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Identification of an octanucleotide motif sequence essential for viral protein, DNA, and progeny virus biosynthesis at the origin of DNA replication of porcine circovirus type 2

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Abstract

A plasmid-based transfection system capable of generating infectious porcine circovirus type 2 (PCV2) was established. This system was then used in mutagenesis studies to investigate the involvement of a "conserved" nonanucleotide (which constitutes a portion of the loop sequence) located at the origin of DNA replication of PCV2 with respect to viral protein synthesis, DNA self-replication, and progeny virus production. The results demonstrated that an octanucleotide (AGTATTAC) embedded in the loop sequence is essential for virus replication. This octanucleotide can be further condensed to an essential core element (ECE) represented by AxTAxTAC. The positions specified by the indicated nucleotides are critical for viral DNA replication and stable infectious virus production, and they cannot be substituted by other bases, while the positions indicated by x can accept variable bases and yield stable progeny viruses.
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Keywords: Porcine circovirus; Origin of DNA replication

Introduction

Porcine circovirus (PCV) belongs to the genus *Circovirus* of the *Circoviridae* family (McNulty et al., 2000; Pringle, 1999), which also includes duck circovirus, psittacine beak-and-feather disease virus, goose circovirus, canary circovirus, and pigeon circovirus (Hattermann et al., 2003; Phenix et al., 2001; Ritchie et al., 1989; Todd et al., 1991, 2001; Woods et al., 1993). Two genotypes of PCV have been identified. PCV type 1 (PCV1) was first detected as a contaminant of the porcine kidney PK15 cell line (CCL-33) distributed by the American Type Culture Collection (Tischer et al., 1974), and PCV type 2 (PCV2) was initially identified in a Canadian swine herd in 1991 (Clark, 1996; Harding, 1996). Serologic surveys indicated that both types of PCV are widespread in swine (Allan and Ellis, 2000; Segales and Domingo, 2002). Whereas PCV1 is nonpathogenic, PCV2 has been implicated as the etiological agent of a swine disease, named postweaning multisystemic wasting syndrome (PMWS) (Allan and Ellis, 2000; Clark, 1996; Harding, 1996).

PCV has an ambisense, single-stranded, closed circular genome (Tischer et al., 1982) that encodes proteins by the encapsidated viral DNA, and by the complementary DNA of the replicative intermediate (RF) synthesized in the host. The genome sequences of several PCV1 (1759 bases) and PCV2 (1768 bases) isolates (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1997, 1998; Morozov et al., 1998; Niagro et al., 1998) have been determined. The potential formation of a palindromic structure as a result of interactions between the inverted repeats (palindrome) at the origin of DNA replication (Ori) and similarities among the putative proteins essential for virus replication indicate that PCV DNA may replicate via the rolling-circle replication (RCR) stem-loop cruciform model proposed for the *Mastrevirus* genus of the *Geminiviridae* family (Mankertz and Hillenbrand, 2001; Palmer and Rybicki, 1998) or via a recently proposed RCR "melting-pot" model (Cheung, 2004b). The Rep proteins of geminivirus and PCV contain the three conserved RCR motifs (RCR-I, -II, and -III) and NTP-

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binding core homologous to the Rep proteins of other prokaryotic and eukaryotic RCR systems (Ilyina and Koonin, 1992; Mankertz et al., 1998). However, in contrast to geminiviruses, PCV DNA replication requires two proteins (Rep and Rep') instead of just one multifunctional Rep protein (Cheung, 2003c, 2004a; Mankertz and Hillenbrand, 2001; Palmer and Rybicki, 1998). It has been demonstrated that the Rep protein of geminivirus binds to a "conserved nonanucleotide" (TAATATT↓AC) and nicks this cognate recognition site between the seventh T and the eighth A (indicated by ↓) to initiate plus-strand DNA replication (reviewed in Gutierrez, 1999; Hanley-Bowdoin et al., 2000; Palmer and Rybicki, 1998).

The overall DNA sequence homology within the PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68–76%. Sequence conservation between the capsid and Rep genes of PCV1 and PCV2 differs considerably. The Rep gene region is greater than 85% homologous, while the capsid-gene region is only 62% homologous (Mankertz et al., 2003). The Ori of PCV1 has been mapped to a 111-base-pair fragment (Mankertz et al., 1997). PCV1 (CTG*TAGTATT↓AC*) and PCV2 (T*AAGTATT↓AC*) each contain a nonanucleotide (the underlined portion of the loop sequence), which is similar to that of geminiviruses, and they are flanked by a pair of palindromic sequences (indicated as stem) (Fig. 1). It has been reported that the nonanucleotide and palindromic sequences are critical for geminivirus DNA replication (Kammann et al., 1991; Orozco and Hanley-Bowdoin, 1996) as well as for PCV DNA replication (Cheung, 2004b; Mankertz et al., 1997). In vitro protein-binding experiments showed that Rep recognizes the right arm of the palindrome while Rep' recognizes the proximal 6-nucleotide (nt) tandem repeat sequences at the Ori, but neither protein recognizes the nonanucleotide sequence (Mankertz and Hillenbrand, 2002; Steinfeldt et al., 2001). However, sequence-specific binding at the right arm of the flanking palindrome by the Rep protein was not observed in in vivo experiments (Cheung, 2004b).

