCV2 showed a general decrease in infectivity titers from 36 to 96 hpi compared to those for PCV1 (Fig. 2C), although this was not as large as the increase observed for the PCV2 chimeric viruses.

Differences in the *rep* gene and Ori of PCV1 and PCV2 affect virus replication efficiencies. In general, chimeric viruses based on the PCV1 backbone replicated to higher infectivity titers at an earlier time, and the titers remained higher throughout the study. Chimera PCV1-Ori2 achieved significantly higher infectivity titers than PCV2 at 36, 48, 72, and 84 hpi ($P \leq 0.035$). The virus titers produced by the chimera PCV1-*rep*2 were significantly higher than those of PCV2 at 48, 60, 84, and 96 hpi ($P \leq 0.042$) but were not significantly different from those of PCV1 (Fig. 2C). The significant difference in net changes of viral genomic copy numbers ($P = 0.027$) (Fig. 1B) from 12 to 96 hpi between chimeras PCV1-Ori2 and PCV1-*rep*2 is consistent with our finding that the production of infectious virus by chimeric viruses containing the PCV1 *rep* gene was significantly enhanced.

Possible explanations for the differences in replication effi-

ciencies observed in this study include changes in gene expression and genome replication and altered interactions between cellular and viral proteins. The promoters for *cap* and *rep*, located within *rep* and the Ori, respectively (3), were exchanged in the chimeric viruses in this study. Rep is known to repress the *rep* promoter; thus, differences in binding between PCV1 Rep and PCV2 Rep could affect the levels of expression from heterologous promoters (20). Differences in the abilities of chimeric viruses to replicate viral genomic DNA could alter the amount and timing of infectious-virus production. However, the observed differences in infectivity titers are unlikely due to viral-DNA replication efficiencies alone, since the wild type and most chimeric viruses produced similar amounts of viral DNA genomes (Fig. 1B and data not shown).

PCV Cap is known to interact with Rep and several cellular proteins and thus may influence intracellular transport, encapsidation, and/or transcription of viral genes (17, 30). In our study, chimeric viruses with Cap and Rep of PCV2 were less efficient in the production of infectious virus. Compared to that produced by PCV1, significantly less infectious virus was produced at 36 and 84 hpi ($P \leq 0.035$) by chimera PCV2-*rep*1 and at 36, 48, and 96 hpi ($P \leq 0.039$) by chimera PCV2-Ori1 (Fig. 2B). Furthermore, chimeric PCV1 viruses with either *rep*2 or Ori2 produced significantly more infectious virus ($P \leq 0.043$) at most time points after 24 hpi than PCV2, but infectious-

virus production was not significantly different from that of PCV1 (Fig. 2C). Interestingly, the replication efficiency of the PCV1-*cap2* chimera from our previous studies was decreased in comparison to those of PCV2 and chimera PCV2-*cap1* (15), and two amino acid changes in Cap were shown to increase the replication efficiency of PCV2 *in vitro* (14). Thus, complex interactions between Cap, Rep, and cellular factors with viral DNA elements, including the Ori, are likely responsible for the observed differences in production of infectious virus.

In summary, by using a live chimeric-virus system, we provided direct proof of the results of earlier studies with reporter gene-based assays that the replication factors are completely exchangeable between PCV1 and PCV2. Most importantly, we demonstrated that both the *rep* gene and the Ori of PCV1 enhance replication of the PCV2-based chimeric viruses. It remains to be seen, however, whether or not enhanced replication ability *in vitro* has any effect on the *in vivo* pathogenicity of these chimeric viruses. Nevertheless, the results have important implications for engineering a chimeric PCV2 virus with enhanced replication capability for future vaccine development.

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