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# Rolling-Circle Replication of an Animal Circovirus Genome in a Theta-Replicating Bacterial Plasmid in *Escherichia coli*

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**A bacterial plasmid containing 1.75 copies of double-stranded porcine circovirus (PCV) DNA in tandem (0.8 copy of PCV type 1 [PCV1], 0.95 copy of PCV2) with two origins of DNA replication (Ori) yielded three different DNA species when transformed into *Escherichia coli*: the input construct, a unit-length chimeric $PCV1_{Rep}$/$PCV2_{Cap}$ genome with a composite Ori but lacking the plasmid vector, and a molecule consisting of the remaining 0.75 copy $PCV1_{Cap}$/$PCV2_{Rep}$ genome with a different composite Ori together with the bacterial plasmid. Replication of the input construct was presumably via the theta replication mechanism utilizing the $ColE_1$ Ori, while characteristics of the other two DNA species, including a requirement of two PCV Oris and the virus-encoded replication initiator Rep protein, suggest they were generated via the rolling-circle copy-release mechanism. Interestingly, the PCV-encoded Rep′ protein essential for PCV DNA replication in mammalian cells was not required in bacteria. The fact that the Rep′ protein function(s) can be compensated by the bacterial replication machinery to support the PCV DNA replication process echoes previous suggestions that circular single-stranded DNA animal circoviruses, plant geminiviruses, and nanoviruses may have evolved from prokaryotic episomal replicons.**

*Porcine circovirus* (PCV) is a member of the genus *Circovirus* of the *Circoviridae* family, which includes a group of diverse animal viruses with small, closed-circular, single-stranded (ss) DNA that replicate their genomes through double-stranded (ds) intermediates (28, 34). The animal circoviruses are closely related to the plant circoviruses, now renamed nanoviruses (27, 29). Nucleotide sequence analysis showed that the PCV genome is intermediate between the genomes of plant geminivirus and nanovirus (30). Based on analysis of replication initiator protein (Rep) sequences, it has also been suggested that the PCV genome was the result of a recombination event between a nanovirus and an animal picorna-like RNA virus (13).

Two genotypes of PCV have been identified, PCV type 1 (PCV1) and PCV2, and their genomes share 68 to 76% sequence homology. PCV has an ambisense circular genome that encodes proteins both by the encapsidated viral DNA and by the minus-strand genome of the replication intermediate synthesized in the host. Two coding regions of opposite polarity, the Rep protein on the right and the capsid protein (Cap) on the left, are separated at their 5′ ends by the origin of DNA replication (Ori) intergenic region (IR) of approximately 80 nucleotides (Fig. 1). Sequence and structural motif similarities suggest that PCV replicates via a rolling-circle replication (RCR) mechanism in a manner similar to the *Geminiviridae* family (see reviews in references 14, 16, and 32) with modifications at the Ori proposed by the RCR "melting pot" model (3, 4), which is consistent with the four-stranded DNA structures detected among short inverted repeat sequences when examined by electron and atomic force microscopy (20, 21, 31). These similarities include the following: (i) a Rep protein that contains three conserved RCR motifs (RCR-I, -II, and -III) and an nucleoside triphosphate-binding (P-loop) core homologous to the Rep proteins of other prokaryotic and eukaryotic RCR systems (18, 23) and (ii) the Ori-IR of PCV and geminivirus contain a similar nonanucleotide sequence (TAGTATTAC for PCV1, AAGTATTAC for PCV2, and TAATATT↓AC for geminivirus [the ↓ indicates a nick site]) flanked by a pair of inverted repeat (palindromic) sequences capable of forming a stem-loop structure during DNA replication. It has been demonstrated that the nonanucleotide of geminivirus is cleaved at the indicated nick site by the virus-encoded Rep to initiate plus-strand DNA replication (15, 17, 22, 23).

The minimal Ori of PCV1 has been mapped to a 111-bp fragment (24) which includes the 83-nucleotide (nt) Ori-IR (Fig. 1). The current model for PCV DNA replication postulates that the closed-circular ssDNA genome is first converted to a superhelical dsDNA replication intermediate. Interestingly, replication of geminivirus or nanovirus DNA in plant cells requires just one multifunctional initiator Rep protein, while replication of PCV DNA in mammalian cells requires two virus-encoded proteins, Rep and Rep′ (the Rep complex) (2, 5, 25). The *Rep′* RNA is derived from the *Rep* RNA via internal splicing. The spliced *Rep′* RNA codes for a protein identical to Rep in the amino portion but different in the carboxyl portion because splicing changes the translational frame. The Rep complex recognizes and binds the direct hexanucleotide (H) tandem repeats and the right arm of the stem-loop structure (38), destabilizes and unwinds the Ori sequence, and then nicks the octanucleotide $A_1G_2T_3A_4T_5T_6\downarrow A_7C_8$ (designated Oc8, or O nucleotides) (6, 7) within the nonanucleotide sequence 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 Oc8 motif sequence ($A_1x_2T_3A_4x_5T_6\downarrow A_7C_8$) and the hexanucle-

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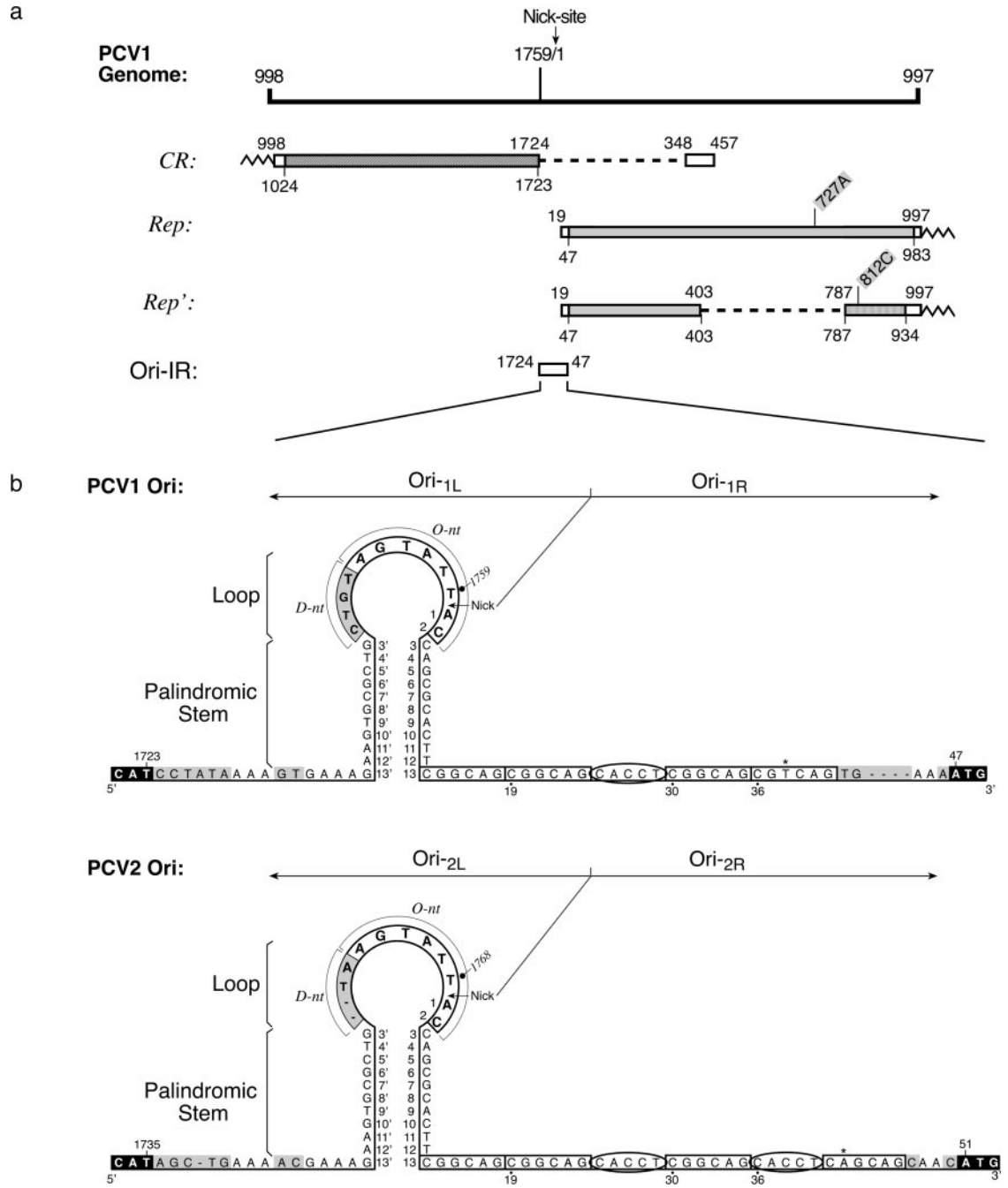


