Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 346 (2006) 32 – 39

VIROLOGY

www.elsevier.com/locate/yviro

Regeneration of the replication-associated proteins tandem direct repeat recognition nucleotide sequence at the origin of DNA replication of porcine circovirus type 1

Andrew K. Cheung *

Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, PO Box 70, Ames, IA 50010, USA

Received 3 July 2005; returned to author for revision 8 September 2005; accepted 4 October 2005
Available online 21 November 2005

Abstract

Four copies of a hexanucleotide (H) sequence are located to the right of the palindrome at the origin of DNA replication of the porcine circovirus type 1 (PCV1) genome. These sequences are organized in two direct tandems, the proximal H1/H2 and the distal H3/H4 repeats, and they have been shown to be binding sites for the essential Rep and Rep′ proteins. Previous work demonstrated that infectious PCV1 virion can accommodate a variable number of H sequences at the origin of DNA replication. In this work, mutational analysis was conducted to elucidate the critical core element within the hexanucleotide with respect to self-DNA replication and progeny virus synthesis. It was found that while a single H sequence abutting the palindrome is sufficient for PCV1 viability, a tandem repeat arrangement is the more stable and thus preferred configuration. Within the H sequence, selected nucleotides at specific positions are critical for Rep-associated protein recognition and for viral DNA replication.
Published by Elsevier Inc.

Keywords: Porcine circovirus; Origin of DNA replication; Direct tandem repeats

Introduction

Porcine circovirus (PCV) is a member of the genus *Circovirus* of the Circoviridae family. This family consists of a diverse group of animal viruses that possess a small, closed circular, single-stranded DNA genome that replicate through double-stranded intermediates (McNulty et al., 2000; Pringle, 1999). The PCV virion is icosahedral, non-enveloped and 17 nm in diameter (Tischer et al., 1982). Two genotypes of PCV have been identified. PCV type 1 (PCV1) is non-pathogenic, while PCV type 2 (PCV2) has been implicated as the etiological agent of postweaning multisystemic wasting syndrome in swine (Allan and Ellis, 2000; Clark, 1996; Harding, 1996; Segales and Domingo, 2002). The genome nucleotide (nt) sequences of a number of PCV1 and PCV2 isolates have been determined (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1997, 1998; Morozov et al., 1998; Niagro et al., 1998). It has been suggested that the PCV genome is an intermediate between geminivirus and plant circovirus (renamed nanovirus) (Niagro et al., 1998; Randles et al., 2000) and resulted from recombination between a plant nanovirus and an animal picorna-like RNA virus (Gibbs and Weiller, 1999).

The minimal origin of DNA replication (Ori) of PCV1 has been mapped to a 111-bp fragment (Mankertz et al., 1997) which includes the large intergenic region at the Ori (Ori-IR) (Fig. 1). The current model for PCV DNA replication postulates that the closed circular single-stranded DNA genome is first converted to a superhelical double-stranded DNA replication intermediate. The virus-encoded Rep and Rep′ proteins (Rep-complex) essential for DNA replication are expressed (Cheung, 2003, 2004a; Mankertz and Hillenbrand, 2001). The Rep-complex recognizes and binds the hexanucleotide (H) tandem direct repeats and the right-arm of the presumed stem-loop structure formed by a pair of inverted repeats (palindrome) (Steinfeldt et al., 2001). This complex then destabilizes, unwinds, and nicks (indicated by ↓) the octanucleotide motif sequence ($A_1x_2T_3A_4x_5T_6{\downarrow}A_7C_8$) (condensed from the $\underline{T}A_1G_2T_3A_4T_5T_6A_7C_8$ nonanucleotide)

* Fax: +1 515 663 7458.
E-mail address: acheung@nadc.ars.usda.gov.

0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2005.10.004

(Cheung, 2004c, 2005a) between T_6 and A_7 to generate a free 3'-OH end for initiation of plus-strand DNA replication. It has been demonstrated that the replication proteins (Mankertz et al., 2003) and the Ori-IRs (including the loop sequences) of PCV1 and PCV2 are interchangeable (Cheung, 2004c; Mankertz et al., 2003); and that the conserved octanucleotide motif sequence embedded in each respective loop sequence is essential for DNA replication (Cheung, 2004c, 2005a). Apparently, the Ori-flanking stem-loop structure of PCV is non-essential for initiation but is likely a signal for termination of DNA replication (Cheung, 2004b, 2004d).

