Journal of Virology, Apr. 2004, p. 4268–4277 Vol. 78, No. 8
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.8.4268–4277.2004

Detection of Template Strand Switching during Initiation and Termination of DNA Replication of Porcine Circovirus

Andrew K. Cheung*
Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa 50010

Received 23 September 2003/Accepted 22 December 2003

Nucleotide substitution mutagenesis was conducted to investigate the importance of the inverted repeats (palindrome) at the origin of DNA replication (Ori) of porcine circovirus type 1 (PCV1). Viral genomes with engineered mutations on either arm or both arms of the palindrome were not impaired in protein synthesis and yielded infectious progeny viruses with restored or new palindromes. Thus, a flanking palindrome at the Ori was not essential for initiation of DNA replication, but one was generated inevitably at termination. Among the 26 viruses recovered, 16 showed evidence of template strand switching, from minus-strand genome DNA to palindromic strand DNA, during biosynthesis of the Ori. Here I propose a novel rolling-circle "melting-pot" model for PCV1 DNA replication. In this model, the replicator Rep protein complex binds, destabilizes, and nicks the Ori sequence to initiate leading-strand DNA synthesis. All four strands of the destabilized inverted repeats exist in a "melted" configuration, and the minus-strand viral genome and a palindromic strand are available as templates, simultaneously, during initiation or termination of DNA replication. Inherent in this model is a "gene correction" or "terminal repeat correction" mechanism that can restore mutilated inverted-repeat sequences to a palindrome at the Ori of circular DNAs or at the termini of circularized linear DNAs. Potentially, the melted state of the inverted repeats increases the rate of noncomplementary or illegitimate nucleotide incorporation into the palindrome. Thus, this melting-pot model provides insight into the mechanisms of DNA replication, gene correction, and illegitimate recombination at the Ori of PCV1, and it may be applicable to the replication of other circular DNA molecules.

Porcine circovirus (PCV), *Psittacine beak-and-feather disease virus*, *Goose circovirus*, *Canary circovirus*, and *Pigeon circovirus* (30, 33, 39–41) belong to the genus *Circovirus* of the *Circoviridae* family (20, 31). Two genotypes of PCV have been identified. Whereas PCV type 1 (PCV1) is nonpathogenic, PCV2 type 2 (PCV2) has been implicated as the etiological agent of a new disease, named postweaning multisystemic wasting syndrome (1). It has been suggested that animal circoviruses were derived from a plant virus (probably a plant nanovirus) that switched hosts (via an insect vector) to infect a vertebrate and then recombined with a vertebrate-infecting virus (probably a single-stranded RNA virus such as a calicivirus) (8).

It is now well established that during geminivirus replication (reviewed in references 10, 12, and 29), the closed circular single-stranded DNA genome is converted to a superhelical double-stranded DNA intermediate. The virus-encoded Rep binds and nicks (indicated by an arrow) between the 7th T and the 8th A of a "conserved" nonanucleotide (TAATATT ↓ AC) at the origin of DNA replication (Ori) to initiate plus-strand DNA replication. This nonanucleotide is present among all members of the *Geminiviridae* family, and it is flanked by two inverted-repeat (palindromic) sequences, which presumably can base pair together to form a cruciform structure during DNA replication. Previous studies have indicated that an Ori-flanking palindrome is essential for geminivirus DNA replication (15, 16, 28, 32).

Like geminiviruses, PCV has a closed circular single-stranded DNA genome (38). The genome sequences of a number of PCV1 and PCV2 isolates have been determined (7, 11, 21, 22, 25, 27), and nucleotide sequence analysis has indicated that the PCV genome is an intermediate between the genomes of geminivirus and plant circovirus (27). Structural and sequence similarities of these viruses suggest that PCV DNA replicates in a manner similar to that of the *Mastrevirus* genus of the *Geminiviridae* family, via the rolling-circle replication (RCR) stem-loop cruciform model (17, 29). These similarities include the following: (i) Rep proteins of geminivirus and PCV contain the three conserved RCR motifs (RCR-I, -II, and -III) and a nucleoside triphosphate-binding core homologous to those of the Rep proteins of other prokaryotic and eukaryotic RCR systems (14, 18), and (ii) the Ori of PCV1 has been mapped to a 111-bp fragment (19) which contains a nonanucleotide (TAGTATT ↓ AC) (Fig. 1) similar to that of geminiviruses. This sequence is flanked by an 11-nucleotide (nt) palindrome that has the potential to form a stem-loop cruciform structure. In contrast to that of geminiviruses, PCV DNA replication requires two proteins, Rep and Rep′, instead of just one multifunctional Rep protein (4, 5, 29). In vitro experiments showed that PCV1 Rep binds to the right arm of the presumed stem-loop, while both Rep and Rep′ bind to two adjacent, almost perfect 6-nt (CGGCAG) tandem direct repeats located at nt 13, 19, 30, and 36 (Fig. 1) (36).

