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Comparative analysis of the transcriptional patterns of pathogenic and nonpathogenic porcine circoviruses

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Abstract

The RNAs of porcine circovirus type 1 (PCV1) synthesized in PK15 cells were characterized. A total of 12 RNAs were detected. They include the viral capsid protein RNA (CR), a cluster of eight Rep-associated RNAs (designated Rep, Rep′, Rep3a, Rep3b, Rep3c-1, Rep3c-2, Rep3c-3, and Rep3c-4), and three NS-associated RNAs (designated NS462, NS642, and NS0). Members of the Rep-associated RNA cluster all share common 5′- and 3′-nucleotide sequences and they share common 3′-nucleotide sequence with the NS-associated RNAs. Rep, capable of coding for the full-length replication-associated protein, appears to be the primary transcript that gives rise to the other seven Rep-associated RNAs by alternate splicing. NS462, NS642, and NS0 appear to have been transcribed from three different promoters present inside ORF1, independent from the Rep promoter. Based on sequence alignment analysis, both the nonpathogenic PCV1 and the pathogenic porcine circovirus type 2 (PCV2) (with nine RNAs: Rep, Rep′, Rep3a, Rep3b, Rep3c, NS515, NS672, and NS0) utilize comparable genetic elements similarly located along the genome for viral gene expression. The Rep, Rep′, Rep3a, Rep3b, and NS0 of PCV1 and PCV2 are considered equivalent entities in their respective systems. However, quantitative and qualitative differences (splice junction variation) were observed among the Rep3c- and NS-associated RNAs. This work provides a general framework and genetic basis to investigate the biologic properties (and differences) of PCV1 and PCV2.

Keywords: Circoviridae; RNAs of porcine circoviruses

Introduction

The *Circoviridae* family includes a diverse group of small, single-stranded, closed circular DNA viruses (McNulty et al., 2000). Members of the *Circovirus* genus (Pringle, 1999) include porcine circovirus (PCV), psittacine beak-and-feather disease virus, goose circovirus, canary circovirus, and pigeon circovirus (Phenix et al., 2001; Ritchie et al., 1989; Todd et al., 1991, 2001; Woods et al., 1993). Based on a conserved stem-loop structure at the origin of DNA replication and similarities among the putative proteins essential for viral DNA replication, it was suggested that the animal circoviruses are closely related to plant nanoviruses (Mankertz et al., 1997; Meehan et al., 1997; Niagro et al., 1998).

The PCV virion is icosahedral, nonenveloped, and 17 nm in diameter (Tischer et al., 1982). It has an ambisense genome that encodes proteins by the encapsidated viral DNA and by the complementary DNA of the replicative intermediate synthesized in the host. Two genotypes of PCV, PCV type 1 (PCV1) and PCV type 2 (PCV2), have been identified. PCV1 is widespread in swine, but it has not been associated with any animal disease. PCV2 is the causative agent of a new disease, named postweaning multisystemic wasting syndrome (PMWS), which has been reported worldwide (Allan and Ellis, 2000). The overall DNA sequence homology among PCV1 and PCV2 isolates is greater than 90%, while the homology of PCV1 and PCV2 isolates is 68–76%.

The genome sequences of a number of PCV1 and PCV2 isolates (Fenaux et al., 2000; Hamel et al., 1998; Meehan et

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al., 1997, 1998; Morosov et al., 1998; Niagro et al., 1998) have been determined; however, the genetic basis for PCV2 pathogenicity remains unclear. In an attempt to elucidate the mechanisms of PCV pathogenesis, the genes expressed by PCV1 and PCV2 were examined. Previous work showed that nine RNAs (CR, Rep, Rep′, Rep3a, Rep3b, Rep3c, NS515, NS672, and NS0) were synthesized in PCV2-infected PK15 cells (Cheung, 2003). To date, only a partial transcription map of PCV1 with three RNAs (CR, Rep and Rep′) is available (Mankertz et al., 1998a,b). In this study, the same strategies used to investigate the RNAs of PCV2 were used to examine the transcription pattern of PCV1 during virus replication in PK15 cells. The data are presented in similar fashion to facilitate comparison between the two viruses.

Results

Molecular cloning of the PCV1 genome

Low molecular weight cellular DNA was isolated (Bloom et al., 1983) from the CCL-33 cell line and amplified with primers N992R and N993F that contain a *Bam*H1 site. After restriction enzyme digestion, the DNA was inserted into the *Bam*H1 site of the Bluescript plasmid. A PCV1 clone, designated PCV1/AC1, was selected and sequenced. The genomic sequence was 1759 nt (GenBank Accession No. AY184287) and was more than 99% homologous to other PCV1 sequences in the data base. This sequence was aligned and compared with the sequence of PCV2/688 (GenBank Accession No. AY094619) (Fig. 1).

