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