Nucleotide sequence homology between the large intergenic regions (LIR) of PCV1 and PCV2 (from nt 1736 through 1768/1 to nt 50) (Fig. 1A) is 75%, but sequence homology in a 60 nt stretch (from nt 1741 to 36) immediately surrounding the presumed nick site is greater than 90%, and they share many similar DNA sequence motifs. Notably, the loop sequence of each virus is flanked by a pair of 11-nt inverted repeats, and an array of 6-nt (CGGCAG) and 5-nt (CACCT) direct repeats (Fig. 1B). Recent in vitro transient expression assays demonstrated that the Rep proteins of PCV1 and PCV2 can recognize the LIR sequences of these two viruses interchangeably (Mankertz et al., 2003). Although the "loop" at the Ori of PCV1 (CTGT***AGTATT↓AC***) and PCV2 (TA***AGTATT↓AC***) differ in size (12 vs. 10 nt) and slightly in sequence, an identical "octanucleotide" (underlined) is embedded within each loop sequence. This sequence is essential for PCV1 DNA replication because mutations engineered into the octanucleotide of PCV1 (from T***A***GTATT↓AC to C***T***GTATT↓AC) resulted in complete shut down of PCV1 DNA replication in vitro (Mankertz et al., 1997).

In this work, experiments were conducted to examine the interchangeability of the loop sequences between PCV1 and PCV2 in vivo and to investigate the importance of each nt of the PCV2 loop sequence with respect to viral protein synthesis, DNA self-replication, and progeny virus production.