Northern blot analysis

DNase 1 treated total cell RNAs (Fig. 2) isolated from PCV2/688-infected PK15 cells (lane 1), PCV1-infected PK15 cells (lane 2), and the PCV1-persistently infected CCL-33 cells (lane 3) were hybridized with a single-stranded riboprobe containing DNA sequence from nt 1013 to nt 858 (a sequence complementary to all ORF1 RNAs transcribed in the rightward orientation—see below). Previous work with PCV2 showed that the 1000-, 750-, and 450-nt RNAs correspond to Rep, Rep′, and NS0, respectively (Cheung, 2003). In the PCV2 sample, NS0 was more abundant than Rep′; but in both PCV1 samples, NS0 was less abundant than Rep′.

PCR analysis

To investigate the minor RNA species synthesized by PCV, RNA samples were subjected to PCR (before and after digestion with DNase 1) and Marathon cDNA cloning [after digestion with DNase 1 and poly(A) selection] analyses. Specific oligonucleotide primers were selected for (A) RT-PCR or (B) PCR on cDNA. The PCR products were analyzed by agarose gel electrophoresis and the nucleotide sequence of each product was determined after cloning into a bacterial plasmid. Apart from CR, the other 11 RNAs detected were assigned to two groups. Those that share common 5′ and 3′ sequences with Rep were designated Rep-associated RNAs and those that share only 3′ sequences with Rep were designated NS-associated RNAs.

RT-PCR

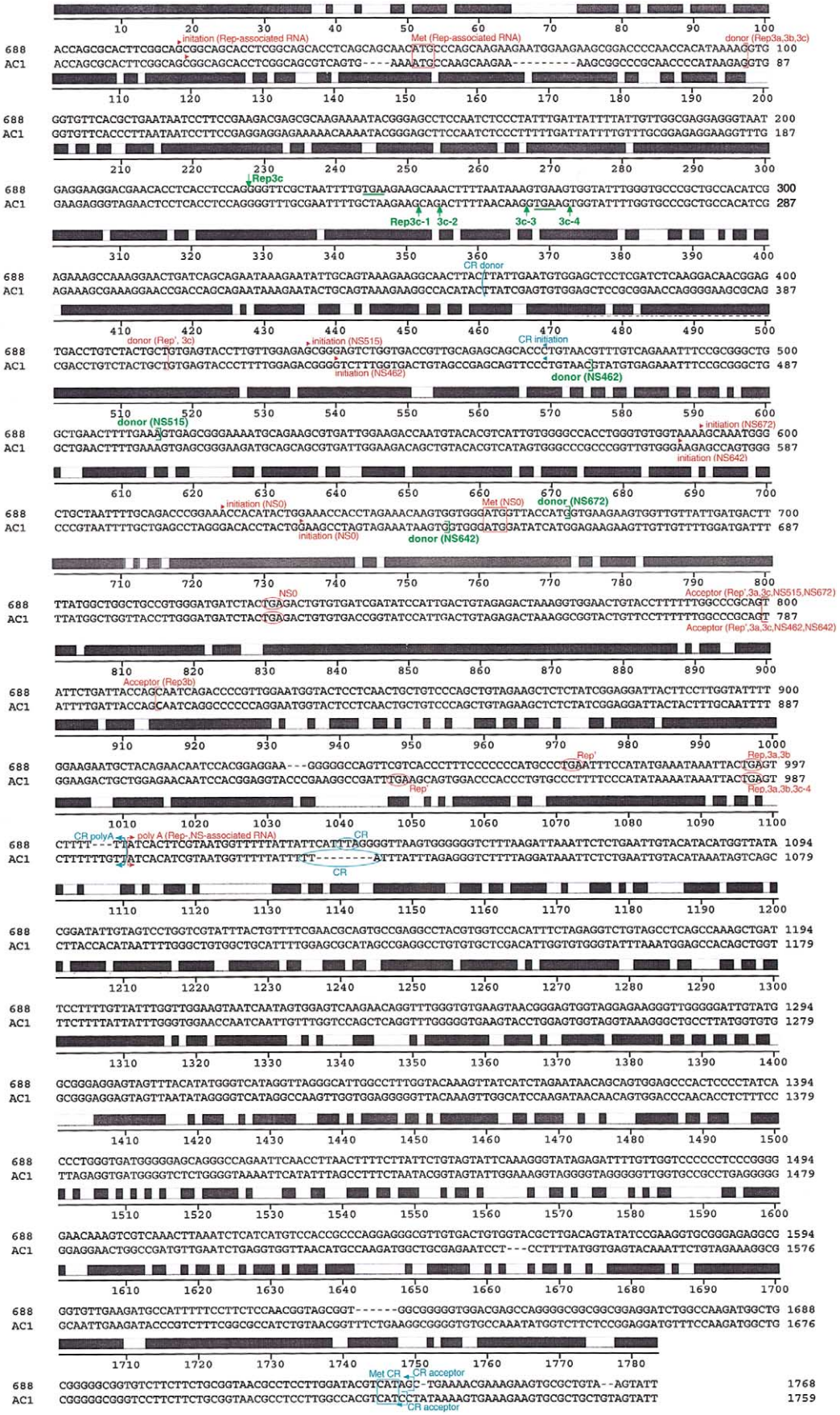
In addition to the capsid RNA (CR) and the three RNAs detected by Northern blot (Rep, Rep′, and NS0), six minor RNAs (Rep3a, Rep3b, and four versions of Rep3c) were uncovered in the PCV1 RNA samples.