In vitro experiments showed that PCV1 Rep, but not Rep′, binds the right-arm of the stem-loop structure at the PCV1 Ori (Steinfeldt et al., 2001); while both Rep and Rep′ bind the adjacent proximal tandem H sequences at nt 13–18/19–24 (CGGCAG/CGGCAG = H1/H2) and the distal, almost perfect, tandem at nt 30–35/36–41 (CGGCAG/CGT*CAG = H3/H4). The H1/H2 and H3/H4 tandem repeats are separated by a 5-nt sequence (cacct = y), which is similar to the 5-nt (cactt = x) of the palindrome preceding H1/H2. Previous work (Cheung, 2005b) showed that the A-rich sequence to the left of the stem-loop structure is not essential for virus replication, and not all 4 copies of the H sequence are required for PCV1 viability. Mutations introduced into H1/H2 and/or H3/H4 resulted in progeny viruses containing a variety of h-like/H sequences.

In this study, a series of modified genomes containing a tandem H3/H4 sequence (2-H genotype), a single H sequence (1-H genotype) or no H sequence (0-H genotype) were analyzed to determine the critical motif within the H sequence.

Results

Mutagenesis of viral genomes with 2-H genotype

A mutant genome (Ca9), with deletion of the H1/H2.y sequence, was engineered from wild-type J1 (Fig. 1B). Additional mutations (space insertion, single-nucleotide substitution, double-nucleotide substitution, nucleotide insertion or deletion) were then introduced into Ca9 to disrupt the remaining H3/H4 tandem repeat.

(i) Space insertion

Two mutant genomes (C101 and C103) with 1 or 3 A nucleotides inserted between the stem-loop and H3/H4 were constructed (Table 1). Immunochemical staining showed that the number of Rep-positive cells exhibited by both mutants was greatly reduced when compared to Ca9. Progeny viruses were not readily detected in the transfected cultures, but infectious viruses were recovered from two of the cultures upon additional cell passages. Therefore, a small amount of progeny viruses must have been synthesized initially in these negative cultures. At cell passage 3, the progeny viruses recovered from C101 contained the sequence aGGCAG/H4, while the progeny viruses recovered from C103 contained the sequence aaGCAG/H4. Thus, the engineered A nucleotide in C101 had replaced the first C nucleotide of H3 and 2 of 3 engineered A nucleotides in C103 had replaced the CG dinucleotide of H3, to maintain a 6-nt unit, in the recovered viruses. Although both types of mutant viruses were still detectable at cell passage 8, some of the C101 progeny viruses had already reverted to the parent Ca9 genotype.

(ii) Single-nucleotide substitution

A series of twelve mutant genomes were constructed by replacing each position of H3/H4 with an arbitrarily selected nucleotide, individually (Table 2a). Immunochemical staining showed that all twelve mutant genomes exhibited comparable number of Rep-positive cells and yielded progeny viruses. The viruses recovered from the H4 mutant genomes all retained the engineered mutations. The viruses recovered from the H3 mutant genomes showed that position 1 mutation was retained, position 2 mutation was retained initially but revert to wild type at later cell passages, positions 3 to 6 mutations reverted to wild type readily.