Results

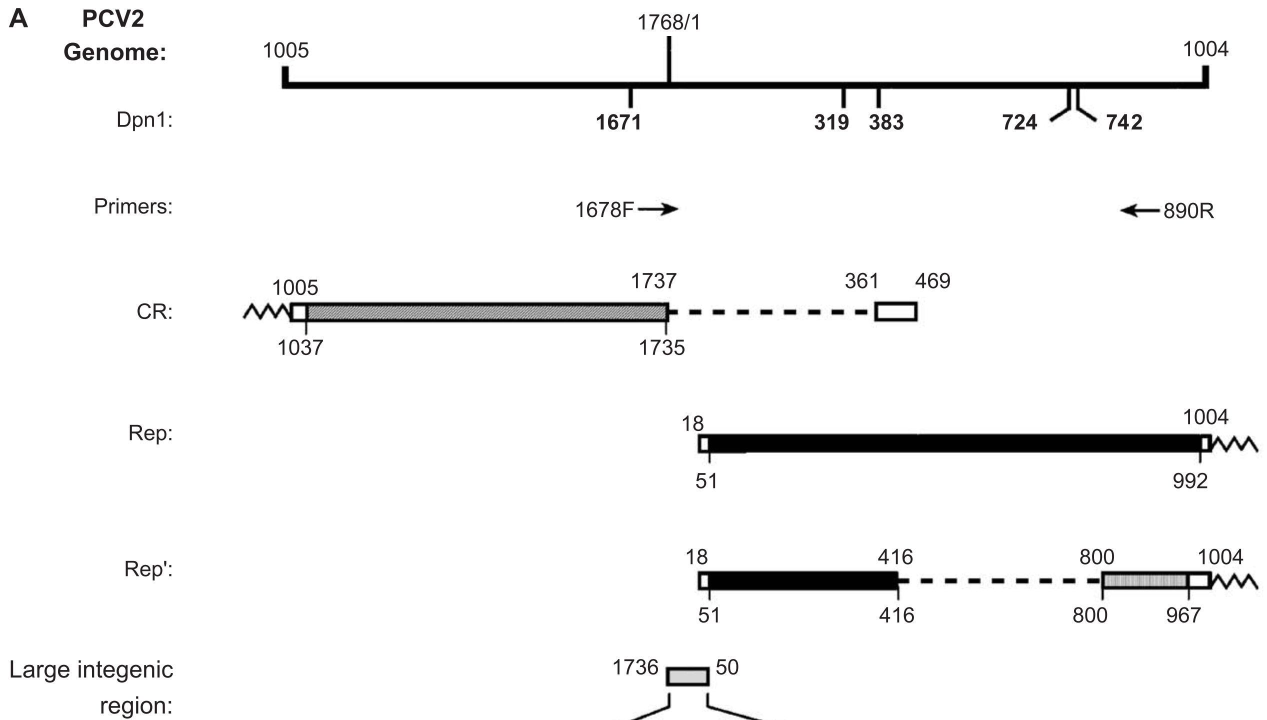
Experimental design

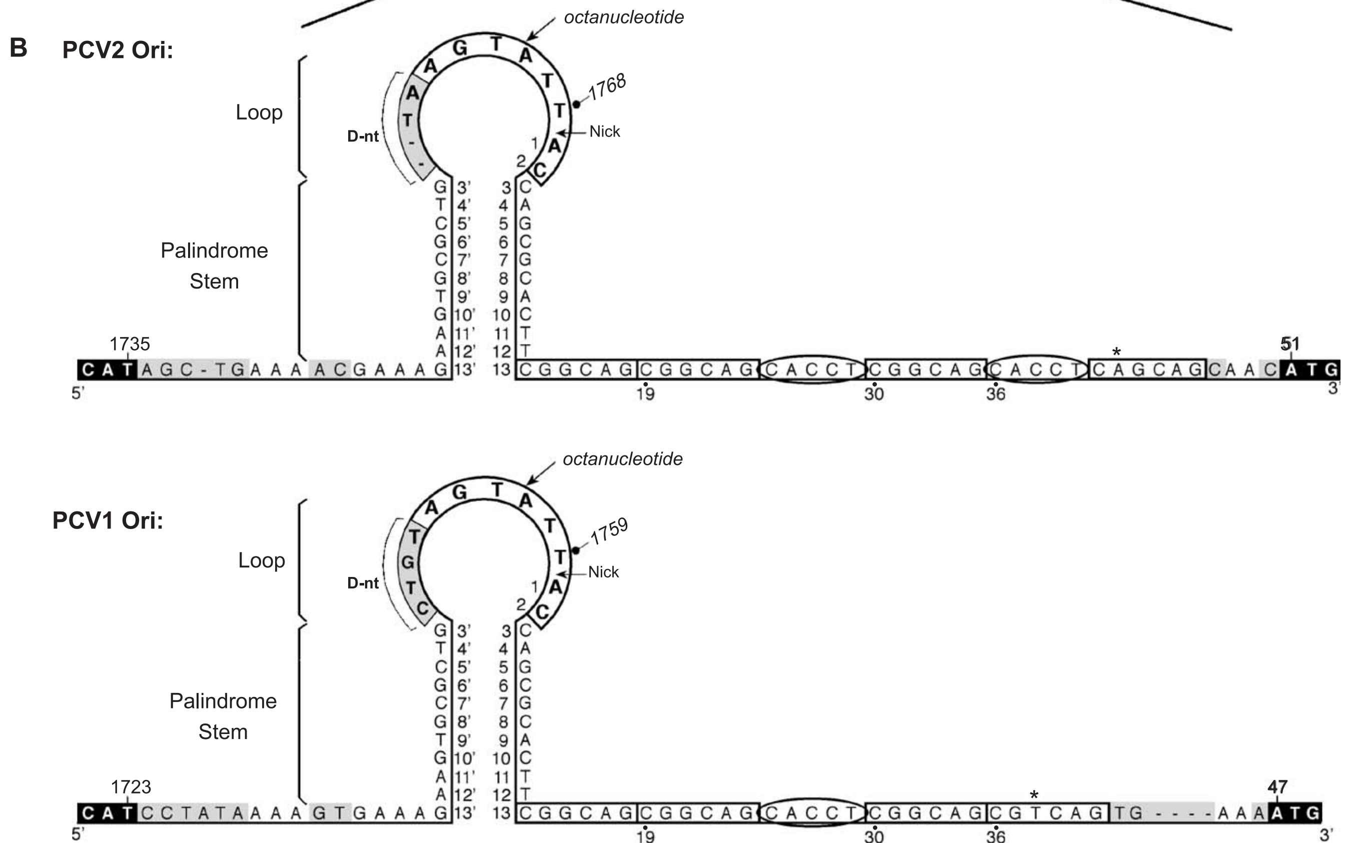
A PCV2 genomic clone derived from PCV2/688 isolate (Cheung, 2003a) was inserted into the *Eco*RI site of Bluescript plasmid (Strategene, California) to generate plasmid-Z1 (pZ1). The excised and circularized double-stranded PCV2 genome from pZ1 was capable of generating infectious progeny viruses upon transfection into PK15 cells (Fig. 2), and this plasmid was used to construct all other mutant plasmids in this study. Plasmids are indicated by prefix p, the excised and recircularized viral genomes are indicated without any prefix and the progeny viruses are indicated by prefix v. A series of mutations were engineered into the loop sequence (T***AAGTATT↓AC***) at the Ori of PCV2 with predetermined oligonucleotide primers. Two types of mutations were engineered. (1) PCV1 and PCV2 loop nt exchange and (2) single nt substitution.

The viral DNAs were excised from the plasmids and then circularized by T4 DNA-ligase before transfection into PK15 cells. At 48 h, one set of transfected cultures was assayed for viral protein synthesis by immunochemical staining with a hyperimmune swine serum (B6) raised against PCV2 that reacts with the Rep-antigens of PCV1 (Cheung, 2004a; Cheung and Bolin 2002). A second set of transfected cultures was assayed for newly synthesized RF DNA (i.e., samples digested with S1 nuclease and Dpn1 restriction enzyme) by PCR. There are five Dpn1 sensitive sites between nt 1447 and 865 in the input PCV2 DNAs propagated in *Escherichia coli* (Fig. 1A). At 7 days post-transfection, a third set of transfected cultures was harvested, froze-thawed three times, and then assayed for infectious virus after inoculation onto fresh PK15 cells. These samples were further passaged in PK15 cells, and periodically, assayed for infectious virus by immunochemical staining. Virus-infected cell DNAs were then isolated and amplified by PCR with PCV-specific primers, 1678F and 890R (Fig. 1A). Each PCR product was subcloned into a TA-cloning plasmid (In Vitrogen, Carlsdad, CA) for nt sequence determination.