FIG. 1. Schematic representation of the origins of DNA replication of PCV1 and PCV2. (A) Transcription patterns of the major PCV1 RNAs (1). Capsid RNA (*CR*) is transcribed leftward. *Rep* and *Rep′* are transcribed rightward. The RNAs are annotated with nucleotide coordinates that indicate the last nucleotide of each respective exon. The coding sequence of each transcript is shaded, and the nucleotide coordinates are indicated below each RNA. Locations of the PCV1 *Rep* mutation (nt 727A) and the PCV1 *Rep′* mutation (nt 812C) are indicated. (B) Schematic representation of the PCV1 and PCV2 plus-strand Ori, indicating potential base-pairing of the flanking palindrome. The genomic sequences of PCV1 (1,759 nt) and PCV2 (1,768 nt) with respect to the presumed nick site (AGTATT ↓ AC) present in the octanucleotide (O-nt) and the D-nt of the loop are in bold letters and enclosed in boxes. The nucleotide coordinates 1, 2, 3, etc. are based on the actual genomic sequence, and the nucleotide coordinates 3′, 4′, 5′, etc. are arbitrarily assigned to show nucleotide complementarity of the flanking palindromic sequences. The Ori nucleotide sequences to the left of the nick site of PCV1 and PCV2 are designated Ori-$_{1L}$ and Ori-$_{2L}$, and sequences to the right of the nick site are labeled Ori-$_{1R}$ and Ori-$_{2R}$. The 6-nt tandem H repeat sequence (CGGCAG, CGGCTG, and CAGCAG) and 5-nt common sequence (CACCT) between PCV1 and PCV2 are enclosed in square and oval boxes, respectively. Nucleotide differences between PCV1 and PCV2 are shaded, and the initiation codons for *Rep* and *CR* are in black boxes.

otide tandem repeat (H1/H2) abutting the stem-loop structure are essential for PCV DNA replication (6–9). After nicking Oc8, the Rep complex is attached to the 5′ end of the displaced genomic strand via the conserved tyrosine residue of motif RCR-III. It is not clear whether Rep or Rep′ mediates the nicking function or which protein is attached to the displaced DNA. The Rep complex is not expected to possess any DNA polymerase activity and, therefore, the actual polymerization of the nascent

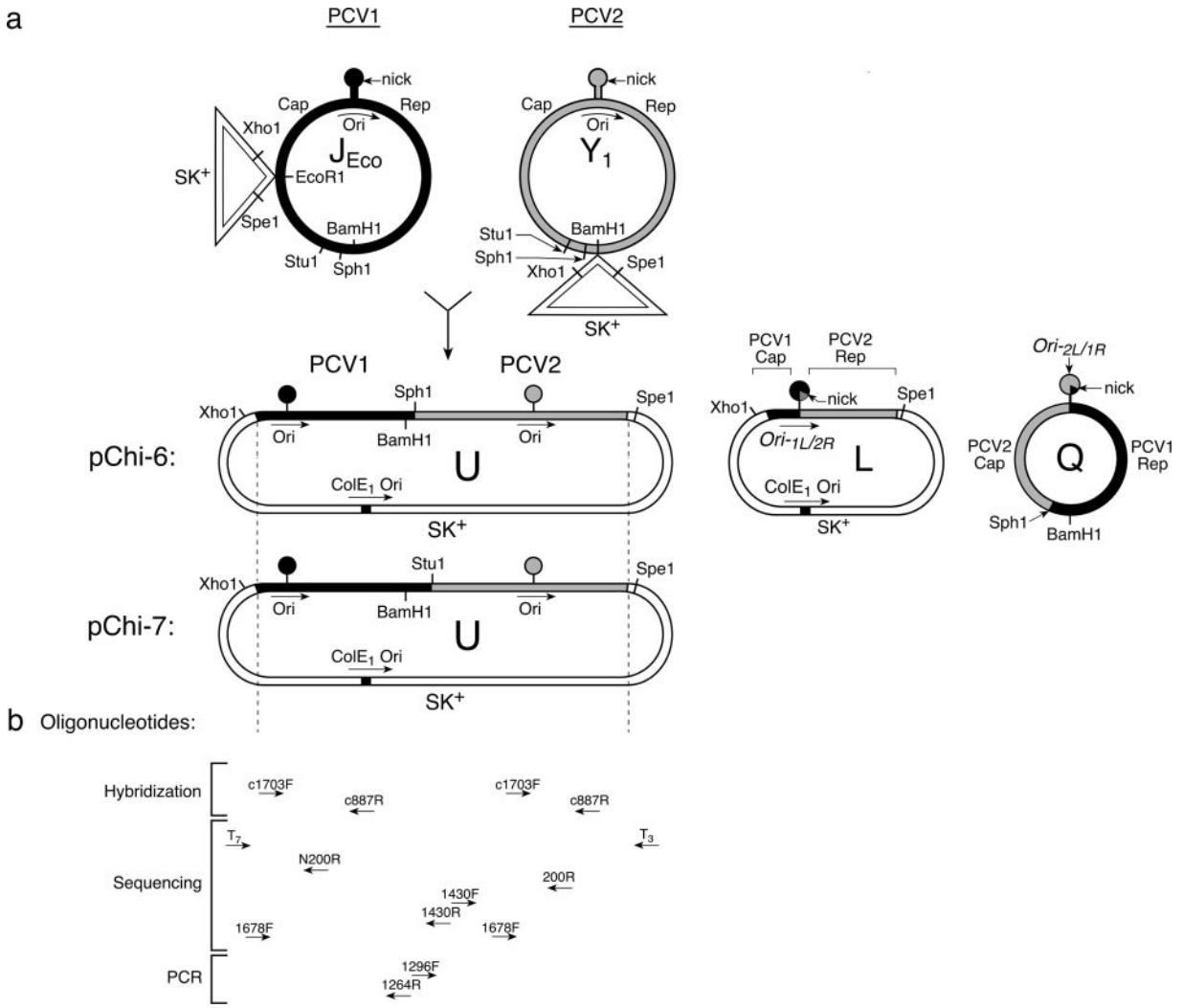


FIG. 2. (a) Schematic representation of pChi-6, pChi-7, and the U, L, and Q DNA species. (b) Locations of oligonucleotides. See Materials and Methods for construction of pChi-6 and pChi-7. PCV1 DNA is indicated in black, PCV2 DNA is shaded, and $pSK^+$ DNA is in the open box. The restriction enzyme sites relevant to this study are denoted. The Xho1 site is present only in the $pSK^+$ sequence, and the Stu1 site is present in both the PCV1 and PCV2 genomes. The BamH1 and Sph1 sites were engineered into the constructs for cloning purposes.

