



Specific functions of the Rep and Rep' proteins of porcine circovirus during copy-release and rolling-circle DNA replication

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ABSTRACT

The roles of two porcine circovirus replication initiator proteins, Rep and Rep', in generating copy-release and rolling-circle DNA replication intermediates were determined. Rep uses the supercoiled closed-circular genome (ccc) to initiate leading-strand synthesis (identical to copy-release replication) and generates the single-stranded circular (ssc) genome from the displaced DNA strand. In the process, a minus-genome primer (MGP) necessary for complementary-strand synthesis, from ssc to ccc, is synthesized. Rep' cleaves the growing nascent-strand to regenerate the parent ccc molecule. In the process, a Rep'-DNA hybrid containing the right palindromic sequence (at the origin of DNA replication) is generated. Analysis of the virus particle showed that it is composed of four components: ssc, MGP, capsid protein and a novel Rep-related protein (designated Protein-3).

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Background

Porcine circovirus (PCV) is a member of the genus *Circovirus* of the *Circoviridae* family, which includes a group of diverse animal DNA viruses with small single-stranded circular genomes (ssc) (Fauquet and Fargette, 2005). For PCV, two virus-encoded replication initiator proteins, Rep and Rep', are required to replicate the ssc genome via the rolling-circle replication (RCR) mechanism (Cheung, 2004b; Mankertz and Hillenbrand, 2001). The Rep proteins of other prokaryotic and eukaryotic RCR systems commonly have four conserved motifs: RC-I (unknown function), RC-II (divalent ion coordination), RC-III (nicking/joining activities) and P-loop (a putative helicase domain) (Ilyina and Koonin, 1992). PCV Rep has all four signature amino acid motifs. Rep' has three of these motifs, RC-I, RC-II, and RC-III (but lacks P-loop) resulting from internal RNA splicing to a different ORF at the 3'-end. To initiate RCR with the supercoiled double-stranded closed-circular genome (ccc), an initiator protein binds the H1/H2 hexanucleotide sequences, nicks the Oc8 recognition sequence ($A_1X_2T_3A_4X_5T_6\downarrow A_7C_8$) ($\downarrow$ indicates the nick site) at the origin of DNA replication (Ori) and covalently links itself to the 5'-end of the nicked-strand via a tyrosine residue present in RC-III (Steinfeldt et al., 2007). In vitro, both Rep and Rep' exhibit nicking/joining activities in concert with Oc8. During nicking, either protein can covalently-link itself to the 5'-end of the nicked-strand. During joining, Oc8 is reconstituted in the presence of a stem-loop structure and the

covalently-attached protein is released (Cheung, 2007; Steinfeldt et al., 2007).

RCR is an asymmetric replication system that can be separated into two phases: leading-strand synthesis (LSS) and lagging-strand or complementary-strand synthesis (CSS). To initiate LSS, a replication initiator protein (Rep) nicks the positive-strand of supercoiled ccc and generates a 3'OH-end that serves as primer for DNA elongation. As the positive-strands of the nicked-circular molecules (nc) elongate, LSS-extension products (LSS^{ep}) that give rise to single-stranded circular genomes (ssc) are synthesized. The ssc molecules serve as templates for CSS in the presence of the minus-genome primers (MGP). After the MGP-coupled ssc (ssc^{MGP}) molecules are converted to double-stranded open-circular molecules (oc) via a series of CSS-extension products (CSS^{ep}), the ends of oc are then joined to form double-stranded relaxed-circular molecules (rel). Upon supercoiling, rel become ccc and serve as templates for the next round of LSS.

