Detection of rampant nucleotide reversion at the origin of DNA replication of porcine circovirus type 1

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Abstract

Mutational analysis was conducted to investigate the involvement of the “loop-sequence” (which is flanked by a pair of 11-nucleotide inverted repeats) at the origin of DNA replication of porcine circovirus type 1 with respect to viral protein synthesis, DNA self-replication and progeny virus production. The results demonstrated that an octanucleotide \( \text{(A}_1\text{G}_2\text{T}_3\text{A}_4\text{T}_5\text{T}_6\text{A}_7\text{C}_8) \) embedded in the loop is essential for viral DNA replication. Similar to previous work with porcine circovirus type 2, this octanucleotide can be further condensed to an essential core element represented by \( \text{AxTAxTAC} \). After transfection, mutations introduced into the positions indicated by \( x \) (positions 2 and 5) were retained in the progeny viruses, while mutations engineered into the positions specified by the indicated nucleotides either did not yield any progeny virus (positions 6, 7, and 8) or they reverted back to wild-type nucleotide to generate infectious progeny viruses (positions 1, 3, and 4). In comparison to porcine circovirus type 2, porcine circovirus type 1 mutant genomes with perturbed octanucleotide sequences exhibited higher propensity to revert to wild-type under similar experimental conditions. The rate and frequency at which some of the nucleotide reversions occurred suggest that base complementarity may not be the governing factor for nucleotide incorporation at the porcine circovirus origin of DNA replication, and that the Rep-associated protein(s) may play a critical role in this process.

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Keywords: Porcine circovirus; Origin of DNA replication

Introduction

The Circoviridae family includes a diverse group of small, closed circular, single-stranded (ss) animal DNA viruses (McNulty et al., 2000; Pringle, 1999). Members of the genus Circovirus are porcine circovirus (PCV), beak-and-feather disease virus, bovine circovirus (GenBank accession # NC002068), canary circovirus, columbid circovirus, goose circovirus, and mulard duck circovirus (Clark, 1996; Harding, 1996; Hattermann et al., 2003; Mankertz et al., 2000; Phenix et al., 2001; Ritchie et al., 1989; Tischer et al., 1982; Todd et al., 1991, 2001; Woods et al., 1993). These animal circoviruses are closely related to the plant circoviruses, now renamed nanoviruses (Meehan et al., 1997; Niagro et al., 1998; Randles et al., 2000). It has been suggested that the PCV genome is an intermediate between plant geminivirus and nanovirus (Niagro et al., 1998) and that PCV was the result of a recombination event between a nanovirus and an animal picorna-like RNA virus (Gibbs and Weiller, 1999).

PCV has an ambisense circular genome (Tischer et al., 1982) that encodes proteins by the encapsidated viral DNA, and by the complementary DNA of the replication intermediate synthesized in the host. 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 a new disease, named postweaning multisystemic wasting syndrome (Allan and Ellis, 2000; Clark, 1996; Harding, 1996; Segales and Domingo, 2002). The genome sequences of a
number of 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. Sequence and structural motif similarities suggested that PCV replicates via the rolling circle replication (RCR) mechanism in a manner similar to the Mastrevirus genus of the Geminiviridae family (Palmer and Rybicki, 1998) with modifications at the origin of DNA replication (Ori) proposed by the newly described RCR “melting-pot” model (Cheung, 2004b, 2004d). These similarities include: (1) Rep proteins of both geminivirus and PCV contain the 3 conserved RCR motifs (RCR-I, -II, and -III) and a NTP-binding (P-loop) core homologous to the Rep proteins of other prokaryotic and eukaryotic RCR systems (Ilyina and Koonin, 1992; Mankertz et al., 1998). Interestingly, PCV DNA replication requires two Rep-associated proteins, Rep and Rep’, while geminivirus DNA replication requires just one multi-functional initiator protein (Cheung, 2003b, 2004a; Mankertz and Hillenbrand, 2001). (2) The minimal DNA Ori of PCV type 1 (PCV1) has been mapped to an 111-base-pair fragment (Mankertz et al., 1997), which contains a nonanucleotide sequence (TAGTATTAC) (Δ indicates the presumed nick-site) similar to that of geminiviruses (TAATAATTAC) and both nonanucleotides are flanked by a pair of inverted repeat (palindromic) sequences.