Exchange of PCV1 and PCV2 loop sequences

The loop sequences of PCV1 (CTGT***AGTATT↓AC***) and PCV2 (TA***AGTATT↓AC***) differ at the 5′-end (designated D


A
PCV2 Genome:
1768/1
1005
1004
Dpn1:
1671
319
383
724
742
Primers:
1678F
890R
CR:
1005
1737
361
469
1037
1735
Rep:
18
1004
51
992
Rep':
18
416
800
1004
51
416
800
967
Large integenic region:
1736
50


B
PCV2 Ori:
octanucleotide
1768
Nick
D-nt
Loop
Palindrome Stem
1735
51
5'
3'
19
30
36
PCV1 Ori:
octanucleotide
1759
Nick
D-nt
Loop
Palindrome Stem
1723
47
5'
3'
19
30
36

position nt), but each contains an identical octanucleotide sequence at the 3′-end (designated O position nt) (Figs. 1B and 2A). Hybrid plasmid pHy1, with PCV1-background/PCV2-loop, was constructed by modifying the 5′ D-nt of a PCV1 genomic clone (pJ1) that was derived from a PCV1/AC1 isolate (GenBank accession number AY184287) with an additional *Bam*HI site at nt 992 and inserted into the *Bam*HI site of Bluescript plasmid (Cheung, 2003c). The excised and circularized double-stranded PCV1 genome from pJ1 has also been shown to generate infectious progeny viruses upon transfection into PK15 cells (Cheung, 2004a). There is a unique Dpn1-sensitive site located at nt 711 of the PCV1 DNAs propagated in *E. coli*. For the reverse hybrid plasmid pHy2a, with PCV2-background/PCV1-loop, the 5′ D-nt of pZ1 was modified. A second reverse hybrid plasmid pHy2b, with deletion of an A nt from the octanucleotide sequence (CTGT-**GTATTAC**), was also constructed.

Immunochemical staining at 48 h post-transfection showed that an abundant number of cells exhibiting nuclear Rep-antigens was observed between J1 and Hy1, and an abundant number of cells exhibiting predominantly cytoplasmic capsid protein was observed between Z1 and Hy2a (Fig. 2B, upper panel). Dpn1-resistant RF viral DNAs (Fig. 2C) and progeny viruses (vJ1, vHy1, vZ1, and vHy2a) were also recovered from these four transfected cultures (Fig. 2B, lower panel). In comparison, Hy2b showed reduced level of viral protein synthesis (less than 10% of Hy2a), and Dpn1-resistant RF viral DNAs and progeny viruses were not detected. At cell passage 3, virus-infected cell DNAs were isolated and nt sequence analysis showed that the engineered input mutations were retained in the hybrid viruses, vHy1 and vHy2a (Fig. 2D).

Single nucleotide substitution mutation

Each nt of the PCV2 loop was assigned an arbitrary position, D1, D2, and O1 through O8 (Fig. 3), which also bears the designation of the mutated viral genomes. Mutant plasmids (pD1, pD2, and pO1 through pO8) were generated by introducing single nt substitution into the PCV2 loop sequence of pZ1. After transfecting the excised and recircularized viral genomes into PK15 cells, the transfected cultures were monitored for viral protein synthesis, DNA replication, and progeny viruses. Two independent experiments were conducted and the results were essentially identical (Table 1).

Viral protein synthesis

At 48 h, one set of transfected cultures was assayed for viral proteins by immunochemical staining with antiserum B6. Similar to Z1, an abundant number of viral antigen-positive cells was observed with D1, D2, O2, O4, and O5 (Fig. 4, upper panel). A reduced number of antigen-positive cells was observed with O1 (10% of Z1) and pO3 (<2% of Z1), while few antigen-positive cells (<1% of Z1) were detected with O6, O7, and O8. In addition, the antigen-positive cells of O3, O6, O7, and O8 were not as darkly stained as Z1, which indicated that these mutant genomes expressed reduced levels of viral antigens.

DNA replication

At 48 h post-transfection, Dpn1-resistant DNA was detected with Z1, D1, D2, O2, O4, and O5 (Fig. 5). Reduced level of newly synthesized viral RF DNA was observed with O1, and no Dpn1-resistant DNA was detected with O3 and O6 through O8. Each PCR product was cloned and the nt sequence at the Ori was determined. Only the input engineered nt were detected with D1, O1, O2, O4, and O5, but both input and wild-type sequences (the engineered C nt reverted to an A nt) were detected with D2.

Progeny virus production

At 7 days, infectious viruses were readily detected in the cultures transfected with D1, D2, O1, O2, O4, and O5 (Fig. 4–lower panel). Whereas progeny virus vD1, vD2, vO2, and vO5 continued to multiply with each additional cell passage, the progeny viruses vO1 and vO4 diminished with each additional cell passage. Both vO1 and vO4 failed to grow beyond cell passage 3 or 4 in two separate experiments. At passage 5, progeny viruses that could be propagated were vD1, vD2, vO2, and vO5.

Seven additional transfections were conducted with O1, O3, and O4 in three separate experiments, and the transfected cultures were monitored for progeny virus production. Two of the experiments gave results identical to that described above. However, in an experiment carried out in duplicate, the progeny viruses recovered from O1 and O4 (designated

Fig. 1. (A) Schematic representation of the PCV2 genome. Locations of the Dpn1 sites and primers used for PCR amplification are indicated below the genome. Transcription pattern of the major PCV2 RNAs are also indicated. *CR* is transcribed leftward. *Rep* and *Rep*′ 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. The large intergenic region of PCV2 is from nt 1736 to nt 50. (B) Schematic representation of the PCV2 and PCV1 plus-strand Ori indicating potential base pairing of the flanking palindrome. The genomic sequences of PCV2 (1768 nt) (GenBank accession number AY094619) and PCV1 (1759 nt) (GenBank accession number AY184287) with respect to the presumed nick site (TAGTATTAC) present in the octanucleotide of the loop are in bold letters and enclosed in boxes. The nt coordinates (1, 2, and 3 ...) are based on the actual genomic sequence and the nt coordinates (3′, 4′, and 5′ ...) are arbitrarily assigned to show nt complementarity of the flanking palindromic sequences. The 6-nt tandem repeat sequence (CGGCAG) and 5-nt common sequence (CACCT) between PCV1 and PCV2 are enclosed in square and oval boxes, respectively. Nucleotide differences are shaded and the initiation codons for *Rep* and *CR* are in black boxes.