Two PCV genotypes, PCV1 and PCV2, have been identified. Previous work showed that a head-to-tail tandem genome construct (HTT) containing two PCV Oris and a Rep gene was capable of excising the unit-length PCV ssc molecule by the copy-release mechanism and the excised ssc was converted to ccc (Cheung, 2006, 2012). As reported, a chimeric HTT (pChi7), containing 1.75 copies PCV1 and PCV2 DNA in tandem inserted into the pBluescript SK⁺ (pSK⁺) bacterial plasmid, when transformed into *Escherichia coli* (*E. coli*) yielded three supercoiled ccc molecules: the parent HTT (designated U), the pSK⁺ plasmid plus the additional flanking PCV DNA sequences (designated L) and the excised double-stranded unit-length PCV genome (designated Q). A derivative construct defective in the Rep genes (pChi7-Rep⁻) failed to exhibit the copy-release mechanism

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yielded only U, while a derivative construct defective in the Rep' genes (pChi7-Rep'⁻) yielded all three U, L and Q molecules. Thus, Rep is capable of initiating copy-release of PCV ssc, with no involvement of Rep', in *E. coli*.

Although both Rep and Rep' are essential for PCV DNA replication in mammalian cells (Cheung, 2004b; Mankertz and Hillenbrand, 2001), the specific steps in which each protein is involved is not known. There are no Rep-deficient or Rep'-deficient viruses available for experimentation. The three previously reported HTTs (pChi7, pChi7-Rep⁻ and pChi7-Rep'⁻) (Cheung, 2006) were employed to determine the specific functions of Rep and Rep', individually, with respect to the mechanisms of copy-release replication and RCR in porcine kidney (PK15) cells.

Results

Identification of PCV replicative intermediates by 2D-gel systems

To assess the ability of Rep and Rep' to generate replicative intermediates, it is essential that the various DNA forms are adequately resolved. Starting with ssc^{MGP} that engages in RCR, the molecule progresses in the following order: ssc^{MGP}, CSS^{ep}, oc, rel, ccc, nc, LSS^{ep} and ssc/ssc^{MGP}. These DNA species migrate to specific locations in 2D gel-systems in the presence of chloroquine. An established 2D gel-system (designated gel-system 1) (Jeske et al., 2001) had been used successfully to display the replicative intermediates present in PCV-infected cells: ds linear (lin), ssc/ssc^{MGP}, oc/nc, ccc and LSS^{ep} (Cheung, 2012).

A modified 2D system (designated gel-system 2) was developed to exhibit CSS^{ep} that connects ssc^{MGP} and oc (Fig. 1). Comparing the results obtained from single-stranded directional probes showed that 1xss (includes ssc and unit-length linear, ssl), 2xss and 3xss are virion-strand molecules. These 2xss and 3xss molecules represent DNA species that failed to terminate at the end of previous round(s) of LSS. With gel-system 1, lin and oc/nc

of PCV-infected cells were well separated (Cheung, 2012). With gel-system 2, lin and oc/nc co-migrated to the same location (Fig. 2A and B). Although oc (minus-strand not covalently closed) and nc (virion-strand not covalently closed) are indistinguishable