viral genome is carried out by cellular enzymes. Upon completion of the first round of DNA synthesis, the Rep complex cleaves Oc8 between the nascent and original DNA. Concomitantly, the Rep complex mediates joining the ends of the displaced genome to reconstitute the original Oc8 and release the Rep complex and a ss viral genome.

Previous work with geminiviruses showed that plasmid constructs containing greater-than-unit-length viral DNA accumulate viral replicative-form DNAs in bacteria indistinguishable from those produced in infected plants (35, 37), and accumulation of the viral DNA species depends on the presence of two Oris in the plasmid constructs. In this study, we examined two constructs, pChi-6 and pChi-7 (Fig. 2), each consisting of 1.75 copies of PCV1/PCV2 DNA with two PCV Oris (specific for the RCR mechanism) inserted into the pBluescript SK(+) ($pSK^+$) bacterial plasmid containing the colicin $E_1$ ($ColE_1$) Ori (specific for a unidirectional theta-replication mechanism) (19, 40). PCV1/PCV2 heterologous tandem constructs were chosen because the components essential for PCV1 or PCV2 DNA replication (the Ori-IR and the Rep complex) are interchangeable (6, 26) and the two viral genomes are different enough to be easily discernible. The results demonstrated that three distinct DNA species of different molecular sizes were generated from each engineered construct utilizing two different replication mechanisms in *Escherichia coli*. In comparison to the homologous geminivirus systems used in previous studies (35, 37), the heterologous tandem constructs employed in this work allowed easier identification of the mechanism for generation of the novel DNA species.

## MATERIALS AND METHODS

**Construction of tandem PCV1/PCV2 chimeric clones.** A PCV1 genomic clone ($J_{Eco}$ with two engineered sites, EcoR1 at nt 1421 and BamH1 at nt 992; GenBank accession number AY184287) and a PCV2 genomic clone ($Y_1$ with an engineered BamH1 site at nt 1015 to 1020; GenBank accession number AY094619) inserted into the $pSK^+$ plasmid (Stratagene, La Jolla, CA) were

employed to engineer two chimeric head-to-tail tandem constructs containing 0.8 copy of PCV1 and 0.95 copy of PCV2 (Fig. 2a). Both genomic clones are capable of producing infectious viruses after excision of the viral DNA from the vector, recircularization, and transfection into PK15 cells (2, 5). To generate pChi-6 or pChi-7, the Sph1-Spe1 or Stu1-Spe1 fragment containing the PCV2 Ori of $Y_1$ was used to replace the small Sph1-Spe1 or Stu1-Spe1 DNA fragment not containing the PCV1 Ori of $J_{Eco}$, respectively. For the generation of mutant versions of pChi-6 and pChi-7, the mutations were engineered initially into $J_{Eco}$ or $Y_1$ and then the appropriate DNA fragments were cloned together.

**Extraction of DNA or RNA.** A Miniprep plasmid DNA kit (Promega, Madison, WI) and total cell DNA and RNA kits (QIAGEN, Valencia, CA) were used to isolate nucleic acids from overnight bacterial cultures. Bacterial strains TOP10, TOP10F′, and DH5α were purchased from Invitrogen (Carlsbad, CA), and XL-Blue was purchased from Stratagene (La Jolla, CA). Total mammalian PK15 cell DNA was isolated using the STAT-60 DNA extraction kit purchased from TEL-TEXT B, Inc. (Friendswood, TX). Prior to analysis, the total DNA and RNA samples were treated with RNase A or DNase I, respectively. DNAs were isolated from a 1% agarose gel (after electrophoresis) using the Geneclean kit (Q-Biogene, Irvine, CA).

**Southern blot analysis.** Blot preparation and hybridization with $^{32}$P-labeled probes were carried out as previously described (10). Nick translation and 5′-labeling kits (Amersham, Piscataway, NJ) were used to generate various $^{32}$P-labeled probes for hybridization. Positions of the oligonucleotide probes are shown in Fig. 2b.

**Transfection, DNA mutagenesis, and PCR.** The methodologies for DNA transfection, mutagenesis, and PCR have been described previously (2). The oligonucleotides used (Fig. 2b) included the following: c1703F, TCTTCTGCG GTAACGCCTCCT; c887R, AGTAATCCTCCGATAGAGAG; T7, TAATAC GACTCACTATAGGG; T3, ATTAACCCTCACTAAAGGGA; 1678F, CAAG ATGGCTGCGGGGGC; N200R, CAAACCTTCCTCTCCGCA; 200R, ATTA CCCTCCTCGCCAAC; 1430R, GCATGAATTCTGGCCCTGCTCCCCCA; 1430F, GCATGAATTCAACCTTAATTTTCTT; 1296F, GTATGGCGGGAG GAGTAGTT; 1264R, TACTTCACACCCAAACCT. The suffix (F or R) of the oligonucleotide indicates the orientation of the primer. F indicates the forward direction, to the right, while R indicates the reverse direction, to the left.

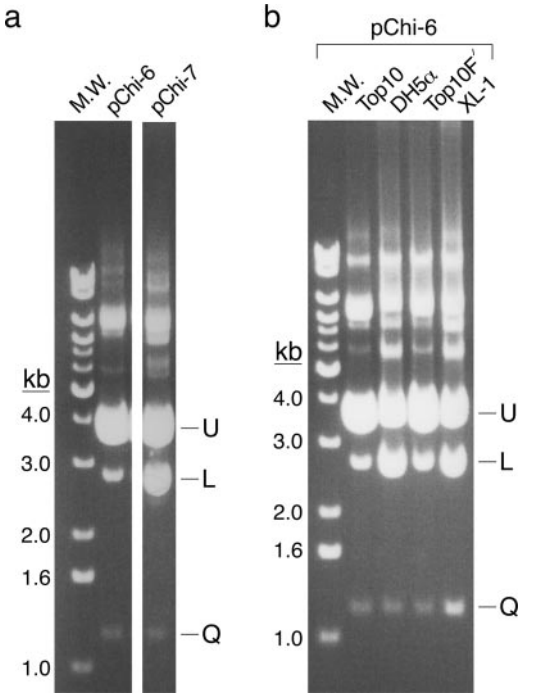


FIG. 3. Agarose gel electrophoresis of plasmid DNA recovered from *E. coli* TOP10 cells transformed with pChi-6 and pChi-7 (a) and plasmid DNA recovered from various *E. coli* strains transformed with pChi-6 (b). The predominant closed-circular ds molecules are labeled U, L, and Q.