CR. Primers 427R/N993F and 455R/N993F gave PCR products of 800 base-pairs (bp) (Fig. 3a). Both DNA fragments contained the CR splice junction at nt 348:1724.

NS0 and Rep. Primers 718F and 995R gave a single fragment of 280 bp (Fig. 3b) and the DNA sequence was collinear with the viral genome. This PCR product could have derived from the unspliced full-length Rep or NS0 or both.

Rep′, Rep3a, Rep3b, and Rep3c. PCR amplification with PCV2-infected (using primers 47F and 890R) and two PCV1-infected cell RNAs (using primers N47F and 890R) were conducted in parallel for comparison (Fig. 4). Except for a more prominent 300-bp band in the PCV1 samples, as compared to a very faint 350-bp band in the PCV2 sample (Cheung, 2003), the banding patterns were quite similar among these three samples. The PCV1 fragments were cloned and sequenced. Similar to results obtained for PCV2, nt sequences from the 800-nt bands in the sample not treated with DNase 1 (U), (indicated by ➤), were collinear with the genomic sequence. This 800-nt band was not present in the DNase 1 treated sample (T). Thus, the 800-nt fragments were derived from contaminating viral DNAs. From the 500-bp fragment, three of three clones contained the Rep′ spliced junction at nt 403:787. From the 200-bp fragment, three of five clones contained the Rep3a splice junction at nt 84:787 and two of five clones contained the Rep3b spliced junction at nt 84:802. The 600-bp fragment gave clones of Rep′, Rep3a, and Rep3b sequences, indicating that it contained Rep′/Rep3a and Rep′/Rep3b hybrids. The 240-bp fragment gave clones of Rep3a and Rep3b sequences, indicating that it contained Rep3a/Rep3b hybrids.

While the faint PCV2 350-nt fragment gave clones of only one double-spliced Rep3c species, the 300-nt PCV1 fragment gave four double-spliced Rep3c clones with slight variations. For the first splice junction, the donor site was identical for all four Rep3c variants, but the acceptor site for each variant was different (Rep3c-1 at nt 84:239, Rep3c-2 at nt 84:242, Rep3c-3 at nt 84:254, and Rep3c-4 at nt 84:260). All four variants contained the same second splice junction at nt 403:787. Of the nine cDNA clones recovered, the


initation (Rep-associated RNA)
Met (Rep-associated RNA)
donor (Rep3a,3b,3c)
Rep3c
Rep3c-1
3c-2
3c-3
3c-4
CR donor
donor (Rep', 3c)
initation (NS515)
CR initation
initation (NS462)
donor (NS462)
donor (NS515)
initation (NS672)
initation (NS642)
initation (NS0)
Met (NS0)
donor (NS672)
donor (NS642)
NS0
Acceptor (Rep',3a,3c,NS515,NS672)
Acceptor (Rep',3a,3c,NS462,NS642)
Acceptor (Rep3b)
Rep'
Rep,3a,3b
Rep,3a,3b,3c-4
CR polyA
poly A (Rep- NS-associated RNA)
CR
Met CR
CR acceptor

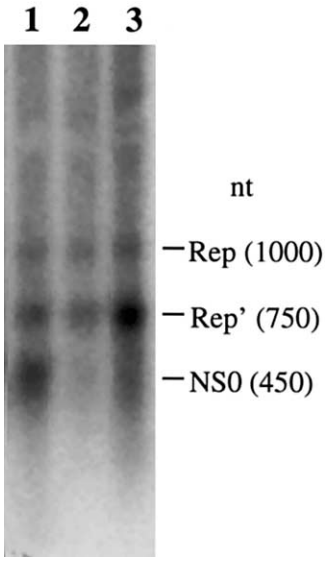


Fig. 2. Northern blot analysis. DNase 1 treated total cell RNAs isolated from three different sources were hybridized with a single-stranded directional riboprobe. Lanes (1) PCV2/688-infected PK15 cells at 48 h p.i.; (2) PCV1-infected PK15 cells at 48 h p.i.; and (3) CCL-33 cells.

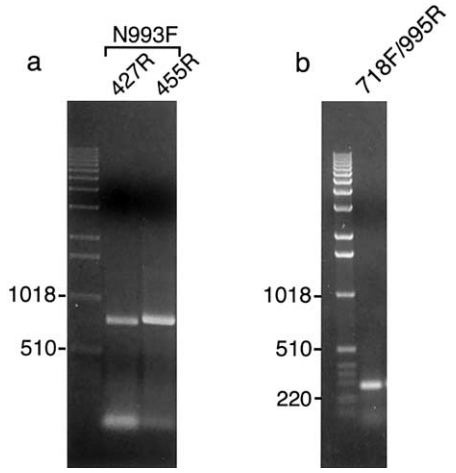


Fig. 3. CR and NS0/Rep. RT-PCR was carried out with DNase 1 treated PCV1 RNAs from 48 h p.i. (a) is CR-specific and (b) is NS0/Rep-specific. The primers used are indicated on top of each lane. Molecular weight standards are shown in the left lanes.

distribution of Rep3c-1:Rep3c-2:Rep3c-3:Rep3c-4 was 1:6:1:1, respectively.