In this work, mutational analysis was conducted to investigate the importance of the 11-nt palindrome of PCV1 with respect to protein synthesis and progeny virus production.

* Mailing address: National Animal Disease Center, P.O. Box 70, Ames, IA 50010. Phone: (515) 663-7497. Fax: (515) 663-7458. E-mail: acheung@nadc.ars.usda.gov.

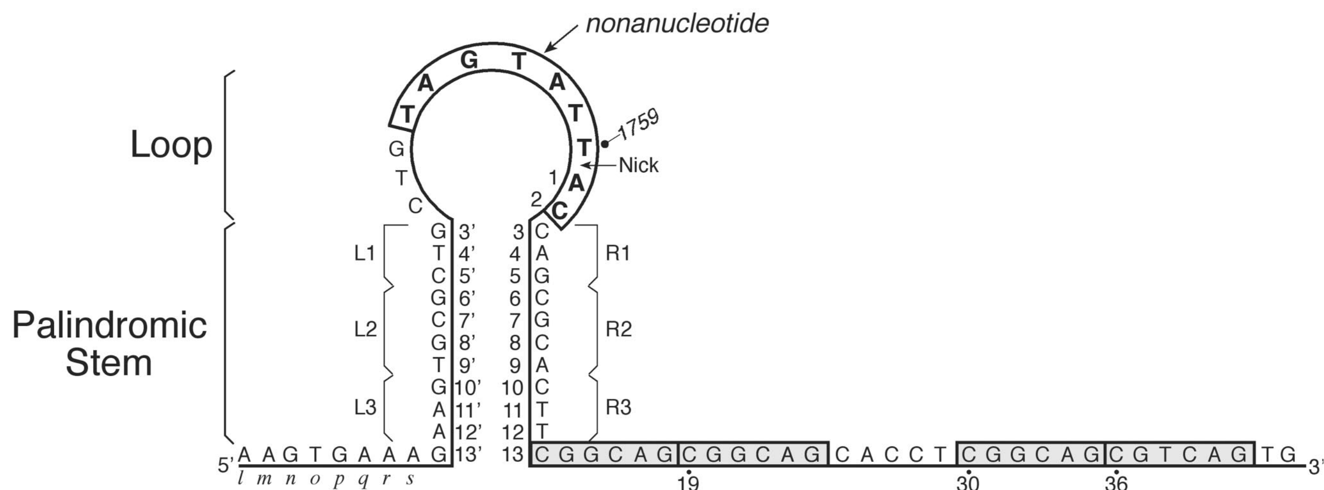


FIG. 1. Schematic representation of the PCV1 plus-strand Ori indicating potential base pairing of the flanking inverted-repeat sequences. The genome sequence (1,759 nt) and coordinates (numbered 1, 2, 3, etc.) are based on GenBank accession number AY184287. The nucleotide coordinates (numbered 3′, 4′, 5′, etc.) and italicized letters *l* through *s* are arbitrarily assigned to show nucleotide complementarity of the palindromic sequences and to facilitate sequence identification in the recovered viruses, respectively. The nonanucleotide containing the presumed nick site (TAGTATT ↓ AC) is boxed. The palindrome is arbitrarily divided into six regions (R1 to R3 in the right arm and L1 to L3 in the left arm). The 6-nt (CGGCAG) tandem repeats located at nt 13, 19, 30, and 36 (not perfect at nt 38) are shaded.

MATERIALS AND METHODS

Virus, cells, and antiserum. A PCV1 isolate (PCV/AC1) (GenBank accession number AY184287) (3), a PCV1-free PK15 cell line, and a hyperimmune swine serum raised against PCV2 that reacts with the Rep-associated proteins of PCV1 (5, 6) were used.

Oligonucleotide primers. The primers for PCR, CCAAGATGGCTGCG GGGG and GTAATCCTCCGATAGAGAGC, were located at nt 1665 to 1682 and at nt 874 to 855 of PCV1/AC1 (3), respectively. The primer sets for mutagenesis were designed to contain 18 to 21 identical nucleotides on either side of the predetermined mutation (3 to 4 nt) as previously described (4).