The current model for PCV DNA replication postulates that the closed circular ss-DNA genome is first converted to a superhelical ds-DNA replication intermediate. The virus-encoded Rep and Rep’ proteins essential for DNA replication are expressed (Cheung, 2003b, 2004a; Mankertz and Hillenbrand, 2001). Rep (and/or Rep’) recognizes and binds the octanucleotide sequence (A1G2T3A4T5A6T7AC) located at the Ori. The Rep-associated proteins destabilize and unwind the Ori sequence, nick the octanucleotide between the 6th T and the 7th A to generate a free 3'-OH end for initiation of plus-strand DNA replication. It has been demonstrated that the large intergenic regions (Mankertz et al., 2003) or the loop-sequences of PCV1 (12 nt) (CTGTAGTATTAC) and PCV2 (10 nt) (TAAGTATTAC) are interchangeable (Cheung, 2004c) and that the conserved octanucleotide motif sequence (AXTAXTAC) embedded in the loop-sequence is essential for PCV2 DNA replication (Cheung, 2004c). The Rep-associated proteins also interact with the inverted and direct repeat sequences flanking the Ori. In vitro experiments showed that PCV1 Rep binds to the right-arm of the presumed stem-loop structure at the Ori (Fig. 1), while both Rep and Rep’ bind to two adjacent, almost perfect, 6-nt (CGGCAG or CGTCA) tandem direct repeats located at nt 13, 19, 30 and 36 (Fig. 1) (Steinfeldt et al., 2001). However, in vivo experiments suggest that Rep does not bind the right-arm sequence in a sequence-specific manner, but interacts only with the C nt at positions 3 and 10 present in the right-arm of the palindrome (Cheung, 2004b). Recent reports also showed that the Ori-flanking palindrome is non-essential for initiation but is likely a signal for termination of DNA replication (Cheung, 2004b, 2004d).

In this study, mutational analysis was conducted to investigate the importance of each nt of the PCV1 loop-sequence with respect to viral protein synthesis, DNA self-replication and progeny virus production. The results showed that some nt alterations introduced into the loop are acceptable and progeny viruses were recovered while others were not tolerated and rapidly reverted to wild-type. However, nt changes engineered into the immediate area of the presumed nick-site are invariably lethal.

Results

Experimental design

A PCV1 genomic clone (J1), capable of producing infectious viruses upon transfection into PK15 cells after excision and re-circularization of the viral genome (Cheung, 2003a, 2004a), was employed to construct the mutant genomes used in this study. A schematic representation of the plus-strand Ori together with the major transcripts is shown in Fig. 1. The octanucleotide sequence encompassing the presumed nick-site between the first nt (position 1) and the last nt (position 1759) is boxed. The loop nt that are different between PCV1 and PCV2 are designated D-nt, while the 8 identical nt (octanucleotide) are designated O-nt. Based on this arbitrary assignment, genomic sequence nt 1 of PCV1 is position O7 in this communication. Mutations were introduced into the loop-sequence using the QuickChange Site-Directed Mutagenesis Kit (Strategene, San Diego, CA). Three types of mutation were engineered: (1) single-nt substitution, (2) double-nt substitution and (3) insertion. The excised and re-circularized viral genomes were transfected into parallel sets of PK15 cell cultures. At 48 h posttransfection, one set of transfected cultures was assayed for viral protein synthesis by immunochromatographic staining (Cheung, 2004a, Cheung and Bolin, 2002). At 7 days posttransfection, a second set of transfected cultures was harvested, freeze-thawed 3 times and then assayed for infectious virus after inoculation onto fresh PK15 cells. The progeny viruses were further passaged in PK15 cells. After confirmation by immunochromatographic staining, virus-infected cell DNA was isolated and amplified by PCR with PCV1-specific primers, 1665F and 874R. Each PCR product was subcloned into a TA-cloning plasmid (Invitrogen, Carlsbad, CA) for nt sequence determination.

Single-nucleotide substitution mutations

Each PCV1 loop nt (CTGTAGTATTAC) was assigned an arbitrary position: D1 through D4 (CTGT) and O1
through O8 (AGTATTAC). A series of mutants with single-nt substitution at each position were constructed. The results of two independent experiments are summarized in Table 1.

**Viral protein synthesis**

At 48 h, one set of transfected cultures was assayed for viral proteins by immunochemical staining (Fig. 2A—upper panel of each mutant genome). An abundant number of viral antigen-positive cells similar to that of the parent J1 genome were observed with the D-nt, O2, O4, and O5 mutant genomes. The number of antigen-positive cells exhibited by O1 and O3 were severely reduced (<1% of J1), while O6 through O8 showed few to no antigen-positive cells (<0.1% of J1).

**Progeny virus production**

At 7 days, the transfected cultures were assayed for progeny viruses. In both experiments, progeny viruses were recovered from D1 through D4, O2, O4, and O5, incon-
sistent from O1 and O3, and none from O6 through O8 (Fig. 2A—lower panel of each mutant genome). However, upon additional cell passages, infectious viruses were readily recovered from all the O1 and O3 samples (Fig. 2A—inserts, Table 1B), indicating that a small amount of progeny viruses must have been produced in the O1- and O3-transfected cultures.