PCR on cDNA

In this experiment, other than the full-length Rep, two minor RNAs (NS462 and NS642) were identified.

The 3′ portion. Each upstream primer (N597F, 491F, or N406F) was amplified with the downstream 995R primer (Fig. 5a). In all three cases, there was a prominent upper band generated. These upper DNA fragments all contained nt sequences collinear with the viral genome, indicting that they were derived from Rep. No lower band was detected with primers 491F/995R. The lower bands generated by N597F/995R and N406F/995R contained the NS462 splice junction at nt 462:787 and the splice junction of NS642 at nt 642:787, respectively.

The 5′ portion. Primer N47F was paired up with 737R (Fig. 5b). A prominent fragment that contained nt sequence collinear with the viral genome was observed. This PCR product could only be derived from Rep.

The 5′-initiation and 3′-poly(A) sites

The cDNAs generated by the Marathon RACE experiment were subjected to PCR amplification with the adaptor primers, AP1 or AP2, and selected viral primers (Fig. 6). The PCR products obtained were analyzed by agarose gel electrophoresis and cloned into bacterial plasmids and their DNA sequences were then determined.

The 3′ poly(A) sites

CR. Nested-PCR with AP1/N1444R and AP2/N1363R generated a 450-bp band that gave two clones with 3′-terminal nt at 998 and 1000 (lane 1).

Rep′. Nested-PCR with AP1/286F and AP2/321F generated a 400-bp band that gave a clone with 3′-terminal nt at 997 (lane 2). Presumably, this poly(A) site was used by all the Rep- and NS-associated RNAs.

The 5′initiation sites

CR. Nested-PCR with AP1/N1236F and AP2/N1521F generated a 350-bp band that gave two clones with 5′-terminal nt at 457 (lane 3).

Rep′. PCR with AP1/890R generated a 550-bp band that gave three clones with 5′-terminal nt at 19, 23, and 24 (lane 4).

NS462. Nested-PCR with AP1/890R and AP2/SP462L6 (splice site-specific) gave two NS462 clones with 5′-terminal nt at 440 and 442 (lane 5).

Fig. 1. Comparison of the genomic sequences of PCV1 (AY184287) and PCV2/688 (AY094619). The black boxes on top of the aligned sequences indicate nt identity and the white boxes indicate nt difference. RNAs transcribed to the right (Rep- and NS-associated) are annotated in red and on top of the sequence, while the capsid RNA (CR) is annotated in blue and below the sequence in the leftward orientation. Splice sites deemed different between PCV1 and PCV2 are annotated in green. Initiation codons are in boxes and termination codons are in oval circles. The 5′-initiation nt for each transcript is indicated by (➤). The first acceptor site for Rep3c is indicated by an arrow (up or down) and the termination codons for PCV2 Rep3c, PCV1 Rep3c-1, 3c-2, and 3c-3 are underlined.

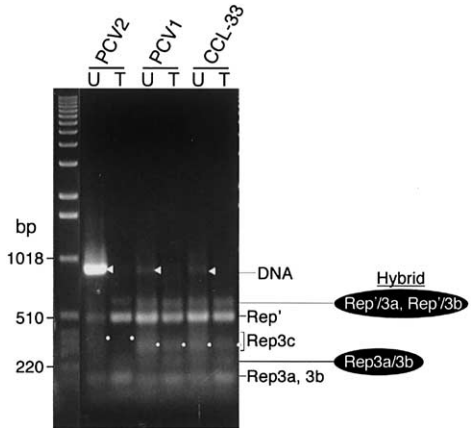


Fig. 4. Rep′, Rep3a, Rep3b, and Rep3c. RT-PCR was carried out with DNase 1 treated (T) or untreated (U) RNA samples from 48 h p.i. The primers used, the PCR products generated, and the corresponding viral RNA from which each product was derived are indicated. Molecular weight standards are shown in the left lane. The PCR product derived from viral DNA contamination is labeled DNA and indicated by (➤).

NS642. Nested-PCR with AP1/890R and AP2/SP642L6 (splice site-specific) gave two NS642 clones with 5′-terminal nt at 588 and 611 (lane 6). In addition, nested-PCR with nonsplice site-specific primers, AP1/890R and AP2/865R, gave a NS642 clone with 5′-terminal nt at 624 (data not shown).

NS0. Nested-PCR with AP1/803R and AP2/737R gave a clone with 5′-terminal nt at 637 (lane 7).