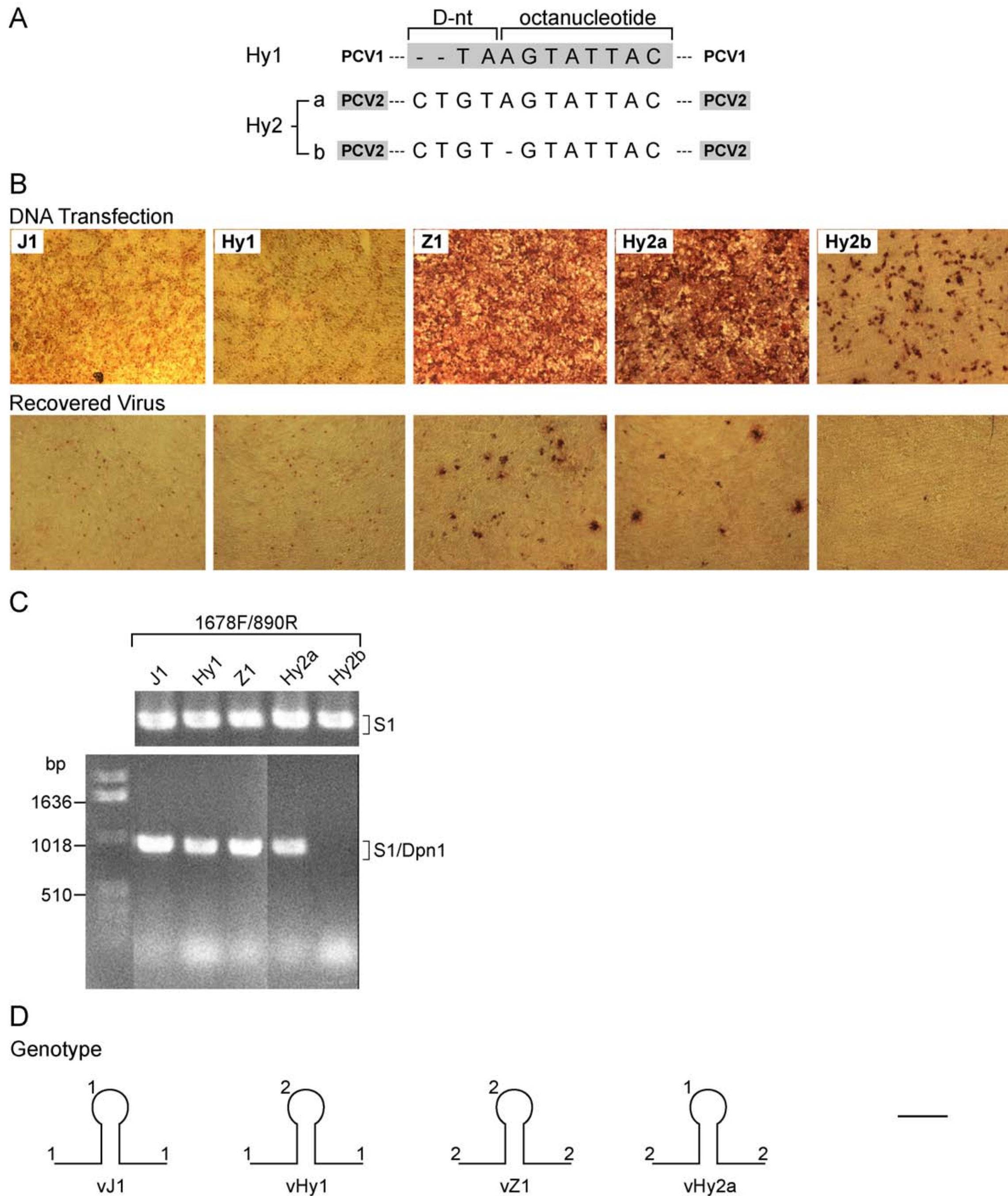


Fig. 2. (A) Composition of the loop sequences in the hybrid plasmids. (B) Immunochemical staining of viral antigens in PK15 cells transfected with plasmid-derived viral DNAs (upper panel) or recovered progeny viruses (lower panel). The viral DNA used for each transfection is indicated in each panel. (C) PCR assay for newly synthesized PCV RF DNA in transfected cultures, after S1 (upper panel) or S1/Dpn1 (lower panel) digestion. The viral genomes used for transfection are indicated on top of each lane. The PCR primers for both PCV1 and PCV2 DNAs are 1678F and 890R. (D) Schematic representation of the nt sequence present in the recovered viruses. PCV1-specific sequence is represented by number 1 and PCV2-specific sequence is represented by number 2.

svO1 and svO4) decreased initially and then increased. These viruses were stable beyond the fifth cell passage.