### Genotype of progeny viruses

Virus-infected cell DNA at designated cell passages was isolated. Sequence determination was performed after PCR amplification and subcloning (Table 1). The viral genomes recovered from D1, D2, D4, O2, and O5 contained either the engineered input mutations or wild-type nt, those recovered
from D3 only contained the input engineered mutation, and those recovered from O1, O3, and O4 reverted to wild-type nt at these positions, exclusively. Interestingly, while position O1 reverted to the wild-type A nt in all the O1 progeny viruses, additional nt modifications were detected in position D2 of virus b, position D3 of virus c, and position O2 of virus a. Taken together, the octanucleotide motif (AxTAxTAC) emerged as an essential core element (ECE) for PCV1 DNA replication.

Double-nucleotide substitution and insertion mutations

Additional mutations targeting the ECE were introduced into J1. Two double-nt substitution mutant genomes, Mut3 (CTGCTGTATTAC) with modifications at D4-O1 and Mut17 (CTGTAGTATTCAC) with modifications at the presumed O6-O7 nick-site, were engineered (Table 2). Incidentally, the double-nt substitution mutation present in Mut17 was reported to render PCV1 DNA non-functional in a complementation in vitro replication assay (Mankertz et al., 1997). Two insertion mutant genomes, Mut4 (CTGTACGTATTAC) with addition of a C nt between positions O4-O5 and Mut5 (CTGTACCCGTATTAC) with addition of 3 Cs at the same location were constructed. All four mutant genomes exhibited severe reduction in the number of Rep-associated antigen-producing cells upon transfection into PK15 cells (Figs. 2B and C); however, progeny viruses were recovered from Mut4 and Mut17. Although variation was detected in other positions of the loop (viruses a, b, c, and d), the Mut4 and Mut17 recovered viruses (wild-type or wild-type-like variants) all regenerated the ECE motif sequence.

Fig. 2. Immunochemical staining of viral antigens in PK15 cells after transfection at 48 h with: (A) single-nt substitution mutant genomes, (B) double-nt substitution mutant genomes and (C) insertion mutant genomes, and their corresponding progeny viruses. The viral genome used for transfection is indicated in each panel. The small insert panels showed detection of progeny viruses at later cell passages.
The above experiments demonstrated that the mutations engineered into positions O1 through O5 reverted to wild-type nt and were retained in the recovered progeny viruses quite readily. To confirm this observation, D3 and O1 through O5 mutant genomes were selected for further investigation. Each mutant genome preparation was transfected into 6–10 PK15 cell cultures. The transfected cultures were monitored for protein synthesis and infectious virus production at cell passage 3 (Table 3). Similar to the results obtained above, an abundant number of antigen-positive cells were observed with D3, O2, O4, and O5, while few antigen-positive cells were detected with O1 and O3 (<1% of J1). Progeny viruses were recovered readily from D3, O2, O4, and O5; and upon additional cell passages with O1 and O3. Sequence analysis showed that wild-type nt reversion did not occur with the progeny viruses recovered from D3. For O1, O3 and O4 mutant genomes, nt reversion occurred 100% of the time and all the recovered viruses contained wild-type nt at these positions only. Again, the O1 mutant genome yielded several virus variants with different D-nt but identical ECE motif sequence. For O2 and O5 mutant genomes, in addition to yielding progeny viruses containing the engineered mutation in all the recovered samples, nt reversion was detected in 4 of 10 O2 experiments and 10 of 10 O5 experiments.

Discussion

The 12 nt loop-sequence of PCV1 (CTGT_AGT_AATTAC) can be divided into 2 parts with respect to virus replication: the non-essential D-nt and the essential O-nt. The D-nt can be substituted by a different base
without any effect on viral protein synthesis or progeny virus production. The O-nt constitute an ECE that can be represented by the octanucleotide motif AxTAxTAC. The variable positions labeled x (O2 and O5) can accept different nt, while the critical positions (O1, O3, O4, and O6 through O8) appear to prefer the nt specified by the motif. Interestingly, the effect of each critical nt on PCV1 replication was not identical. Mutations introduced into O1 and O3 caused severe reduction of viral protein synthesis and only a small amount of wild-type progeny viruses was synthesized initially. Mutations introduced into O4 did not affect protein synthesis and wild-type progeny viruses were readily recovered. Mutations engineered into positions O6, O7, or O8 (that encompass the presumed nick-site) resulted in severe reduction in protein synthesis and no progeny virus production. These observations are in agreement with previous work (Cheung, 2004c) which showed that PCV2 also contains an identical octanucleotide ECE and corresponding mutations introduced into the PCV2 loop-sequence resulted in similar viral protein synthesis patterns. However, in contrast, the engineered mutations at positions O1, O3, and O4 of PCV1 reverted back to wild-type more readily than the corresponding mutations introduced into PCV2. For example, wild-type viruses were recovered from 12 of 12 transfections with O3 mutant genomes of PCV1 (this study) and none from 9 of 9 transfections with O3 mutant genomes of PCV2 (Cheung, 2004c). Whether this phenomenon is related to the differences between the loop-sequences (specific nt and/or size) or the Rep-associated proteins of these two viruses is not clear.