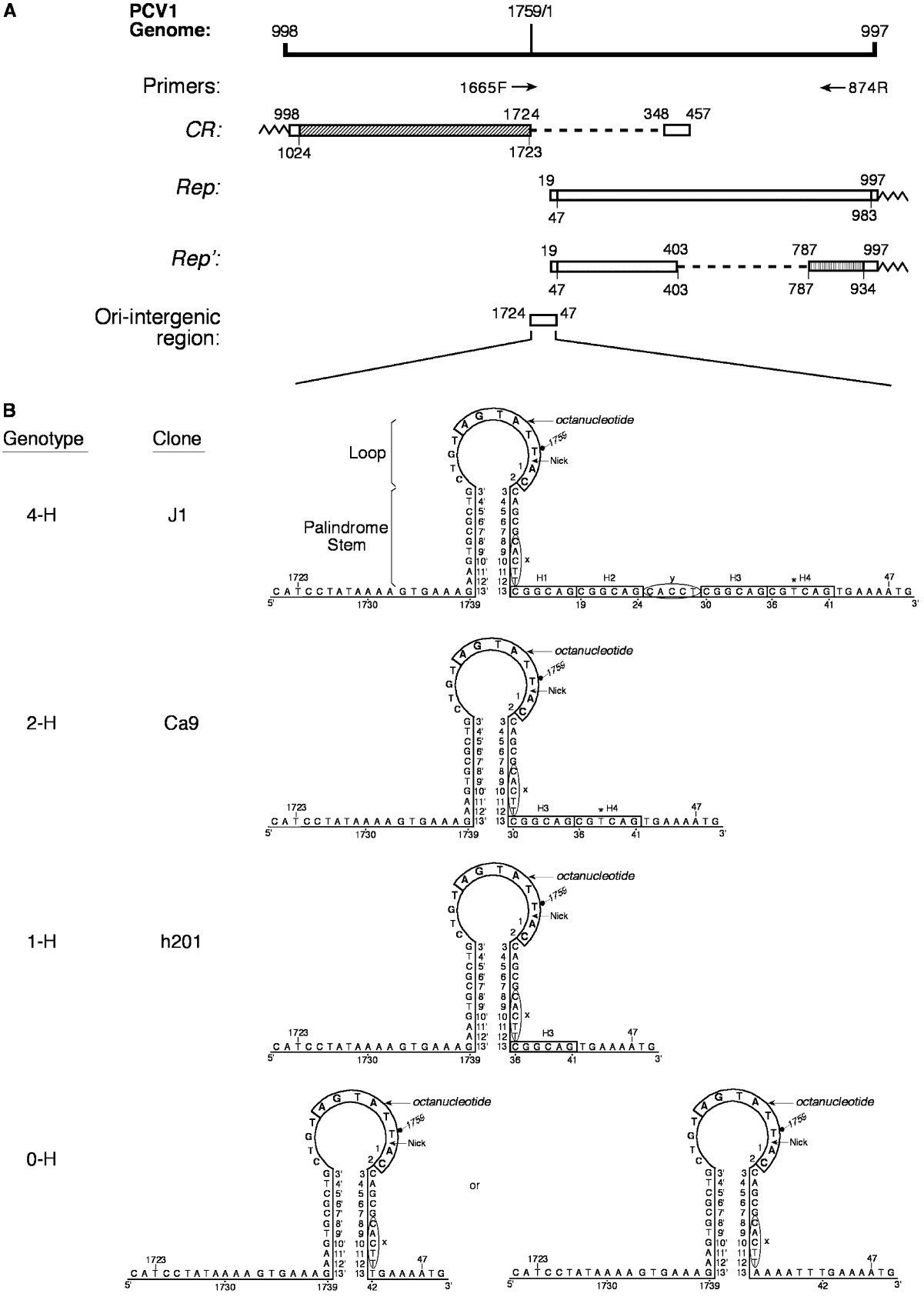
Interestingly, the effect of various base substitutions at a specific position can be quite different. Four genomes (Ca9, h116, h126, and h123) each with a different nucleotide at position 3 of H3 yielded a different set of progeny viruses initially (Table 2b). Sequence analysis of the recovered viruses showed that the G nucleotide in Ca9 was stable, a T nucleotide was stable (h126), an A nucleotide was accommodated at early passages (h116), but a C nucleotide was quickly changed to a T or G (h123). Eventually, all the variant viruses reverted to the parent H3/H4 Ca9 genotype. Taken together, positions 3–6 of H3 appeared to be the critical core element of the H3/H4 tandem, and a C nucleotide at position 3 of H3 was not tolerated.

(iii) Disruption of the H3/H4 tandem motif by double-nucleotide substitution, insertion, or deletion in H4

Four mutant genomes with double-nucleotide substitution (h114 = H3/aaTCAG), deletion (d136 = H3/-GTCAG or d137-8 = H3/C- -CAG) or insertion (i138-9 = H3/CGTttCAG) were engineered into H4 of Ca9 (Table 3). The number of Rep-positive cells exhibited by these genomes, except h114 (50–80% of Ca9), was comparable to Ca9 and progeny viruses were readily recovered from all the mutant genomes. In general, most of the recovered viruses retained the engineered mutations, including insertion or deletion, at early cell passages. At later cell passages, 8 of 10 recovered viruses (virus-m, -a, -b, -e, -g, -p, -q and -r) duplicated the H3 sequence, and they still retained the engineered mutations. The other 2 viruses (virus-k and -c) contained H3/h-like sequences. Thus, the presence of a stringent tandem H motif was not required for PCV1 viability.