## RESULTS

**Detection of three distinct DNA species derived from pChi-6 and pChi-7.** Two PCV1/PCV2 chimeric constructs, pChi-6 and pChi-7, were engineered utilizing a PCV1 genomic clone $J_{Eco}$ and a PCV2 genomic clone Y1 (Fig. 2a). Plasmid DNAs were isolated from pChi-6- or pChi-7-transformed TOP10 bacterial cultures and analyzed by agarose gel electrophoresis. Multiple DNA bands belonging to three molecular species denoted as U (*u*pper), L (*l*ower), and Q (*q*uestionable) were detected (Fig. 3a). All three DNA species recovered from bacteria transformed with pChi-6 or pChi-7 were purified from agarose gel and retransformed into TOP10 cells. Whereas ample ampicillin-resistant colonies were recovered from U or L, no ampicillin-resistant colonies were recovered from Q. Individual ampicillin-resistant colonies from each transformation were selected and analyzed. The results showed that the L-transformed bacteria yielded L DNA, while the U-transformed bacteria yielded U, L, and Q DNAs (data not shown). The U DNA species of pChi-6 was also transformed into several other *recA1 E. coli* strains of different genetic backgrounds (TOP10F′, DH5α, and XL1-Blue) that were designed to reduce the occurrence of nonspecific recombination in cloned DNA (Invitrogen, Carlsbad, CA). Although the relative ratios of molecular DNA species in each bacterial strain were different, U, L, and Q were present (Fig. 3b). The DNA samples isolated from TOP10 cells were selected for further investigation.

**Identities of U, L, and Q. (i) Restriction enzyme and Southern blot analyses.** Plasmid DNA isolated from TOP10 cells transformed with pChi-6 was analyzed before and after digestion with restriction enzymes (Fig. 4a). Digestion of the DNA sample with Xho1 (which cuts pSK$^+$ once) (lane 2) yielded linearized U (lin-U) and lin-L molecules of 6.2 kb and 4.3 kb, respectively. Q was not cut by Xho1, which suggested that it may not contain any bacterial DNA (confirmed by Southern blot analysis and DNA sequencing). Digestion of the DNA sample with the restriction enzyme Stu1 (which cuts the viral sequence once) (lane 3) yielded lin-U and lin-Q of 6.2 kb and 1.76 kb, respectively. Since L was not cleaved by Stu1, it may not contain the PCV2 capsid DNA sequence that harbors the Stu1 site (confirmed by nucleotide sequencing).

Southern blot analysis was carried out with $^{32}$P-labeled DNA probes. As shown, the nick-translated pSK$^+$ probe did not hybridize to Q but hybridized to multiple plasmid DNA-containing species in the undigested sample (lane 1), which upon digestion with Xho1 condensed to lin-U and lin-L (lane 2). As expected, hybridization results confirmed that Stu1 digestion yielded lin-U but did not cut L (lane 3). In comparison, the 5′-labeled PCV-specific directional probes, c1703F and c887R, hybridized to U, L, and Q (lane 1). The lack of hybridization of the pSK$^+$ probe to Q indicates that it contains only PCV DNA and no plasmid vector sequences, which is consistent with the observation that they are not digested by Xho1 (lane 2). Upon digestion with Stu1, Q was linearized (lin-Q) to the genome length of 1.76 kb (lane 3). In an attempt to identify the ssDNA species of U, L, or Q, total DNA isolated from an overnight bacterial culture was analyzed alongside the plasmid preparation by Southern blot analysis (Fig. 4b). Hybridization was

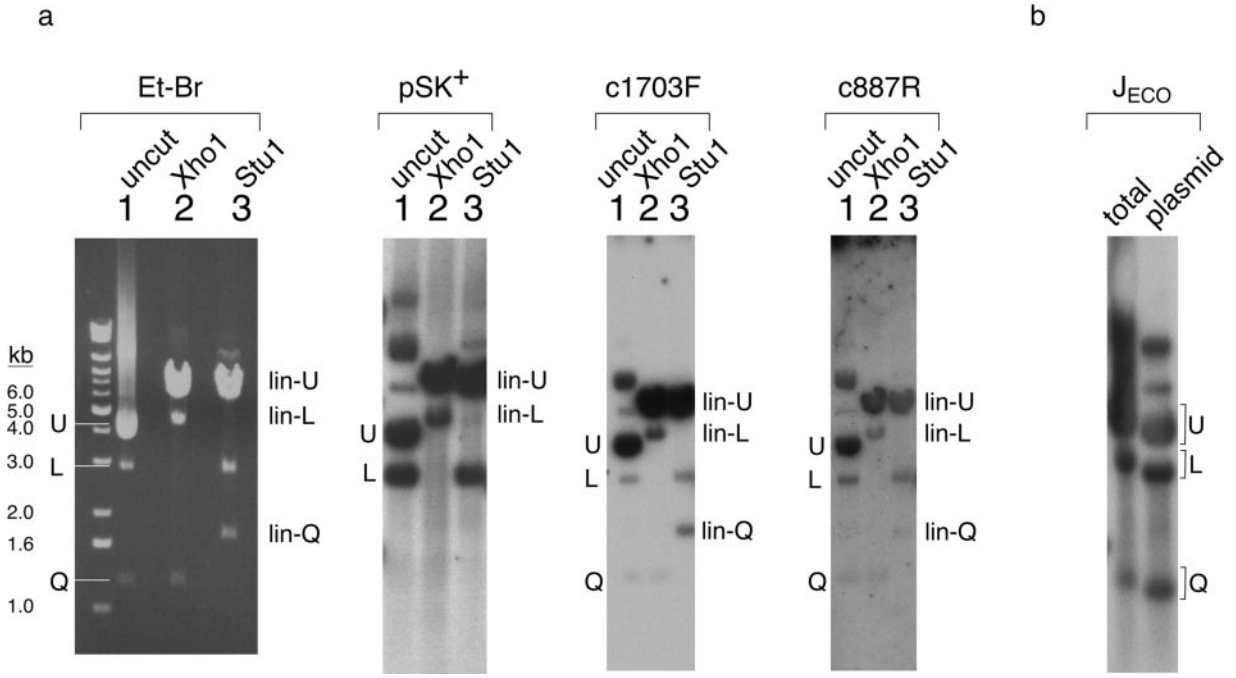


FIG. 4. Restriction enzyme digestion and Southern blot analysis of pChi-6. (a) Agarose gel stained with ethidium bromide (Et-Br) and blots hybridized with nick-translated probes $pSK^+$ or 5′-end-labeled probes (c1703F and c887R) are indicated on the top of each panel. Uncut and restriction enzyme cut DNA is indicated on the top of each lane. (b) Total bacterial or plasmid DNA was hybridized with the nick-translated $J_{Eco}$ probe. The closed-circular ds molecules are labeled U, L, and Q, and the linearized DNA species are indicated with the prefix lin-.

carried out with the PCV1 genomic clone $J_{Eco}$ probe, but no additional DNA species was detected.