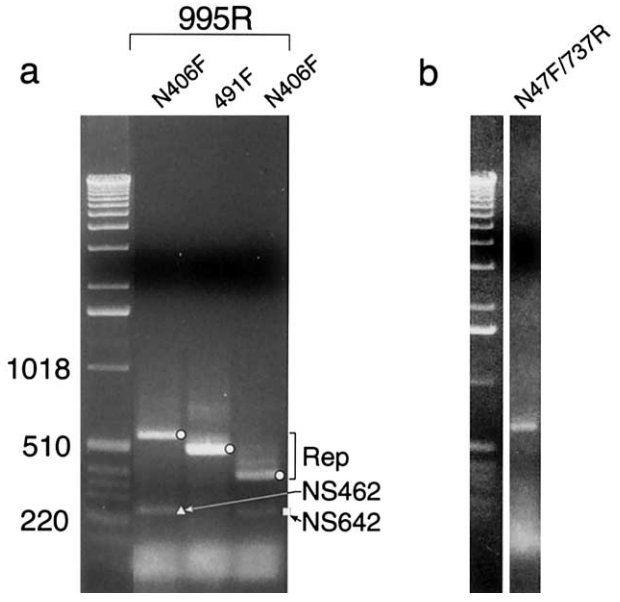


Fig. 5. Rep, NS462, and NS642. Marathon cDNA was amplified with the indicated primers to obtain (a) the 3′-portion of all three RNAs and (b) the 5′-portion of Rep. The primers used are indicated at the top of the lane. Molecular weight standards are shown in lanes 1. Symbols used to identify Rep (○), NS462 (△), and NS642 (□) are indicated.

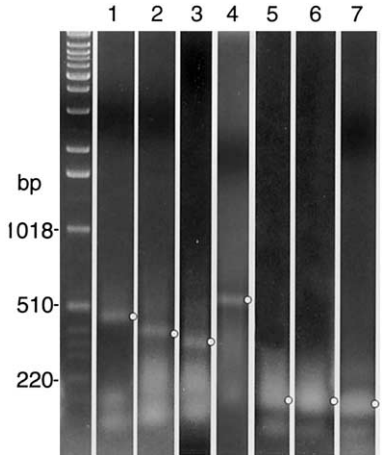


Fig. 6. Determination of the 5′- and 3′-terminal nt of the RNAs by the adaptor-assisted cDNA strategy and after cloning the PCR products into bacterial plasmids. The primers used were as follows: (lane 1) AP1/N1444R and AP2/N1363R; (lane 2) AP1/286F and AP2/321F; (lane 3) AP1/N1236F and AP2/N1521F; (lane 4) AP1/890R; (lane 5) AP1/890R and AP2/SP462L6; (lane 6) AP1/890R and AP2/SP642L6; and (lane 7) AP1/803R and AP2/737R.

Discussion

In this study, a transcription map for PCV1 was established. Twelve transcripts were identified during PCV1 replication in PK15 cells and are summarized in Fig. 7. Only one RNA, CR, is encoded by the complementary DNA strand (transcribed leftward) and the other 11 RNAs are encoded by ORF1 of the virion DNA strand (transcribed rightward). These 11 RNAs were divided into two groups. The Rep-associated RNAs (Rep, Rep′, Rep3a, Rep3b, Rep3c-1, Rep3c-2, Rep3c-3, Rep3c-4) all share common nt sequences at the 5′ and 3′ ends. Presumably, Rep is the primary transcript that gives rise to the other Rep-associated RNAs by alternate splicing. All the splice sites described in this communication contain the consensus dinucleotides (GT . . . AG) at the inside boundaries of the splice junction. The NS-associated (NS462, NS642, and NS0) RNAs all share common nt at the 3′ end among themselves and with the Rep-associated RNAs. Although all 11 RNAs contain 200 common nt at the 3′ end, these nt were used differently by each transcript. The RNAs that utilize this sequence to code for any amino acid (aa) include Rep, Rep′, Rep3a, Rep3b, and Rep3c-4.

CR

The initiation site is located at nt 457; the splice junction is at nt 348:1724, and the termination site is at nt 997. The 5′-untranslated sequence extends 1 nt into the second exon and the coding region is between nt 1723 and 1024 for 233 aa residues. For comparison, the 5′-untranslated sequence of PCV2 CR extends 2 nt into the second exon to maintain the consensus dinucleotide configuration at the inside boundary of the splice junction.