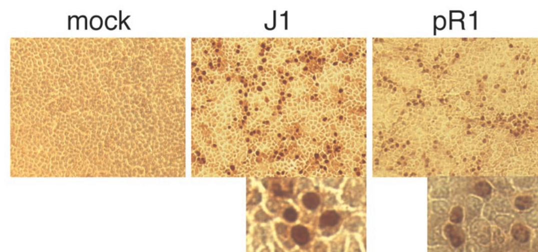
DNA mutagenesis, transfection, immunochemical staining, and PCR. The methodologies used for DNA mutagenesis, transfection, immunochemical staining, and PCR have been described previously (4).

RESULTS

Construction of mutant plasmids. A PCV1 genomic clone (3), with an additional BamHI site (GGATCC) at nt 992 between the Rep and the capsid regions, was inserted into the BamHI site of the Bluescript plasmid to generate plasmid J1. The excised and circularized double-stranded viral genome from this construct was capable of producing infectious PCV1 upon transfection into PK15 cells (Fig. 2) (5). Plasmid J1 was then used to generate subsequent mutant plasmids. A schematic representation of the single-stranded plus-strand Ori is outlined in Fig. 1, and a series of mutations was engineered into arbitrarily assigned regions (starting at nt 3 of the genomic sequence) of the right arm (regions R1 to R3), the left arm (regions L1 to L3), both arms, or consecutive regions of the palindrome to destabilize the potential stem-loop structure. The "conserved" nonanucleotide (TAGTATT ↓ AC) encompassing the presumed nick site between the first (position 1) and the last (position 1759) nucleotide was indicated. To assist in tracing the template used during DNA synthesis, the relevant viral sequences were also assigned arbitrary positions. A collection of 15 mutant plasmids with modified PCV1 genomes were constructed (Fig. 3). Prior to transfection into PK15 cells, the PCV1 genomes were excised from the Bluescript plasmid (Stratagene, San Diego, Calif.) with BamHI and circularized with T4 DNA ligase. At designated times posttransfection, the cultures were assayed for viral protein synthesis and infectious progeny virus production.

Viral protein synthesis. At 48 h, one set of transfected cultures was assayed for Rep-associated protein by immunochemical staining with a hyperimmune swine serum (5).

(a) Transfected DNA



(b) Recovered Virus

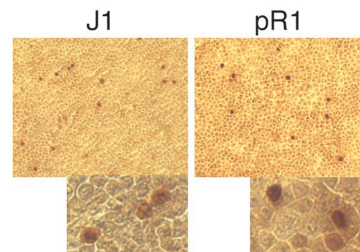


FIG. 2. Immunochemical staining of Rep-associated antigens in PK15 cells that were either transfected with viral genomes excised from plasmid DNAs (a) or infected with recovered viruses (b). Only parent and mutant genomes from plasmids J1 and pR1, respectively, are shown.