(iv) Disruption of the H3/H4 tandem motif by double-nucleotide substitution in H3

Five mutant genomes containing substitutions (h111 = ttGCAG/H4, h112 = CttCAG/H4, h103 = CGcgAG/H4, h113 = CGttAG/H4, h115 = CGGCtt/H4) were engineered into the H3 sequence of Ca9 (Table 4). Immunochemical staining showed that the number of Rep-positive cells was


A
PCV1 Genome:
998
1759/1
997
Primers:
1665F
874R
CR:
998
1724
348
457
1024
1723
Rep:
19
997
47
983
Rep':
19
403
787
997
47
403
787
934
Ori-intergenic region:
1724
47
B
Genotype
Clone
4-H
J1
2-H
Ca9
1-H
h201
0-H
Loop
Palindrome Stem
octanucleotide
Nick
or

slightly affected by the mutations in h113 and h115 (80–90% of Ca9), moderately affected in h111 (40–60% of Ca9), and severely affected in h103 and h112 (1–5% of Ca9). Progeny viruses were recovered from h111, h113, and h115 but not from h103 and h112. Interestingly, mutations introduced at the same location with different nucleotides, h103 and h113, yielded very different results. Whereas h113 exhibited ample number of Rep-positive cells and with production of progeny viruses, h103 exhibited severely reduced number of Rep-positive cells and yielded no progeny virus. Sequence determination showed that all the recovered viruses maintained the 6-nt unit configuration and accommodated several variant H motifs such as h-like/H4, H3/H4 or h-like/H4/H4. Unexpectedly, wild-type PCV1 (with the sequence H1/H2.cacct.H3/H4) was detected among the recovered viruses of h111 at later cell passages.

Table 1
Space insertion mutagenesis between the stem-loop and H3/H4 of Ca9

	Ca9	C101			C103		
	H3/H4	aH3/H4			aaaH3/H4		
Rep+ cell (% Ca9)	100	3			<1		
Recovered virus	+	−	−	−	−	−	−
Passage 1	+	+	−	−	−	−	−
Passage 2	+	+	−	−	−	−	+
Passage 3	+	+	−	−	−	−	+
Genotype							
Passage 4	d^{10}	A^{10}					AA^{11}
Passage 8	d	A^8d^{10}					AA

d = Ca9, A = aGGCAG/H4, AA = aaGCAG/H4, − = negative, + = positive. The number of examples (subclones) of each genotype, determined by sequencing cloned PCR fragments, is indicated by superscript. In subsequence experiments, superscript is not included if only one genotype of virus is observed.

Mutagenesis of viral genomes with 1-H genotype

A mutant genome (h201) with only one copy of the H sequence (H3 = CGGCAG) (Fig. 1B) was constructed. Additional mutations (space insertion or single-nucleotide substitution) were then introduced into h201.

(i) Space insertion

Three mutant genomes (D101, D102 and D103) with 1, 2, or 3 A nucleotides inserted between the stem-loop structure and H3 were engineered into h201. In comparison with Ca9, these mutant genomes exhibited greatly reduced number of Rep-positive cells and did not yield any progeny virus.

(ii) Single-nucleotide substitution mutation

Each nucleotide of the H3 sequence of h201 was replaced with an arbitrarily selected nucleotide, individually (Table 5). In comparison with Ca9, all these mutant genomes exhibited variable number of Rep-positive cells, and progeny viruses were readily recovered. Although progeny viruses with a single H3 sequence (virus-s) or h-like/H4 sequence (virus-a and -c) were detected at early cell passages, all the them (including h201) reverted to the H3/H4 Ca9 genotype by passage 7.

Mutagenesis of viral genomes with 0-H genotype

Two 0-H mutant genomes were engineered. The H3 sequence was either deleted from h201 or replaced with the sequence AAAATT (Fig. 1B). In comparison with h201, the number of Rep-positive cells was severely reduced, and progeny virus was not recovered. Substitution of the AAAATT sequence at each position, individually, with a wild-type nucleotide also did not yield any progeny virus.