Genotypes of progeny viruses

Nucleotide sequence determination was carried out with virus-infected cell RF DNAs at cell passages 2, 3, and 5 for experiment 1 and at cell passage 3 for experiment 2 (Table 1). Among the stable viruses, the engineered mutant nt were detected with vD1, vO2, and vO5, but both mutant and wild-type nt were detected with vD2. For the unstable viruses vO1 and vO4 of experiments 1 and 2 (assayed at early cell passages), they only contained the engineered input mutations.

	D-nt		O-nt							
Position	1	2	1	2	3	4	5	6	7	8
								↓		
PCV2	T	A	A	G	T	A	T	T	A	C
pD1	C	•	•	•	•	•	•	•	•	•
pD2	•	C	•	•	•	•	•	•	•	•
pO1	•	•	C	•	•	•	•	•	•	•
pO2	•	•	•	C	•	•	•	•	•	•
pO3	•	•	•	•	C	•	•	•	•	•
pO4	•	•	•	•	•	C	•	•	•	•
pO5	•	•	•	•	•	•	C	•	•	•
pO6	•	•	•	•	•	•	•	C	•	•
pO7	•	•	•	•	•	•	•	•	C	•
pO8	•	•	•	•	•	•	•	•	•	G

Fig. 3. Single nt substitution mutations introduced into the loop sequence of PCV2. The octanucleotide core sequence is shaded and the presume nick site is indicated by an arrow.

For the stable svO1 viruses, two variant loop sequences [TAAGTATTAC (wild-type) and TACTATTAC or (wild-type and AGTAGTATTAC)] were recovered from each transfected culture. From the two O4 transfected cultures, only wild-type loop sequence was detected among the progeny svO4 viruses.

Discussion

In this work, a plasmid-based (Z-series) transfection system capable of yielding stable infectious PCV2 was established. In a previous study (Cheung, 2003c), PCV2 genomic clones (Y-series) that contained an engineered *Bam*HI site at nt 1015–1020 (modified from taatgg to ggatcc) in the small intergenic region between the capsid and Rep proteins were capable of yielding progeny viruses upon transfection into PK15 cells; however, the recovered viruses could not be propagated beyond a few cell passages. In the present study, pZ1 was generated by cleaving the PCV2 genomic DNA at the unique *Eco*RI site (located at nt 1427) and then inserting it into the *Eco*RI site of Bluescript plasmid. The excised and circularized viral DNAs used for transfection were 100% wild-type sequences and the recovered viruses, vZ1, were stable. Thus, an intact small intergenic region is essential for the generation of stable PCV2 in PK15 cells.

Mutational analysis was conducted to investigate the importance of the PCV2 loop sequence in viral protein synthesis, self-DNA replication, and progeny virus production. The results of the first two experiments were

Table 1
Summary of single nt substitution mutation experiments

		D-nt		O-nt							
	Position	**1**	**2**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Expt 1	Protein (%)	100	100	<10	<100	<1	100	100	<1	<1	<1
	DpnI^{R} DNA	++	++	+	++	-	++	++	-	-	-
	genotype:	i^{7}	$i^{5}w^{5}$	i^{8}	i^{12}	nd	i^{5}	i^{9}	nd	nd	nd
	Virus recovery	++	++	+	++	-	++	++	-	-	-
	passage 1:	↑	↑	↓	↑	-	↓	↑	-	-	-
	passage 2:	↑	↑	↓	↑	-	↓	↑	-	-	-
	genotype:	i^{3}	$i^{4}w^{2}$	i^{3}	i^{3}	nd	i^{5}	i^{6}	nd	nd	nd
	passage 3:	↑	↑	-	↑	-	↓	↑	-	-	-
	genotype:	i^{8}	$i^{1}w^{6}$	nd	i^{9}	nd	i^{6}	i^{5}	nd	nd	nd
	passage 5:	↑	↑	-	↑	nd	-	↑	nd	nd	nd
	genotype:	i^{8}	$i^{2}w^{6}$	nd	i^{6}	nd	nd	i^{5}	nd	nd	nd
Expt 2	Protein (%)	100	100	<5	<100	<1	80	100	<1	<1	<1
	DpnI^{R} DNA	++	++	+	++	-	++	++	-	-	-
	genotype:	i^{8}	$i^{6}w^{4}$	i^{7}	i^{11}	nd	i^{6}	i^{8}	nd	nd	nd
	Virus recovery	++	++	+	++	-	++	++	-	-	-
	passage 1:	↑	↑	↓	↑	-	↓	↑	-	-	-
	passage 2:	↑	↑	↓	↑	-	↓	↑	-	-	-
	passage 3:	↑	↑	-	↑	-	↓	↑	-	-	-
	genotype:	i^{6}	$i^{1}w^{3}$	nd	i^{4}	nd	i^{4}	i^{5}	nd	nd	nd
	passage 4:	↑	↑	-	↑	-	-	↑	nd	nd	nd
	consensus:	**x**	**x**	**A**	**x**	**T**	**A**	**x**	**T**	**A**	**C**

+ positive	↑ increase	i input	nd not done
- negative	↓ decrease	w wild-type	

Protein synthesis (%), Dpn1-resistant RF DNA (Dpn^{R}), and genotype (superscript indicates frequency of the sequence detected) were monitored.