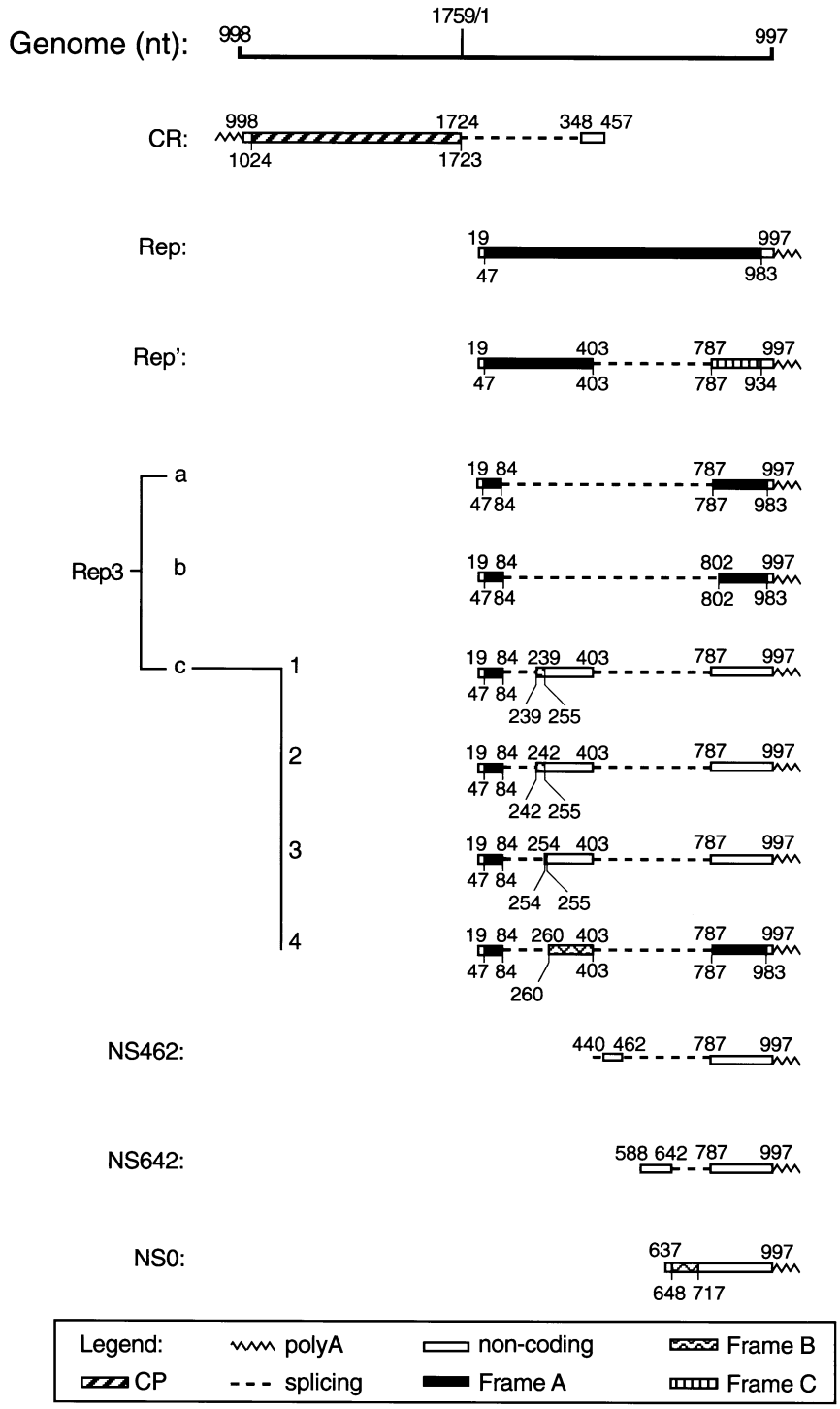


Fig. 7. Schematic representation of the PCV1 RNAs. CR is transcribed leftward and the rest are transcribed rightward. The RNAs are annotated with nt coordinates that indicate the last nucleotide of each respective exon. The coding sequence of each transcript was shaded and their nt coordinates are indicated below each RNA.

Rep and Rep′

Similar to PCV2, a stable full-length Rep RNA was detected in PCV1-infected cells by Northern blot and PCR (Figs. 2 and 5). The initiation site is located at nt 19 and the poly(A) site is located at nt 997. The Rep coding region is 312 aa, from nt 47 to 983, and it is designated Frame A. The deduced aa sequence of Rep has been used to support the theory that PCV was the result of a recombination event between a plant nanovirus and an animal picorna-like RNA virus (Gibbs and Weiller, 1999). Rep′ is a spliced message that codes for a protein of 168 aa residues, and the splice

junction is located at nt 403:787. The first exon is in Frame A, while the second exon is in Frame C. As noted before, the deduced aa sequence of Frame C (PCV1 and PCV2) shares homology with a putative plant MYB transcription factor of *Arabidopsis thaliana* (Cheung, 2003). Thus, Rep′ of PCV1 is equivalent to Rep′ of PCV2 (Cheung, 2003; Mankertz and Hillenbrand, 2001). The deduced exon 1 aa sequence of Rep′, identical to the N-terminal portion of Rep, shares extensive homology with the Reps of several plant nanoviruses (Gibbs and Weiller, 1999; Niagro et al., 1998). It has been reported that the proteins encoded by Rep and Rep′ are required for viral DNA replication (Mankertz and Hillenbrand, 2001).