Discussion

This work confirms and extends previous work that the H sequences at the Ori of PCV1 are very flexible. The number of copies as well as the exact nucleotide sequence can vary. Although wild-type PCV1 has 4 copies of H, mutant viruses containing one, two, or three copies of H have been isolated (Table 3, Cheung, 2004d, 2005a, 2005b). The presence of 2 H sequences in tandem repeat motif (H3/H4), although not absolutely essential, appears to be a stable configuration since 1-H genotype viruses (the h201 mutant genome series) consistently revert to the H3/H4 Ca9 genotype upon passage in cell culture (Table 5). However, a stringent tandem motif is also not required for PCV1 DNA replication, since progeny viruses [virus-AA of C103 (aaGCAG/CGtCAG) and virus-i of h111 (ttGCAG/CGtCAG)] each containing 3 different nucleotides out of 6 between H3 and H4 are viable.

The H3 sequence nearest to the stem-loop appears to be the critical component within the H3/H4 tandem repeat of Ca9 (Table 2a). Single nucleotide substitutions introduced into H4 were retained, while mutations engineered into the last 4 positions of H3 reverted to wild type readily. Within the H3 sequence ($C_1G_2G_3C_4A_5G_6$), positions 1 and 2 can tolerate different nucleotides (e.g., virus-AA of C103 and virus-i of h111), position 3 can accommodate G, T and A (e.g., virus-c, -d, and -y in Table 4) but not a C (e.g., h123), position 4 prefers a C nucleotide but will tolerate a T (e.g., virus-h of h123), and

Fig. 1. (A) Schematic representation of the PCV1 genome. Locations of the primers used for PCR amplification are indicated below the genome. Transcription patterns of the major PCV1 RNAs (Cheung, 2003) are also indicated. Capsid RNA (*CR*) is transcribed leftward. *Rep* and *Rep′* are transcribed rightward. The RNAs are annotated with nt co-ordinates that indicate the last nucleotide of each respective exon. The coding sequence of each transcript is shaded and their nt co-ordinates are indicated below each RNA. (B) The plus-strand Ori-IR indicating potential base pairing of the flanking palindrome of PCV1 (designated 4-H genotype). The genomic sequences of PCV1 (1759 nt) (GenBank accession numberAY184287) encompassing the presumed nick-site (AGTATT↓AC) present in the octanucleotide of the loop are in bold letters and enclosed in a box. The nt co-ordinates (numbered 1, 2, 3, etc.) are based on the actual genomic sequence and the nt co-ordinates (numbered 3′, 4′, 5′, etc.) are arbitrarily assigned to show nt complementarity of the flanking palindromic sequences. The 5-nt *x* and *y* sequences are enclosed in ovals. The 6-nt H sequences (CGGCAG or CGT*CAG) are labeled and enclosed in boxes. The initiation codons for the Rep proteins (at nt 47) and the capsid protein (at nt 1723) are shaded. Mutant genomes with 2-H, 1-H, or 0-H genotype are indicated.

Table 2a
Single-nucleotide substitution mutagenesis of the H3/H4 sequence of Ca9

		H3							H4					
h-genome	Ca9	101	102	116	117	118	119	/	136	137	138	139	140	141
Position		1	2	3	4	5	6	/	7	8	9	10	11	12
		C	G	G	C	A	G	/	C	G	T	C	A	G
Mutation	none	A	C	A	T	C	C	/	T	T	C	T	T	T
Rep+ cell (% J1)	100	100	100	100	100	100	100		100	100	100	100	100	100
Recovered virus	+	+	+	+	+	+	+	/	+	+	+	+	+	+
Passage 3	+	+	+	+	+	+	+	/	+	+	+	+	+	+
Genotype														
Passage 3	d^5/d^5	i^{12}/i^{12}	i^{11}/i^{12}	i^9d^1/i^9	i^1d^6/i^9	i^7d^1/i^6d^4	i^2d^2/i^6d^5	/	i/i	i/i	i/i	i/i	i/i	i/i
Passage 4	d/d	i/i	$i/i^{10}a^1$	i^6d^5/i^6d^6	d/d	i^1d^{11}/d	d/d	/	i/i	i/i	i/i	i/i	i/i	i/i
Passage 8	d/d	i/i	i^3a^{11}/d	d/d	d/d	i^7d^{16}/d	d/d	/	i/i	i/i	i/i	i/i	i/i	i/i
Consensus	–	–	–	G	C	A	G	/	–	–	–	–	–	–

+ = positive, – = not done, i = input, a = CaGCAG/H4, d = Ca9 = H3/H4. Superscript indicates the number of subclones.

positions 5 and 6 are usually occupied by the wild-type nucleotides A and G, respectively. Therefore, nucleotide combinations and their locations are crucial for PCV1 viability. Different mutations introduced at the same position may yield very different results (e.g., h103 and h113).