**(ii) Nucleotide sequences of U, L, and Q.** Since U, L, and Q share common sequences and they were present together in the DNA recovered from pChi-6- or pChi-7-transformed *E. coli*, the strategy to determine the nucleotide sequence of each DNA species from both constructs was as follows. L was separated from U and Q by retransforming agarose gel-purified L DNA into TOP10 cells and determining the sequence of the recovered L DNA using the selected oligonucleotide primers indicated in Fig. 2b. The results with T7 and T3 primers showed that L contained the left-hand-truncated Cap region of PCV1, the right-hand Rep region of PCV2, and the composite Ori-$_{1L/2R}$ (Fig. 2a). Q was separated from U and L and linearized with Pst1 (only cutting PCV1 DNA once) and then inserted into the Pst1 site of $pSK^+$. Primers T7, T3, and 1678F were then used to determine the nucleotide sequence of Q. The results showed that Q was a unit-length chimeric PCV1/PCV2 genome containing the Rep region of PCV1, the Cap region of PCV2, and a composite Ori-$_{2L/1R}$ (Fig. 2a). In essence, U was split at the PCV1 and PCV2 Oc8 motifs to yield L and Q. Primers N200R, 1430F, and 1430R were used to sequence U. Although primers 1430F and 1430R also hybridized to Q present in the DNA recovered from pChi-6- or pChi-7-transformed *E. coli*, the fact that the amount of U was in far excess compared to Q indicated that the sequence obtained would represent U. The results confirmed that U corresponded to the engineered input pChi-6 or pChi-7 construct.

**(iii) Q was generated predominantly via the RCR copy-release mechanism.** If Q were derived from pChi-6 or pChi-7 via homologous recombination, recombination might be expected to occur at various sites throughout the constructs and most likely would result in an Ori containing either PCV1 or PCV2 sequence. On the other hand, if Q were generated via an RCR copy-release mechanism, it would always have a chimeric Ori-$_{2L/1R}$ composed of PCV2 Ori-$_{2L}$ sequence to the left of the nick site and the PCV1 Ori-$_{1R}$ sequence to the right (Fig. 1b). One hundred ampicillin-resistant colonies containing pChi-6 Q DNA inserted into $pSK^+$ were picked and analyzed. The nucleotide sequence at the PCV Ori was determined by using oligonucleotide primer 1678F, which is identical for both PCV1 and PCV2. The results showed that all 100 clones contained the composite Ori-$_{2L/1R}$.

**The production of L and Q from U was Rep specific. (i) Rep, but not Rep′, was essential for L and Q production in bacteria.** To ascertain the role of Rep and Rep′ in the synthesis of L and Q, early termination codons (previously shown to render PCV1 or PCV2 genomes nonreplicative when transfected into mammalian PK15 cells) (2, 5) were introduced into pChi-7. These mutations were engineered into both the PCV1 *Rep* (nt 727A) and the PCV2 *Rep* (nt 740A) sequences to generate pChi-7Rep$^-$. Although *Rep′* RNA is not synthesized in bacteria (data not shown), early termination codons were introduced into *Rep′* of PCV1 and PCV2 to ensure that no functional Rep′ proteins could be produced. Mutations were introduced into *Rep′* of PCV1 (nt 799C) and PCV2 (nt 812C) to generate pChi-7Rep′$^-$. Locations of the PCV1 mutations involved are denoted in Fig. 1a. The results demonstrated that the mutations introduced into *Rep*, but not *Rep′*, abolished L and Q synthesis (Fig. 5a). Thus, a functional *Rep* gene, but not *Rep′*, was essential for the synthesis of both L and Q in bacteria.

Past work conducted with monomeric PCV genomes dem-

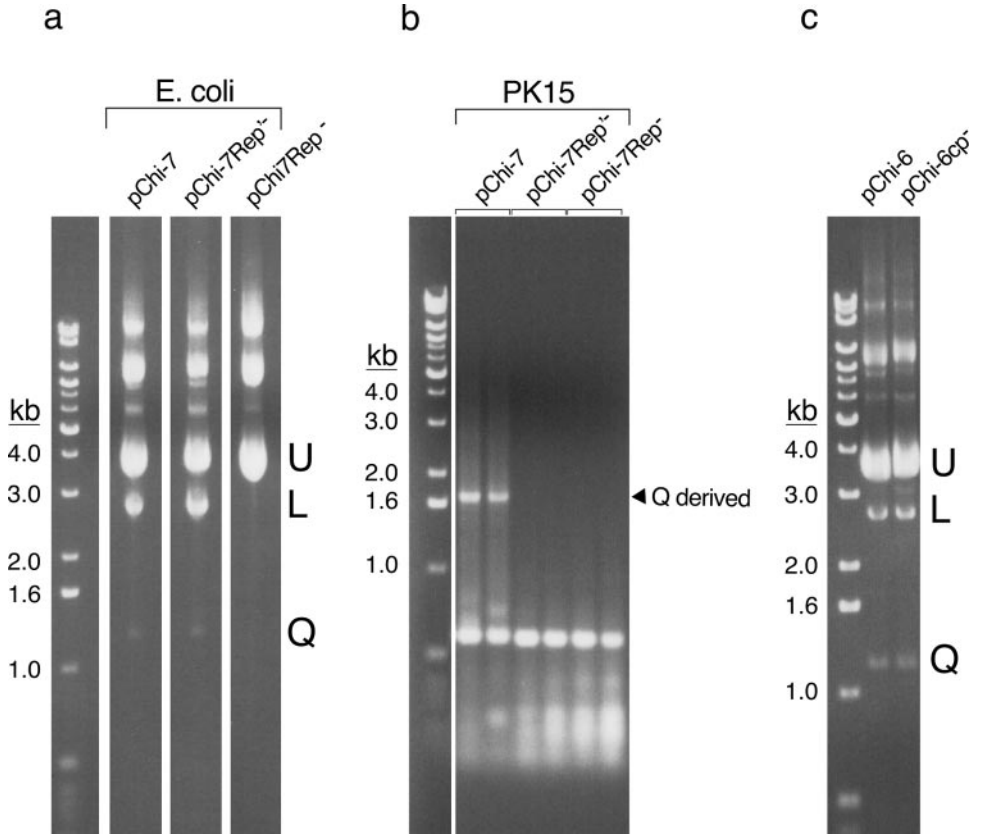


FIG. 5. The role of Rep, Rep′, and Cap in the generation of L and Q from U. (a) Mutational analysis of Rep and Rep′ of pChi-7 in *E. coli*. (c) Mutational analysis of Rep and Rep′ of pChi-7 in PK15 cells. (d) Mutational analysis of the Cap gene of pChi-6 in *E. coli*. The DNA constructs used in each experiment are indicated on top of each lane.

onstrated that both Rep and Rep′ are essential for PCV DNA replication in PK15 cells (2, 5). To determine whether the tandem configuration of pChi-7 may have eliminated the requirement for Rep′ during copy-release replication of Q, experiments were carried out with pChi-7, pChi-7Rep$^-$, and pChi-7Rep′$^-$ in PK15 cells. The U DNA species of all three constructs was purified individually after two consecutive agarose gel electrophoresis, first as supercoils and then as Stu1-linearized DNAs. After recircularization of the linearized DNAs with ligase, the samples were transfected into PK15 cells in duplicate. Total cell DNA was then analyzed by PCR with a pair of diverging primers, 1264R/1296F. These primers would yield a PCR product of approximately 1.7 kb only if circularized Q were generated from the input U DNA. Total cell DNA recovered at 48 h posttransfection showed that pChi-7-transfected PK15 cells yielded a 1.7-kb PCR product, while cells transfected with pChi-7Rep$^-$ and pChi-7Rep′$^-$ failed to yield such a DNA band (Fig. 5b). This 1.7-kb PCR product was purified from the gel, cloned into pSK$^+$, and sequenced with primers T7, T3, and 1678F. The results showed that this PCR product was derived from Q and contained $PCV1_{Rep}$, $PCV2_{Cap}$, and the composite $Ori\text{-}_{2L/1R}$ sequences. Thus, both functional Rep and Rep′ were essential for the synthesis of Q from U in PK15 cells.