Rep3a, Rep3b, and Rep3c

The Rep3a and Rep3b splice junctions are located at 84:787 and 84:802, respectively. The Rep3a and Rep3b proteins are 78 and 73 aa, respectively. Both exon 1 and exon 2 of Rep3a and Rep3b are derived from ORF1 Frame A. Essentially, Rep3a and Rep3b contain 5′ and 3′ aa residues identical to the deduced Rep protein. Since exon 1 of Rep3a and Rep3b are identical, encoding only 13 aa residues, it does not share any homology with the Reps of nanoviruses. Because of the sizable intron, the P-loop motif and much of the subsequent 3′ aa of Rep (Gibbs and Weiller, 1999) are absent. Consequently, the Rep3a and Rep3b proteins contain very limited homology with the 2C-proteins of picornaviruses.

Rep3c RNAs are double-spliced transcripts. There are four Rep3c variants: Rep3c-1, -2, -3, and -4. Organizationally, they differ from each other only slightly at the acceptor site of the first splice junction. Apparently, the Rep3c-3 acceptor site is used more frequently than the other Rep3c sites. It is not clear what determines the choice of acceptor splice sites. Rep3c-1, -2, and -3 codes for small proteins less than 16 aa residues since their coding frames terminate shortly after the first splice junction in Frame B. In contrast, Rep3c-4 codes for a protein of 122 aa residues. The first splice junction at nt 84:254 bypassed the stop codon that terminates the other three Rep3c variants. The first, second, and third exons of Rep3c-4 are in Frames A, B, and A, respectively. BLAST search of the GenBank data base with the deduced aa sequence of exon 2 Frame B did not reveal any significant homology with any known sequences. Similar to PCV2, the last exon of Rep3c-4 is identical to the C-terminal aa sequences of Rep, Rep3a, and Rep3b and shares homology with a putative transposase protein of *Xanthomonas campestris* (Cheung, 2003).

NS-associated RNAs

Three RNAs, NS462, NS642, and NS0, are included in this group. They appear to be independently initiated from three separate promoters, but terminate at an identical location, nt 997. The initiation sites for NS462, NS642, and NS0 are in the vicinity of nt 440, 588, and 637, respectively. The splice junction for NS462 is at nt 462:787 and the splice junction for NS642 is at 642:787. Neither transcript appears to code for a functional protein because no AUG is found in either exon 1. NS0 is not a spliced RNA. It codes for a 23 aa protein in Frame B and shares 61% homology with the deduced aa sequence of PCV2 NS0. BLAST search of the GenBank data base with the deduced PCV1 NS0 aa sequence did not reveal significant homology with any known sequences.

PCV1 vs PCV2

A very complex transcription pattern was demonstrated by PCV1 (with 12 RNAs) and by PCV2 (with nine RNAs) during replication in PK15 cells (Cheung, 2003; Cheung and Bolin, 2002). Based on the aligned sequences presented in Fig. 1, all the transcription initiation and termination signals are situated at comparable locations in the two viral genomes. Rep, Rep′, Rep3a, Rep3b, and NS0 are equivalent entities in their respective systems because the splicing pattern of these RNAs are essentially identical and they code for similar protein products. However, both qualitative and/or quantitative differences were observed with respect to specific RNAs.

For the double-spliced Rep3c RNAs, only one version was detected with PCV2, but four variants (Rep3c-1, -2, -3, and -4) were observed with PCV1. For the first splice junction, the RNAs of both viruses use a comparable donor site but a different acceptor site. For the second splice junction, both viruses use comparable donor and acceptor sites. By RT-PCR, PCV2 Rep3c was barely detectable, while PCV1 Rep3c (collectively) were readily observed. The deduced PCV2 Rep3c (21 aa) and PCV1 Rep3c-1 (18 aa), -2 (17 aa), and -3 (13 aa) proteins are relatively small, but PCV1 Rep3c-4 utilized Frame B of exon 2 to code for a significantly larger novel protein (122 aa residues).

All PCV1 and PCV2 NS-associated RNAs appear to initiate and terminate at comparable sites; however, they also differ either quantitatively or qualitatively. The aligned sequences suggest that PCV1 NS462 and NS642 are equivalent to PCV2 NS515 and NS672, respectively. These RNAs utilize a different donor site but the same acceptor site. Interestingly, none of these RNAs appear to code for any functional protein. For PCV1 or PCV2 NS0, the RNAs are not spliced. Northern blot analysis indicated that the expression levels of NS0 in PCV1-infected and PCV2-infected cells are very different (Fig. 2). Whereas PCV2 NS0 is more abundant than PCV2 Rep′, PCV1 NS0 is less abundant than PCV1 Rep′.

In conclusion, the transcriptional patterns of PCV1 and PCV2 are quite complex. Both viruses maintain a similar gene expression structure by utilizing similar initiation and termination signals at comparable locations of the viral genomes. They encode equivalent protein entities, such as Rep, Rep′, Rep3a, Rep3b, and NS0. However, they also

differ from each other in the gene expression level of specific RNAs (NS0 and Rep3c), as well as splice junction selection, to produce transcripts unique to each virus (Rep3c and NS462, NS642, NS515, and NS672). Although it is not clear how these genetic differences contribute to the biological properties of PCV1 and pCV2, this work provides a general framework and some specific targets for further investigation.