Clearly, the presence of at least 1 "recognizable" H sequence abutting the stem-loop is required for PCV1 viability as evident by the progeny viruses recovered from the h201 mutant genome series (Table 5). In contrast, progeny viruses were not recovered from mutant genomes that have a H sequence but separated from the palindrome (e.g., D1, D2 and D3) or from mutant genomes lacking a recognizable H sequence (e.g., the 0-H mutant genome series). These results are in agreement with the general concept that binding of the H sequences by the Rep-complex is required for PCV1 DNA replication. Based on the mutations introduced and the sequences detected in the recovered viruses in this study, the h-like nucleotide sequences, xxGCAG (virus-i of h111 and virus-AA of C103), xxtCAG (virus-c of h113), xxGtAG (virus-f of h115) and xxaCAG (virus-y of h115) when abutting the palindrome, constitute the Rep-complex recognition core element or "recognizable H sequence" essential for PCV1 DNA replication.

The frequency at which the mutated PCV1 H sequence reverts to wild type or regenerates a second H sequence in the recovered viruses is remarkable. Of particular interest is the consistent regeneration of H3/H4 (CGGCAG/CGTCAG) from the h201 mutant genome series that contain a single (or variant) H3. The regeneration of H4 is not accountable by duplication of the H3 sequence due to slippage of the replication machinery (Graham et al., 1989) because the slippage mechanism would have yielded progeny viruses with H3/H3 genotype and not H3/H4. The fact that PCV1 Rep and Rep′ proteins bind the H sequences and exhibit different specificity (Steinfeldt et al., 2001) suggests that they may play a role in this nucleotide regeneration process.

Materials and methods

Construction of mutant genomes and experimental design

A PCV1 genomic clone (J1), capable of producing infectious PCV1 upon transfection into PK15 cells after excision and re-circularization of the viral DNA (Cheung, 2004a,b), was employed to construct the mutant genomes used in this study. A schematic representation of the plus-strand Ori is denoted in Fig. 1. The octanucleotide sequence encompassing the presumed nick-site between the first nucleotide (position 1) and the last nucleotide (position 1759) is enclosed in a box. A series of nucleotide changes were introduced into the PCV1 genome to generate the predetermined mutations using the QuickChange Site-Directed Mutagenesis Kit (Strategene, San Diego, CA). The excised and re-circularized plasmids were then transfected into duplicate PK15 cell cultures. At 48 h (h) posttransfection, one set of cultures was assayed for Rep-producing cells by immunochemical staining (Cheung, 2004a; Cheung and Bolin, 2002; Nawagitgul et al., 2000). At 7 days posttransfection, a second set of transfected cultures was harvested, freeze thawed 3 times, and then assayed for infectious virus by inoculation onto fresh PK15

Table 2b
Substitution mutagenesis of Ca9 H3 position 3

	$Ca9^G$	$h116^A$		$h126^T$				$h123^C$	
	CGgCAG/H4	CGaCAG/H4 (previous experiment)		CGtCAG/H4				CGcCAG/H4	
Rep+ cell (% Ca9)	100	100		100				50–80	
Recovered virus	+	+	+	+	+	+	+	+	+
Passage 3	+	+	+	+	+	+	+	+	+
Genotype									
Passage 3	d^{10}	i^{12}	$i^{16}d^1$	i^{14}	i^8	i^{16}	i^8	d^5h^6	d^2h^9
Passage 8	d	d	d	i	i	i	i	d	$d^{10}h^2$

+ = positive, i = input, d = Ca9, h = CGtCAG/CGTCAG (H4/H4). Superscript indicates the number of subclones.