**(ii) The viral capsid gene was nonessential for L and Q synthesis.** An early termination codon was engineered at nt 1727 of the PCV2 capsid gene (2) in pChi-6 to ensure that no functional capsid protein could be synthesized. This mutant construct, pChi-6cp$^-$, was capable of producing U, L, and Q in bacteria (Fig. 5c). Thus, PCV capsid was not essential for the generation of L and Q.

**(iii) Oc8 was essential for L and Q synthesis.** The loop sequence of PCV1 consists of four nonessential D nucleotides ($C_1T_2G_3T_4$) and eight essential O nucleotides ($A_1G_2T_3A_4T_5T_6\downarrow A_7C_8$) (Fig. 1). It has been demonstrated that point mutations introduced at O nucleotide position O-6, O-7, or O-8 were lethal, while mutations engineered into the D nucleotides were viable and produced progeny virus when transfected into PK15 cells (6, 7). Here, single-nucleotide substitution mutations (identical to those previously used) were introduced at position D-1, D-2, or D-3 or position O-6, O-7, or O-8 of PCV1 in pChi-7 (Fig. 6a). The single-nucleotide substitutions into D-1, D-2, and D-3 were G, C, and C, respectively, and those for PCV1 O-6, O-7, and O-8 were C, C, and G, respectively. Whereas genomes containing the D-position mutations continued to synthesize U, L, and Q, genomes containing the O-position mutations yielded U only. Separately, an identical single-nucleotide substitution was also introduced into Oc8 of PCV2 at O nucleotide positions O-6, O-7, and O-8 in pChi-7. These constructs also only yielded U. Sequence analysis confirmed that the engineered input mutations were present in all the corresponding recovered DNA species. Thus, the nucleo-

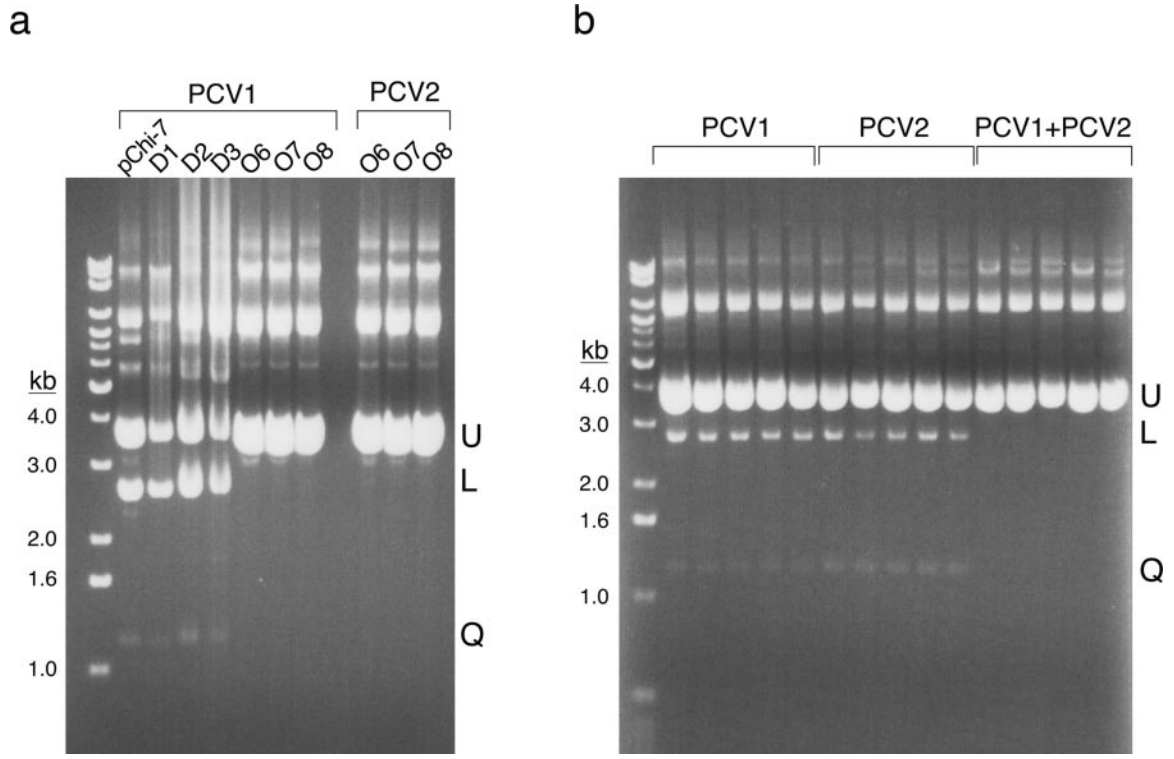


FIG. 6. Mutational analysis of selected *cis*-acting elements essential for L and Q generation from U. (a) Mutation of the Oc8 motif. The position of each mutation is indicated at the top of each lane. (b) Mutation of the H1/H2 tandem repeat of PCV1, PCV2, or both.

tides of Oc8 critical for PCV replication in PK15 cells were also essential for Q and L synthesis in bacteria.

**(iv) The hexanucleotide direct repeat of PCV was essential for L and Q synthesis.** Four copies of a hexanucleotide (CG GCAG or CGGCAG) are present immediately to the right of the stem-loop structure at the Ori of PCV1 and PCV2 (Fig. 1). Previous work demonstrated that at least one "recognizable H motif sequence" abutting the stem-loop structure is essential for PCV1 DNA replication, but a direct tandem repeat (H1/H2) is preferred (8, 9). In this work, a different tandem nucleotide sequence (CGGGGCAC/CGGGGCACC) was used to replace the H1/H2/CACCT sequence present in PCV1, the H1 sequence of PCV2, or the sequences at both locations of pChi-6. After transforming these constructs into TOP10 cells, five individual bacterial clones from each transformation were selected and analyzed. The results showed that mutation of either the H1/H2 CACCT sequence of PCV1 or the H1 sequence of PCV2 did not affect synthesis of L and Q (Fig. 6b). However, when the H sequences at both locations were mutated, the construct failed to generate L and Q but continued to synthesize U.