Materials and methods

Virus and cell

Infectious PCV1 was obtained from a persistently infected porcine kidney PK15 cell line (CCL-33) distributed by the American Type Culture Collection (Tischer et al., 1974). The pathogenic PCV2/688 isolate (Bolin et al., 2001) was propagated in a PCV-free PK15 cell line that was maintained in minimum essential medium with Hank's salts (MEM-H) (Life Technologies, Grand Island, NY) and supplemented with 10% fetal bovine serum.

RNA blot analysis

Total cell RNA was prepared from virus-infected PK15 cells by the guanidinium isothiocyanate and CsCl cushion centrifugation method (Chirgwin et al., 1979; Glisin et al., 1974). Poly(A) RNAs were selected by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). The RNA samples (5 μg) were subjected to electrophoresis on 2.2 M formaldehyde gel and transferred to nylon membranes by positive pressure in 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl with sodium citrate at 0.015 M). Hybridization was carried out in 3.2 M tetramethylammonium (Wood et al., 1985) with a [^{32}P]dUTP-labeled single-stranded riboprobe generated with a genomic clone of PCV2 inserted in the Bluescript plasmid as previously described (Cheung, 2003).

Oligonucleotide primers

The primers were identified by the first nt coordinate of each oligomer with respect to the genomic sequence (Fig. 1). Primers with prefix N are specific to PCV1. Primers without a prefix are common between PCV1 and PCV2 and are numbered according to the PCV2 coordinates. The suffix (F or R) of the oligonucleotide indicates orientation of the primer. F indicates forward direction from nt 1 to 1768, while R indicates reverse direction from nt 1768 to 1. The primers are as follows: 47F: CAACATGCCCAGCAGGAAGAATGGAAG; 286F: CCCGCTGCCACATCGAGAAA; 321F: CAGCAGAATAAAGAATATTGCAGTA; 491F: CCGCGGGCTGGCTGAACTTTTGAAAGT; 718F: GGGATGATCTACTGAGACTG; 427R: AAGGTACTCACAGCAGTAGACAGGTC; 737R: CAGTCTCAGTAGATCATCCC; 803R: GGTAATCTAAATACTGCGGGCCAAAAAAGG; 865R: TCTACAGCTGGGACAGCAGTTG; 890R: GGAAGTAATCCTCCGATAGAGAGC; 995R: CTCAGTAATTTATTTCATATGG; N47F: ATGCCAAGCAAGAAAAGCGG; N406F: TTGCTGAGCCTAGCGACACC; N597F: GAGTACCCTTTTGGAGACGG; N993F: TTGGATCCTTGTTATCACATCGTA; N1236F: GTGAAGTACCTGGAGTGG; N1521F: CAAGATGGCTGCGAGAATC; N455R: TCGGCTACAGTCACCAAAGA; N992R: TTGGATCCAAAAGACTCAGTAATT; N1363R: TCCACTGTTGTTATCTTGGA; N1444R: TTCCAATACTACCGTATTAG; AP1: CCATCCTAATACGACTCACTATAGGGC; AP2: ACTCACTATAGGGCTCGAGCGGC. Splice junction specific primers are SP462L6: CAAAATAGTTACAGGGAACT and SP642L6: CAAAATACACTTATTTCTAC.

PCR

RT-PCR was carried out with 0.1 μg of RNA in the presence of 10 mM Tris–HCl (pH 8.3), 0.2 mM each of dNTP, 100 pM each of the upstream and downstream primer, 2.5 U Taq polymerase, 50 U MMTV reverse transcriptase, and 20 U RNAsin in 50 μl. The reaction mixture was first incubated at 50°C for 30 min and then amplified for 45 cycles at 94°C (10 s), 55°C (30 s), and 70°C (30 s). DNA-PCR was carried out under identical conditions with 0.01 μl of the RT-PCR product and omission of the reverse transcriptase, RNAsin, and 30 min 50°C incubation.

5′- and 3′- RACE cDNA cloning

Adaptor-assisted cDNA cloning was carried out with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Briefly, DNase 1 treated and poly(A)-selected RNAs (1 μg) from PCV1-infected cells at 48 h postinfection (h p.i.) was used as the starting material. First-strand synthesis was conducted with a lock-docking oligo(dT) primer with two degenerate nucleotides at the 3′ end to eliminate any heterogeneity at the poly(A) -tail of the cDNA. Following double-stranded cDNA synthesis, a specialized adaptor was ligated to each end of the cDNA. This adaptor contains two unique sequences (5′-AP1 and 3′-AP2) that can be employed in single- or nested-PCR amplifications with viral specific primers.

DNase 1 treatment

DNase 1 (500 μ/ml) digestion was carried out in a buffer containing 40 mM Tris–HCl (pH 7.5), 6 mM Mg_2Cl, and 10 mM NaCl at 37°C for 30 min.

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