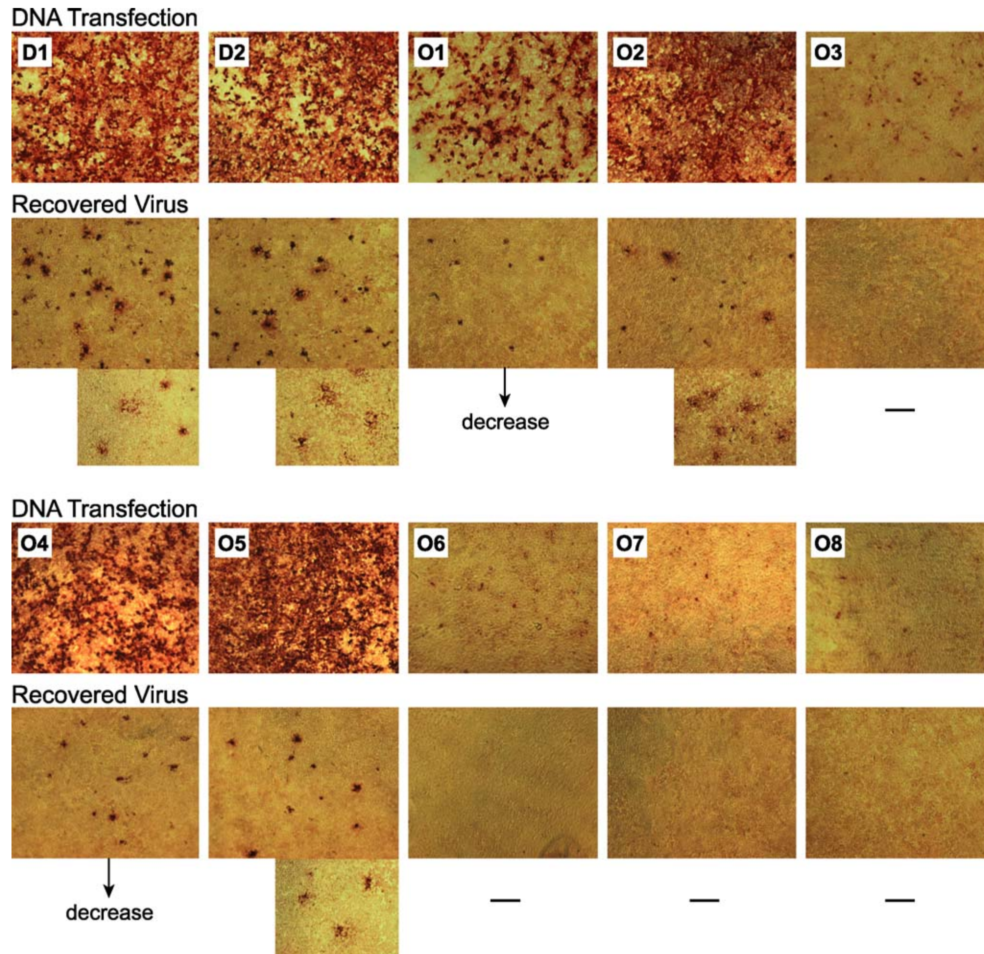


Fig. 4. Immunochemical staining of viral antigens in PK15 cells transfected with plasmid-derived viral DNAs (upper panel) or recovered progeny viruses (lower panel). The insert panels represent recovered progeny viruses at cell passage 5. The viral genomes used for transfection are indicated in each panel.

summarized in Table 1. In general, plasmid-derived viral DNAs that exhibited abundant viral protein synthesis and DNA replication yielded progeny viruses that could be continually propagated in cell culture; however, exceptions (O1 and O4) were observed. Mutant viral genomes (D1, D2, O2, O4, and O5) that exhibited 100% (with respect to Z1) protein synthesis and DNA replication yielded progeny viruses, O1 that showed reduced levels of protein and DNA synthesis also gave progeny viruses, but O3, O6, O7, and O8 that exhibited severe reduction (<1% of Z1) in protein synthesis did not show DNA replication or progeny virus production. Although infectious viruses vO1 and vO4 were recovered from the transfected cultures of experiments 1 and 2 (each contained an engineered mutation), the fact that these viruses could not be propagated in subsequent cell passages suggest that the wild-type nt at positions O1 and O4 are essential for long-term viability of the progeny viruses. Essentially, this notion is supported by the isolation of stable svO1 and svO4 progeny viruses (in the additional experiments) that contained the wild-type or an octanucleotide motif (see below) sequence.

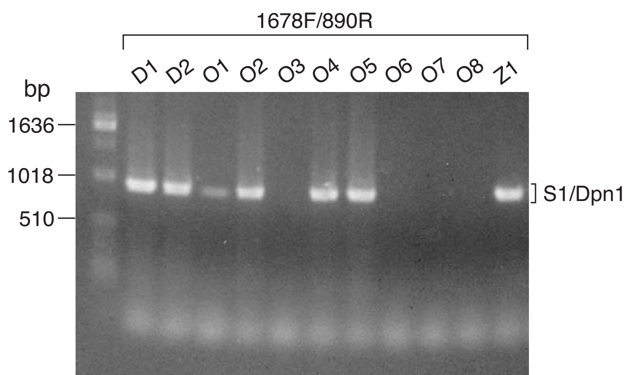


Fig. 5. PCR assay for newly synthesized PCV2 RF DNA in transfected cultures, after S1/Dpn1 digestion. The plasmid used for each transfection is indicated on top of each lane and the primers are 1678F and 890R.