## DISCUSSION

Previous studies have shown that infectious virus can be generated from bacterial constructs containing head-to-tail tandem repeat PCV genomes with two Oris upon transfection into mammalian tissue culture cells or introduction into live animals (11, 12, 36). However, the mechanisms for the derivation of the infectious viral genomes from these constructs have not been elucidated. In this work, we demonstrated that unit-length chimeric $PCV1_{Rep}/PCV2_{Cap}$ genomes (Q) were derived from plasmid constructs containing head-to-tail tandem partial PCV1/PCV2 genomes (together accounting for 1.75 genomes) in *E. coli*. The structure and requirements suggest that the initial generation of these viral genomes was via the RCR copy-release excision mechanism in the presence of a functional Rep protein and two PCV Oris (Fig. 7). Presumably, Rep provides the nicking and joining of Oc8 during initiation and termination of DNA replication, while the bacterial replication machinery carries out the DNA polymerization functions. It is particularly interesting that the generation of Q from pChi-6 or pChi-7 in PK15 cells required both the PCV-specific Rep and Rep′ proteins, while only the Rep protein is required in *E. coli*. We also demonstrated that, with the exception of Rep′, the elements essential for PCV replication in mammalian cells (Rep, Oc8, and H1) were also critical for the generation of Q in *E. coli*.

In this work, two completely different replication mechanisms utilizing two different types of Ori ($ColE_1$ and PCV) were carried out on a single DNA molecule (U) to synthesize three distinct DNA species in *E. coli*. DNA replication utilizing the $ColE_1$ Ori is theta like and unidirectional (19, 40) and involves bacterial proteins only, while DNA replication utilizing the PCV Ori is via the RCR mechanism and involves the PCV-specific Rep protein as well as the bacterial replication machinery. In fact, during DNA synthesis, these two replication systems are moving towards each other on a collision course (Fig. 2). Three distinct DNA species (U, L, and Q) were

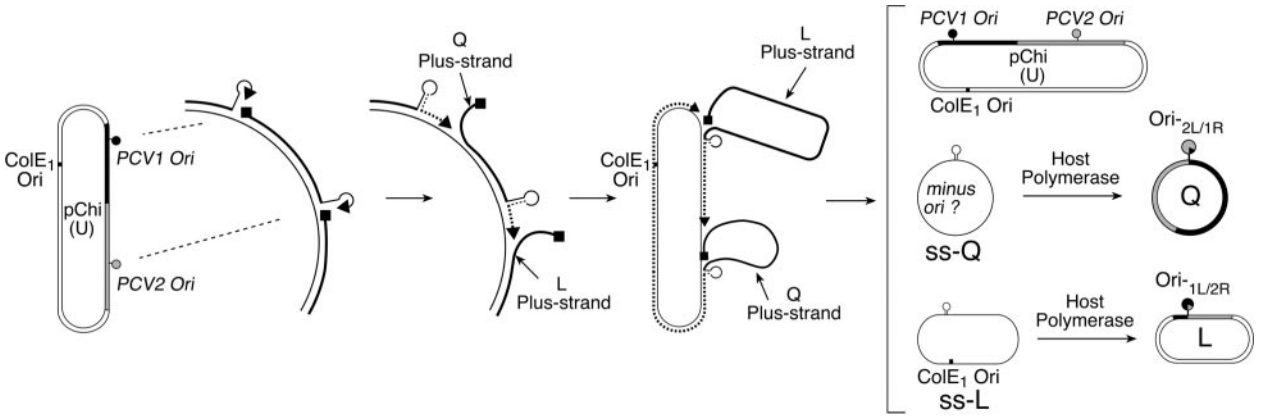


FIG. 7. Model for the generation of Q and L from pChi constructs (or U) by the RCR copy-release mechanism (adapted from reference 35). Expression of the PCV Rep genes in *E. coli* results in the production of active Rep protein (■) that nicks the Oc8 nucleotide within the Ori and attaches itself to the 5′ end of the nicked DNA covalently. Extension of the 3′ end of the nicked DNA by host polymerase (▲) results in displacement of the plus-strand DNA. When the plus-strand DNA containing the second Ori is displaced, Rep nicks the second Oc8 sequence while simultaneously ligating the ends of the ss molecule to reconstitute a new Oc8 sequence. For the production of Q that contains the composite Ori-$_{2R/1L}$, Rep initiates DNA replication at the PCV1 Ori and terminates at the PCV2 Ori. Production of the ds replication intermediate by host enzymes may involve an unidentified minus-strand Ori and an initiation primer. For the production of L that contains the composite Ori-$_{1L/2R}$, Rep initiates DNA replication at the PCV2 Ori and terminates at the PCV1 Ori. The newly synthesized DNA is dotted, and the displaced strand is black.

generated from pChi-6 or pChi-7. For the synthesis of U, only the bacterial replication system utilizing the ColE$_1$ Ori is required.

The generation of L and Q was likely via the PCV Rep-initiated RCR copy-release mechanism in a manner similar to that described for geminiviruses or nanoviruses (35, 39). Conceivably, the synthesis of L and Q can occur simultaneously on the same molecule (Fig. 7). For the generation of Q, DNA synthesis initiates at the PCV1 Ori and terminates at the PCV2 Ori. During initiation, the PCV Rep protein is necessary for cleaving the PCV1 Oc8 sequence to generate a 3′-OH end for leading-strand DNA synthesis, and the polymerization process is carried out by the bacterial replication machinery. During termination, Rep nicks the PCV2 Oc8 sequence and then reconstitutes a chimeric Ori-$_{2L/1R}$ by joining the ends of the displaced chimeric unit-length PCV1$_{Rep}$/PCV2$_{Cap}$ genome and releasing the circular ss Q molecule. However, the displaced circular ss Q species was not detected in *E. coli* here, which is in contrast to the findings obtained with comparable tandem geminivirus plasmid constructs transformed into bacteria (33, 35). The results suggest that the ssDNA species was efficiently converted to dsDNA and that a minus-genome primer is synthesized in bacteria to convert ss Q to ds Q. Theoretically, but not demonstrated, the nascent ds Q species can then replicate itself via the RCR mechanism.

The replication of L is more complicated because it contains both the PCV Ori as well as the ColE$_1$ Ori. To generate ss L, DNA synthesis initiates at the PCV2 Ori and terminates at the PCV1 Ori. The newly generated L DNA would contain the composite Ori-$_{1L/2R}$. Again, ss L was efficiently converted to ds L. Subsequent replication of L can then be via theta-like replication with the ColE$_1$ Ori or, possibly, via the RCR mechanism, utilizing the new chimeric PCV Ori-$_{1L/2R}$.

The ability of *Agrobacterium tumefaciens* or *E. coli* to support the DNA replication process of diverse plant geminiviruses has been described elsewhere (35, 37). Here, we showed that *E. coli* can also support the animal circovirus DNA replication process. In both the plant and animal experimental systems, the production of unit-length viral genome is via the RCR mechanism and depends on the presence of two Oris in a head-to-tail tandem configuration in conjunction with a functional Rep protein. The fact that the plant and animal virus DNA replication processes can be supported by bacterial cellular machinery provides evidence that these circular ssDNA viruses may have evolved from prokaryotic episomal replicons.