This and previous studies (Cheung, 2004c, 2004d) demonstrated that the D-nt of PCV can be expanded or contracted, but the O-nt must conform to the ECE motif to generate stable infectious progeny viruses. For example, O1 and Mut17 (a double-nt mutation at the junction of the D- and O-nt) yielded progeny viruses in which the engineered O1 position reverted to the wild-type A nt with concurrent incorporation of a variety of D-nt. Furthermore, insertion or deletion engineered into the

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**Table 3**

Frequency of wild-type nt reversion at selected positions

<table>
<thead>
<tr>
<th>Position:</th>
<th>D-nt</th>
<th>O-nt</th>
<th>nick site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECE-motif:</td>
<td>x x x x</td>
<td>A x</td>
<td>T A x T A C</td>
</tr>
<tr>
<td>Loop-sequence:</td>
<td>C T G T</td>
<td>A G T A T</td>
<td>A T T A C</td>
</tr>
<tr>
<td>Mutation:</td>
<td>- -</td>
<td>C -</td>
<td>- - -</td>
</tr>
<tr>
<td>Protein (%):</td>
<td>Expt:</td>
<td>100</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**Genotype of viruses recovered from O1:**

O1: CTG-TiGTATTAC
w. CTG-TAGTATTAC (wild-type)
a. tTG/ TAGTATTAC
b. CAG/GAGTGATTAC
c. CTG/gAGTATTAC
d. CT/AGG/AGTATTAC
e. G-G/AGTATTAC
f. CTGtAGTATTAC
g. CTG/gAGTATTAC

Input engineered mutant and wild-type genotypes are indicated by i and w, respectively; and nd indicates experiment not done. Variant viruses recovered from O1 are denoted by a, b, c, d, e, f, and g; and nt that differ from wild-type are in lower case letters and shaded. (−) indicates nt deletion and (·) indicates arbitrary gap introduced to facilitate sequence alignment. The number of examples (subclones) of each genotype, determined by sequencing cloned PCR fragments, is indicated by superscript.
ECE motif either resulted in no production of progeny viruses (Mut3 and Hy2b in Cheung, 2004c) or that the insertion was eliminated (Mut4) to restore the 8-nt ECE ensemble for the generation of infectious progeny viruses. Thus, the octanucleotide ECE motif appears to be specific and maintaining this architecture is essential for PCV viability.

In this study, 5 mutant genomes (O6, O7, O8, Mut3, and Mut5) failed to yield progeny viruses. D3 yielded progeny viruses containing the input engineered mutation in 12 of 12 transfection experiments. The rest of the mutant genomes exhibited either partial or complete reversion to wild-type nt to generate stable progeny viruses. The number of experiments in which O1 (14 of 14), O2 (5 of 12), O3 (12 of 12), O4 (8 of 8), and O5 (12 of 12) reverted to wild-type genotype is rampant (Tables 1 and 3). Partial reversion (i.e., both input and wild-type genotypes were detected) was observed with the O2 and O5 progeny viruses, while complete reversion was observed with the O1, O3, and O4 progeny viruses. Thus, when the critical nt of ECE are perturbed, either progeny viruses are not produced (O6, O7, and O8) or the engineered mutations revert completely to wild-type in the progeny viruses (O1, O3, and O4). It is interesting to note that the D-positions, as well as the two variable O-positions (O2 and O5) can accommodate the input engineered mutations but nt reversion can still occur occasionally. However, it is surprising that when nt reversion occurred, only wild-type nt but not any other bases were detected at these locations in the progeny viruses.

Previous reports with geminiviruses [African cassava mosaic virus (ACMV) and wheat dwarf virus] have shown that variation in the nonanucleotide (TA1A2T3A4C5A6C7T8A9) mosaic virus (ACMV) and wheat dwarf virus] have shown that the input engineered mutations but nt reversion can still occur occasionally. However, it is surprising that when nt reversion occurred, only wild-type nt but not any other bases were detected at these locations in the progeny viruses.

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Oligonucleotide primers

The primers for PCR amplification were (CCAAGAGGGGCGGGG) and (GTAATCCTCCGATAGAGCCAGAC) located at nt 1665 (forward orientation) and nt 874 (reverse orientation) of the PCV1 genome, respectively. The primer sets for mutagenesis were synthesized by placing 15–18 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, and PCR have been described previously (Cheung, 2003b).

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References