Table 3
Disruption of the Ca9 H3/H4 tandem motif by substitution, deletion or insertion in H4

	H3/aaTCAG		H3/-GTCAG		H3/C--CAG		H3/CGTttCAG	
	h114		d136		d137-8		i138-9	
Rep+ cell (% Ca9)	50	80	100	100	100	100	100	100
Recovered virus	+	+	+	+	+	+	+	+
Passage 3	+	+	+	+	+	+	+	+
Genotype								
Passage 3	k^6	$i^2k^2m^7$	i^9	i^{12}	i^9	$i^{10}a^1b^1$	i^{10}	i^{12}
Passage 8	k	k	$c^6e^2i^1$	$a^7b^3c^2g^1$	p	p^3q^9	i^1r^{11}	i^1r^{11}

Input genome	Recovered virus
h114	k = H3/CaTCAG
	m = H3/H3/aaTCAG
d136	a = H3/cactt/H3/-GTCAG
	b = H3/H3/cactt/H3/-GTCAG
	c = H3/-cTCAG
	e = H3/g/H3/-GTCAG
	g = H3/t/H3/-GTCAG
d137-8	p = H3/H3/C--CAG
	q = H3/t/H3/C--CAG
i138-9	r = H3/H3/CGTttCAG

+ = positive, i = input. Superscript indicates the number of subclones.

cells. To propagate recovered progeny viruses, 24-h-infected PK15 cells were treated with 300 mM glucosamine for 30 min (Tischer et al., 1987), and the infection was allowed to proceed for 7 days for each round of virus passage. At designated cell passage and after confirmation by immunochemical staining, total cell DNAs were isolated and amplified by PCR with PCV1-specific primers. Each PCR product was subcloned into a TA-cloning plasmid (In Vitrogen, Carlsbad, CA), and multiple bacterial clones were randomly selected for nucleotide sequence determination.

Virus, cell, and serum

PCV1 isolate (PCV/AC1) (GenBank accession numberAY184287) (Cheung, 2003) and a hyperimmune swine serum produced against PCV2 that reacts with the Rep-associated proteins of PCV1 (Cheung, 2004a; Cheung and Bolin, 2002) were used. PCV1-free PK15 cell lines were maintained in MEM-Hank's balanced salt solution (MEM-H) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum.

Table 4
Disruption of the Ca9 H3/H4 tandem motif by double-nucleotide substitution in H3

	ttGCAG/H4	CttCAG/H4	CGcgAG/H4	CGttAG/H4	CGGCtt/H4
	h111	h112	h103	h113	h115
Rep+ cell (% Ca9)	40–60	<1	<5	80–90	80–90
Recovered virus	+	–	–	+	+
Passage 3	+	–	–	+	+
Genotype					
Passage 3	$i^{11}/a^3i^3/a^9b^2/d^3i^2$	nd	nd	$c^8/c^6/c^4e^8$	$f^3d^1/g^{10}y^1/g^3y^3$
Passage 4	$a^{10}i^1/a^9i^3/a/d$	nd	nd	c/c/c	$d/g^1y^{11}/y$
Passage 8	$a/a^6w^3/a^1j^{10}/d$	nd	nd	c/c/c	d/y/y

Input genome	Recovered virus
h111	i = TTGCAG/H4
	a = TGGCAG/H4
	b = CTGCAG/H4
	d = H3/H4
	w = TTGCAG/H4/H4
	j = H1/H2/cacct/H3/H4
h113	c = CGTCAG/H4 = H4/H4
	e = CCGCAG/H4
h115	f = CGGTAG/H4
	d = H3/H4
	g = GGGCAG/H4
	y = CGACAG/H4

+ = positive, – = negative, i = input, nd = not done. Superscript indicates the number of subclones.

Table 5
Disruption of H3 sequence of h201 by single-nucleotide substitution

1-H genotype: disruption of H3 sequence of h201 by single-nucleotide substitution							
	h201	h211	h212	h213	h214	h215	h216
	CGGCAG	C	G	G	C	A	G
Mutation	–	T	T	T	T	T	T
Rep+ cell (% Ca9)	100	30–60	30–50	60–80	60–80	50–70	80–100
Recovered virus	+	+	+	+	+	+	+
Passage 3	+	+	+	+	+	+	+
Genotype							
Passage 3	s^7d^2/s^2d^2	d^5/d^6	d^6	d^6	d^{11}/a^{10}	d^9/d^{12}	s^5c^1/s^2d^4
Passage 7	d/d	–	–	–	–/d	d/d	d/d

s = h201 = CGGCAG (H3), d = H3/H4, a = CcGCAG/H4, c = CGGtAG/H4, – = not done. Superscript indicates the number of subclones.

Oligonucleotide primers

The primers for PCR amplification were (CCAAGATGGCTGCGGGGG) and (GTAATCCTCCGATAGAGAGC) located at nt 1665 (forward orientation) and at nt 874 (reverse orientation) of the PCV1 genome, respectively (Fig. 1). The primer sets for mutagenesis were synthesized by placing 13–19 flanking nt on either side of the predetermined mutation.