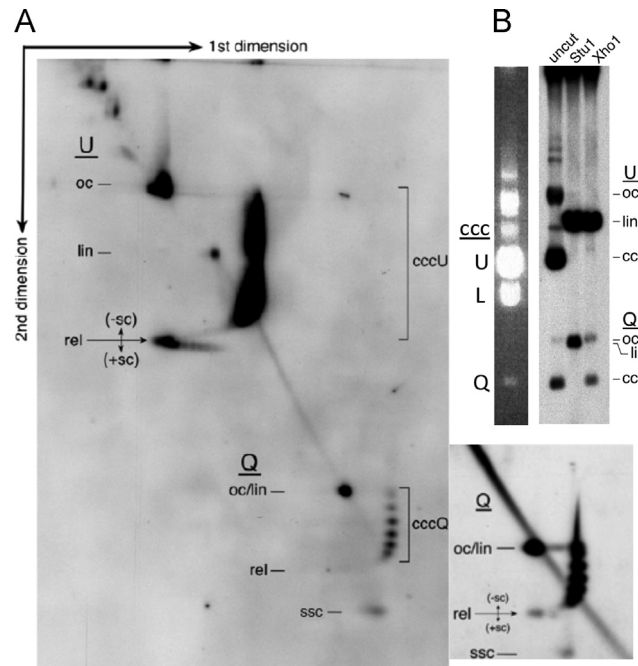


Fig. 2. Migration patterns of the pChi7 HTT replicative intermediates propagated in *E. coli*. (A) Location of relQ in gel-system 2. The U-related and Q-related replicative intermediates are indicated: oc, lin, ccc and rel with positive superhelicity (+sc) or negative superhelicity (-sc). Twenty times more DNA was used in the insert panel. (B) Co-migration of PCV oc and lin in 1% agarose gel. Left panel: ethidium bromide stained pChi7 U, L and Q ccc-DNA species. Right panel: plasmid DNAs were linearized with one cut enzyme, Stu1 or Xho1. Stu1 cuts U and Q, while Xho1 cuts U but not Q. Whereas ocU and linU were well separated, ocQ and linQ migrated to a similar location. Probe-Q was used.

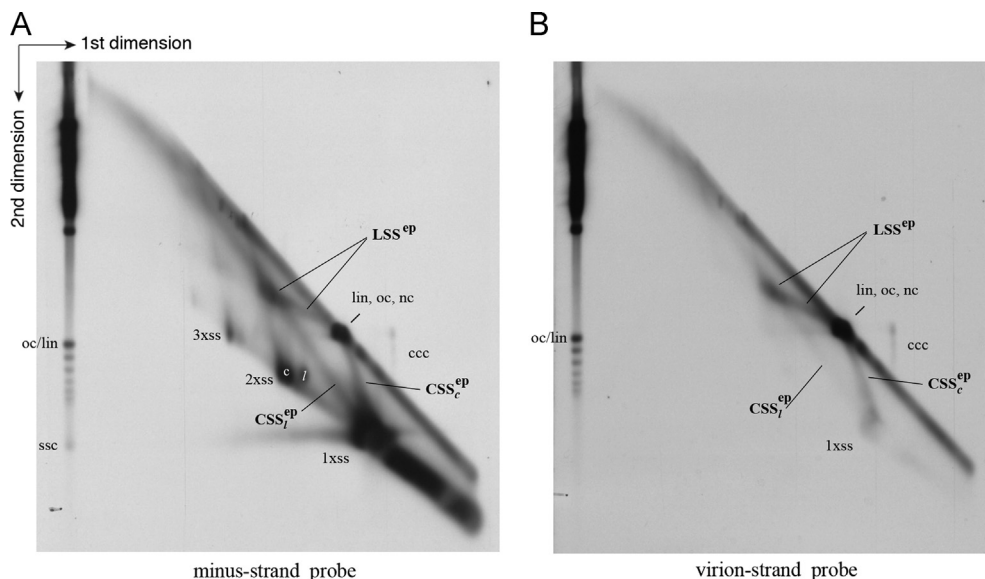


Fig. 1. Resolution of RCR intermediates of PCV2 acutely-infected PK15 cell DNA using gel-system 2 southern blot analysis. (A) The minus-strand probe-1352R detects the virion-strand and (B) the virion-strand probe-1091F detects the complementary-strand. The RCR intermediates are indicated: 1xss includes single-stranded unit-length circular (ssc) and linear (ssl) molecules with or without the minus-genome primer (MGP). Beginning with the ssc containing a MGP (ssc^{MGP}), through complementary-strand synthesis extension products (CSS^{ep}), open-circle (oc), relaxed circle (rel) (below detection limit), supercoiled closed-circular (ccc), nicked-circular (nc) to leading-strand synthesis extension products (LSS^{ep}) and then back to ssc/ssc^{MGP}. Other RCR by-products include unit-length linear 1xssl, unit-length ds linear (lin), dimeric ss circular or linear (2xss-c or -l), trimeric circular or linear (3xss-c or -l), and CSS^{ep} molecules. pChi7Rep⁻ plasmid DNA propagated in *E. coli* was used as molecular weight marker on the left of each panel. The single-stranded probes used are indicated.

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