Nucleotide sequence analysis showed that vD1, vO1, vO2, vO4, and vO5 all contained their respective engineered mutations, although the recovered viruses from D2 were a mixture of two viruses that contained either the initial engineered mutation or the reverted wild-type nt at early

as well as at late cell passages. Thus, the loop sequence of PCV2 at positions D1, D2, O2, and O5 can accept variable nt, while positions O1, O3, O4, O6, O7, and O8 appear to require wild-type nt to generate stable infectious viruses. In the generation of stable svO1 and svO4 progeny viruses, the input mutant nt either reverted to wild-type loop sequence or mutated to an octanucleotide motif sequence with variable D-nt. Taking together, an octanucleotide sequence motif, AxTAxTAC, at the Ori of PCV2 emerges as the essential core element (ECE) for protein synthesis, DNA replication, and stable progeny virus production. In the ECE, there are six critical nt (each represented by an actual nt) and two variable nt (represented by x). Interestingly, a variable nt has also been observed in the conserved nonanucleotide of a geminivirus (Schneider et al., 1992).

Previous sequence alignment analysis demonstrated that both the nonpathogenic PCV1 and the pathogenic PCV2 utilize comparable genetic elements similarly located along their respective genomes for viral gene expression (Cheung, 2003a, 2003b; Cheung and Bolin, 2002). The fact that the Ori sequences of both PCVs are quite similar and that the loop of PCV1 (CTGT***AGTATT↓AC***) and PCV2 (TA***AGTATT↓AC***) both contain an identical ECE, it is not surprising that the loop sequences of these two viruses are functionally interchangeable with respect to protein synthesis, DNA replication and hybrid progeny virus generation. This result corroborates with the findings of a recent study which showed that the Rep proteins of PCV1 and PCV2 can use the LIR sequences of the two viruses interchangeably in transient expression assays (Mankertz et al., 2003). The results also lend support to the expectation that the Rep-protein complex probably binds the PCV2 octanucleotide ECE and nicks it between the sixth T and seventh A to initiate DNA replication, a situation that resembles geminivirus DNA replication closely. It is expected that the octanucleotide plays a similar role in PCV1 because nt deletion (e.g., Hy2b) or base substitution (Mankertz et al., 1997) involving a critical nt of the ECE resulted in complete shut down of viral DNA replication.

In four independent experiments (including two additional experiments not mentioned above), reversion of the engineered C nt at position 2 to the wild-type A nt was observed in a portion of the transfected DNAs and then later in a mixed population of recovered viruses vD2. Interestingly, nt reversion was not observed among other noncritical bases of the loop (positions D1, O2, and O5). Thus, the nt reversion that occurred at position 2 may not be a random event. Previous work (Cheung, 2004b) showed that during initiation of PCV1 DNA replication, two positions of the flanking palindrome at the Ori (i.e., the 3rd and 10th positions of the right arm) would revert to a C nt, regardless of the DNA templates available. Although it was speculated that the Rep-protein complex may play a role in determining which nt to incorporate at these "strategic" positions, the mechanisms involved in nt reversion at the Ori of PCV remained to be elucidated.

Materials and methods

Genomic clone, cell, and antiserum

The PCV2/688 isolate (GenBank accession number AY094619) capable of inducing PMWS (Bolin et al., 2001) was used to generate the genomic clone pZ1 of this study. A PCV1 genomic clone (pJ1) (GenBank accession number AY184287) with an additional *Bam*HI site (GGATCC) at nt 992 (between the 3′ ends of the Rep- and capsid-regions) inserted into the *Bam*HI site of Bluescript plasmid (Cheung, 2003b, 2004a), a PCV1-free PK15 cell line, and a hyperimmune swine serum (B6) raised against PCV2 that reacts with the Rep-antigens of PCV1 (Cheung, 2004a) were used.

Oligonucleotide primer

The primers for PCR amplification are identified by the first nt coordinate of each oligomer with respect to the genomic sequence, and their locations are indicated in Fig. 1A. The suffix F indicates forward (rightward) direction, while suffix R indicates reverse (leftward) direction. The primers are 1678F (CCAAGATGGCTGCGGGGG) and 890R (GTAATCCTCCGATAGAGAGC). These primers can also be used to amplify PCV1 DNA and they are similarly located in the PCV1 genome. Only one strand of the mutagenic primer set is listed and the mutated nt are indicated in upper case letter:

D1: gaagtgcgctgGaagtattacca; D2: aagtgcgctgtCagtattaccag; O1: agtgcgctgtaCgtattaccagc; O2: agtgcgctgtaaCtattaccagcg; O3: agtgcgctgtaagCattaccagcgc; O4: agtgcgctgtaagtCttaccagcgcactt; O5: cgctgtaagtaCtaccagcgcac; O6: tgcgctgtaagtatCaccagcgcacttcg; O7: gcgctgtaagtattCccagcgcacttcgg; O8: cgctgtaagtattaGcagcgcacttcggc; Hy1: gaaagaagtgcgctgTAagtattaccagcgcac; Hy2a: gaaagaagtgcgctgCTGTagtattaccagcgcac; and Hy2b: gaaagaagtgcgctgCTGT-gtattaccagcgcac.

DNA mutagenesis, transfection, immunochemical staining, DNA preparation, and PCR

The methodologies for DNA mutagenesis, transfection, immunochemical staining, and PCR have been described previously (Cheung, 2003c).

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