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## REFERENCES

1. **Cheung, A. K.** 2003. Comparative analysis of the transcriptional patterns of pathogenic and non-pathogenic circoviruses. Virology **310:**41–49.
2. **Cheung, A. K.** 2003. The essential and non-essential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology **313:**452–459.
3. **Cheung, A. K.** 2004. Detection of template strand switching during initiation and termination of DNA replication of porcine circovirus. J. Virol. **78:**4268–4277.
4. **Cheung, A. K.** 2004. 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.
5. **Cheung, A. K.** 2004. 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.
6. **Cheung, A. K.** 2004. 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.
7. **Cheung, A. K.** 2005. Detection of rampant nucleotide reversion at the origin of DNA replication of porcine circovirus type 1. Virology **333:**22–30.
8. **Cheung, A. K.** 2005. Mutational analysis of the direct tandem repeat sequences at the origin of DNA replication of porcine circovirus type 1. Virology **339:**192–199.
9. **Cheung, A. K.** 2006. Regeneration of the replication-associated proteins tandem direct repeat recognition nucleotide sequence at the origin of DNA replication of porcine circovirus type 1. Virology **346:**32–39.
10. **Cheung, A. K., and S. R. Bolin.** 2002. Kinetics of porcine circovirus type 2 replication. Arch. Virol. **147:**43–58.

11. **Fenaux, M., P. G. Halbur, G. Haqshenas, R. Royer, P. Thomas, P. Nawagitgul, M. Gill, T. E. Toth, and X. J. Meng.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J. Virol. **76:**541–551.
12. **Fenaux, M., T. Opriessnig, P. G. Halbur, and X. J. Meng.** 2003. Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. J. Virol. **77:**11232–11243.
13. **Gibbs, M. J., and G. F. Weiller.** 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc. Natl. Acad. Sci. USA **96:**8022–8027.
14. **Gutierrez, C.** 1999. Geminivirus DNA replication. Cell. Mol. Life Sci. **56:** 313–329.
15. **Hafner, G. J., M. R. Stafford, L. C. Wolter, R. M. Harding, and J. L. Dale.** 1997. Nicking and joining activity of banana bunchy top virus replication protein in vitro. J. Gen. Virol. **78:**1795–1799.
16. **Hanley-Bowdoin, L., S. B. Settlage, B. M. Orozco, S. Nagar, and D. Robertson.** 2000. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit. Rev. Biochem. Mol. Biol. **35:**105–140.
17. **Heyraud-Nitschke, F., S. Schumacher, J. Laufs, S. Schaefer, J. Schell, and B. Gronenborn.** 1995. Determination of the origin cleavage and joining domain of geminivirus Rep proteins. Nucleic Acids Res. **23:**910–916.
18. **Ilyina, T. V., and E. V. Koonin.** 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic Acids Res. **20:**3279–3285.
19. **Inselburg, J.** 1974. Replication of colicin E1 plasmid DNA in minicells from a unique replication initiation site. Proc. Natl. Acad. Sci. USA **71:**2256–2259.
20. **Kato, M., S. Hokabe, S. Itakura, S. Minoshima, Y. L. Lyubchenko, T. D. Gurkov, H. Okawara, K. Nagayama, and N. Shimizu.** 2003. Interarm interaction of DNA cruciform forming at a short inverted repeat sequence. Biophys. J. **85:**402–408.
21. **Kato, M., K. Matsunaga, and N. Shimizu.** 1998. A novel unusual DNA structure formed in an inverted repeat sequence. Biochem. Biophys. Res. Commun. **246:**532–534.
22. **Laufs, J., W. Traut, F. Heyraud, V. Matzeit, S. G. Rogers, J. Schell, and B. Gronenborn.** 1995. In vitro cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. Proc. Natl. Acad. Sci. USA **92:**3879–3883.
23. **Mankertz, A., J. Mankertz, K. Wolf, and H.-J. Buhk.** 1998. Identification of a protein essential for replication of porcine circovirus. J. Gen. Virol. **79:** 381–383.
24. **Mankertz, A., F. Persson, J. Mankertz, G. Blaess, and H.-J. Buhk.** 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. J. Virol. **71:**2562–2566.
25. **Mankertz, A., and B. Hillenbrand.** 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. Virology **279:** 429–438.
26. **Mankertz, A., B. Mueller, T. Steinfeldt, C. Schmitt, and T. Finsterbusch.** 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus type 1 and 2. J. Virol. **77:**9885–9893.
27. **Mayo, M. A.** 2002. Virus taxonomy—Houston 2002. Arch. Virol. **147:**1071–1076.
28. **McNulty, M., J. Dale, P. Lukert, A. Mankertz, J. Randles, and D. Todd.** 2000. Circoviridae, p. 299–303. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
29. **Meehan, B. M., J. L. Creelan, M. S. McNulty, and D. Todd.** 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. **78:**221–227.
30. **Niagro, F. D., A. N. Forsthoefel, R. P. Lawther, L. Kamalanathan, B. W. Ritchie, K. S. Latimer, and P. D. Lukert.** 1998. Beak and feather disease virus and porcine circovirus genomes: intermediates between geminiviruses and plant circoviruses. Arch. Virol. **143:**1723–1744.
31. **Ohta, T., S. Nettikadan, F. Tokumasu, H. Ideno, Y. Abe, M. Kuroda, H. Hayashi, and K. Takeyasu.** 1996. Atomic force microscopy proposes a novel model for stem-loop structure that binds a heat shock protein in the Staphylococcus aureus HSP70 operon. Biochem. Biophys. Res. Commun. **226:** 730–734.
32. **Palmer, K. E., and E. O. Rybicki.** 1998. The molecular biology of mastreviruses. Adv. Virus Res. **50:**183–234.
33. **Pant, V., D. Gupta, N. R. Choudhury, V. G. Malathi, A. Varma, and S. K. Mukherjee.** 2001. Molecular characterization of the Rep protein of the blackgram isolate of Indian mungbean yellow mosaic virus. J. Gen. Virol. **82:**2559–2567.
34. **Pringle, C. R.** 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia. Arch. Virol. **144:**2065–2070.
35. **Ridgen, J. E., I. B. Dry, L. R. Krake, and M. A. Rezaian.** 1996. Plant virus DNA replication processes in Agrobacterium: insight into the origins of geminiviruses? Proc. Natl. Acad. Sci. USA **93:**10280–10284.
36. **Roca, M., M. Balasch, J. Segalés, M. Calsamiglia, E. Viaplana, A. Urniza, K. Hattermann, A. Mankertz, J. Plana-Durán, and M. Domingo.** 2004. *In vitro* and *in vivo* characterization of an infectious clone of a European strain of porcine circovirus type 2. J. Gen. Virol. **85:**1259–1266.
37. **Selth, L. A., J. W. Randles, and M. A. Rezaian.** 2002. Agrobacterium tumefaciens supports DNA replication of diverse geminivirus types. FEBS Lett. **516:**179–182.
38. **Steinfeldt, T., T. Finsterbusch, and A. Mankertz.** 2001. Rep and Rep′ proteins of porcine circovirus type 1 bind to the origin of replication in vitro. Virology **291:**152–160.
39. **Stenger, D. C., G. N. Revington, M. C. Stevenson, and D. M. Bisaro.** 1991. Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. Proc. Natl. Acad. Sci. USA **88:**8029–8033.
40. **Tomizawa, J., Y. Sakakibara, and T. Kakefuda.** 1974. Replication of colicin E1 plasmid DNA in cell extracts. Origin and direction of replication. Proc. Natl. Acad. Sci. USA **71:**2260–2264.