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

The methodologies for DNA mutagenesis, transfection, immunochemical staining, DNA preparation, and PCR have been described previously (Cheung, 2004d).

Acknowledgments

The author thanks S. Pohl, D. Alt, and K. Halloum for their technical assistance and M. Marti and S. Ohlendorf for the manuscript preparation.

References

Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. 12, 3–14.
Cheung, A.K., 2003. Comparative analysis of the transcriptional patterns of pathogenic and non-pathogenic porcine circovirus. Virology 310, 41–49.
Cheung, A.K., 2004a. Identification of the essential and non-essential transcription units for protein synthesis, DNA replication and infectious virus production of porcine circovirus type 1. Arch. Virol. 149, 975–988.
Cheung, A.K., 2004b. Detection of template strand switching during initiation and termination of DNA replication of porcine circovirus. J. Virol. 78, 4268–4277.
Cheung, A.K., 2004c. 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. Virology 324, 28–36.
Cheung, A.K., 2004d. Palindrome regeneration by template strand switching mechanism at the origin of DNA replication of porcine circovirus via the rolling-circle melting-pot replication model. J. Virol. 78, 9016–9029.
Cheung, A.K., 2005a. Detection of rampant nucleotide reversion at the origin of DNA replication of porcine circovirus type 1. Virology 333, 22–30.
Cheung, A.K., 2005b. Mutational analysis of the direct tandem repeat sequences at the origin of DNA replication of porcine circovirus type 1. Virology 339, 192–199.
Cheung, A.K., Bolin, S.R., 2002. Kinetics of porcine circovirus type 2 replication. Arch. Virol. 147, 43–58.
Clark, E.G., 1996. Pathology of the post-weaning multisystemic wasting syndrome of pigs. Proc. West Can. Assoc. Swine Pract., 22–25.
Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. 38, 2494–2503.
Gibbs, M.J., Weiller, G.F., 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc. Natl. Acad. Sci. U.S.A. 96, 8022–8027.
Graham, F.L., Rudy, J., Brinkley, P., 1989. Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. EMBO J. 8, 2077–2085.
Hamel, A.L., Lin, L.L., Nayar, G.P.S., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. 72, 5262–5267.
Harding, J.C., 1996. Post-weaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. Proc. West Can. Assoc. Swine Pract. 1996, 21.
Mankertz, A., Hillenbrand, B., 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. Virology 279, 429–438.
Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.-J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. J. Virol. 71, 2562–2566.
Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C., Finsterbusch, T., 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus type 1 and 2. J. Virology 77, 9885–9893.
McNulty, M., Dale, J., Lukert, P., Mankertz, A., Randles, J., Todd, D., 2000. Circoviridae. In: van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Seventh Report of the International Committee on Taxanomy of Viruses. Academic Press, San Diego, pp. 299–303.
Meehan, B.M., Creelan, J.L., McNulty, M.S., Todd, D., 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. 78, 221–227.
Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. 79, 2171–2179.
Morozov, I., Sirinarumitr, T., Sorden, S., Halbur, P.G., Morgan, M.K., Yoon, K.-J., Paul, P.S., 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. 36, 2535–2541.
Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. 81, 2281–2287.
Niagro, F.D., Forsthoefel, A.N., Lawther, R.P., Kamalanathan, L., Ritchie, B.W., Latimer, K.S., Lukert, P.D., 1998. Beak and feather disease virus and porcine circovirus genomes: intermediates between geminiviruses and plant circoviruses. Arch. Virol. 143, 1723–1744.
Pringle, C.R., 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia. Arch. Virol. 144, 2065–2070.
Randles, J.W., Chu, P.W.G., Dale, J.L., Harding, R.M., Hu, J., Kojima, M.K., Makkouk, M., Sana, Y., Thomas, J.E., Vetten, H.J., 2000. Nanovirus. In: van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Seventh Report of the International Committee on Taxanomy of Viruses. Academic Press, San Diego, pp. 303–309.

Segales, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. Vet. Q. 24, 109–124.

Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2001. Rep and Rep′ proteins of porcine circovirus type 1 bind to the origin of replication in vitro. Virology 291, 152–160.

Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987. Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. Arch. Virol. 96, 39–57.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature 295, 64–66.