A Upper Regions

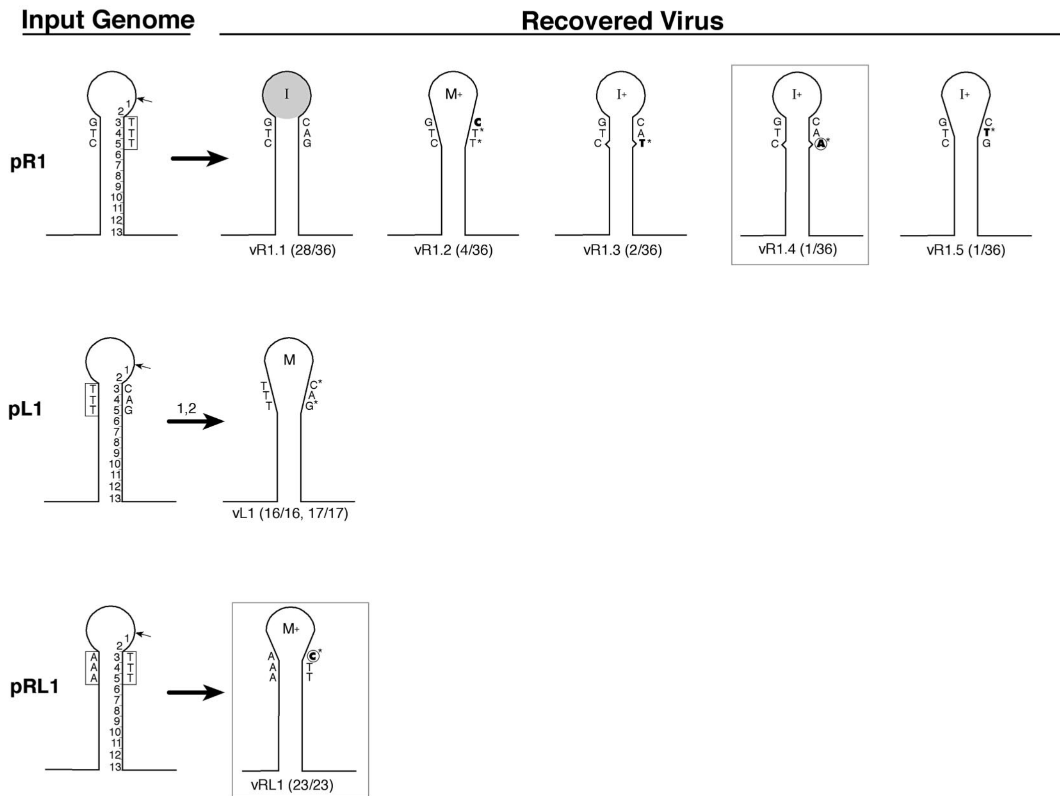


FIG. 3. Progeny viruses derived from PCV1 DNAs containing mutations in the upper (A), central (B), lower (C), and consecutive (D) regions of the 11-nt palindrome. The engineered mutation in the viral genome is boxed. Viruses that can be accounted for by using the minus-strand genome DNA as a template are labeled M or M+ (with mutation). Viruses that can be accounted for by TS during initiation are labeled I (shaded) or I+ (with mutation). Group I viruses can also be accounted for by double TS, i.e., TS during initiation as well as termination. Viruses that can be accounted for by TS during termination are labeled T. Boldfaced letters, point mutations. Asterisks indicate noncomplementary bases with respect to the palindrome; circled nucleotides, "illegitimate" bases. Viruses that contain any illegitimate nucleotides are boxed. The number of examples (specific subclones/total recovered) of each progeny virus, determined by sequencing of cloned PCR fragments, is given in parentheses.

Whereas no viral protein was detected in mock-transfected cells, equivalent numbers of Rep-associated antigen-producing cells were detected with PCV1 genomes excised from plasmid J1 and the 15 mutant plasmids, indicating that Rep-associated protein synthesis was not impaired by the engineered mutations (only results for the parent, J1, and one mutant genome are presented in Fig. 2A).

Progeny virus production. At 7 days, a parallel set of transfected cultures was harvested, freeze-thawed three times, and then assayed for infectious viruses by inoculation into fresh PK15 cells. Progeny viruses were recovered (Fig. 2B) from 14 of 15 mutant genomes. Previous work showed that PCV1 and PCV2 mutants recovered from transfection experiments may not grow beyond cell passage 1 or 2 (5; unpublished data); therefore, the more-stable virus populations at passage 3 were analyzed in this study. However, in two experiments (Fig. 3B), passage-1 viruses were also examined. After confirmation by immunochemical staining, virus-infected cell DNAs were isolated and amplified by PCR with PCV1-specific primers. Each PCR product was subcloned into a TA cloning plasmid (Invitrogen, Carlsbad, Calif.) for nucleotide sequence determination.

The results showed that a collection of viruses with different palindromic sequences were recovered from the transfected cultures (Fig. 3). To ensure that nucleotide variations assigned to the recovered viruses were not errors introduced during PCR, reconstruction controls (i.e., religated input genome mixed with cellular DNAs) were also amplified by PCR and subcloned for sequence determination. Twenty subclones of each reconstructed control were examined, and only the input engineered mutations were observed.

Analysis of the palindromic sequences of progeny viruses. The 15 engineered mutant genomes were organized into four groups: upper regions, central regions, lower regions, and consecutive regions (Fig. 3). The recovered progeny viruses were separated into two categories based on whether template strand switching (TS) (from minus-strand genome DNA to inverted-repeat DNA) took place during synthesis of the 11-nt

B Central Regions

Input Genome

Recovered Virus

FIG. 3—*Continued*.

palindrome. Viruses that did not exhibit TS and used only the minus-strand genome DNA as a template were labeled M. Viruses that exhibited evidence of TS were subdivided into two groups. Those in the first group, which showed TS during initiation of DNA replication, were labeled I; those in the second, which showed TS during termination, were labeled T. In the following analysis, TS was invoked as little as possible to account for the progeny viruses. Viruses containing point mutations with respect to the engineered input sequences were additionally labeled with plus signs. Nucleotides that could not be accounted for by the minus-strand genome DNA template or a palindromic strand template were labeled "illegitimate."

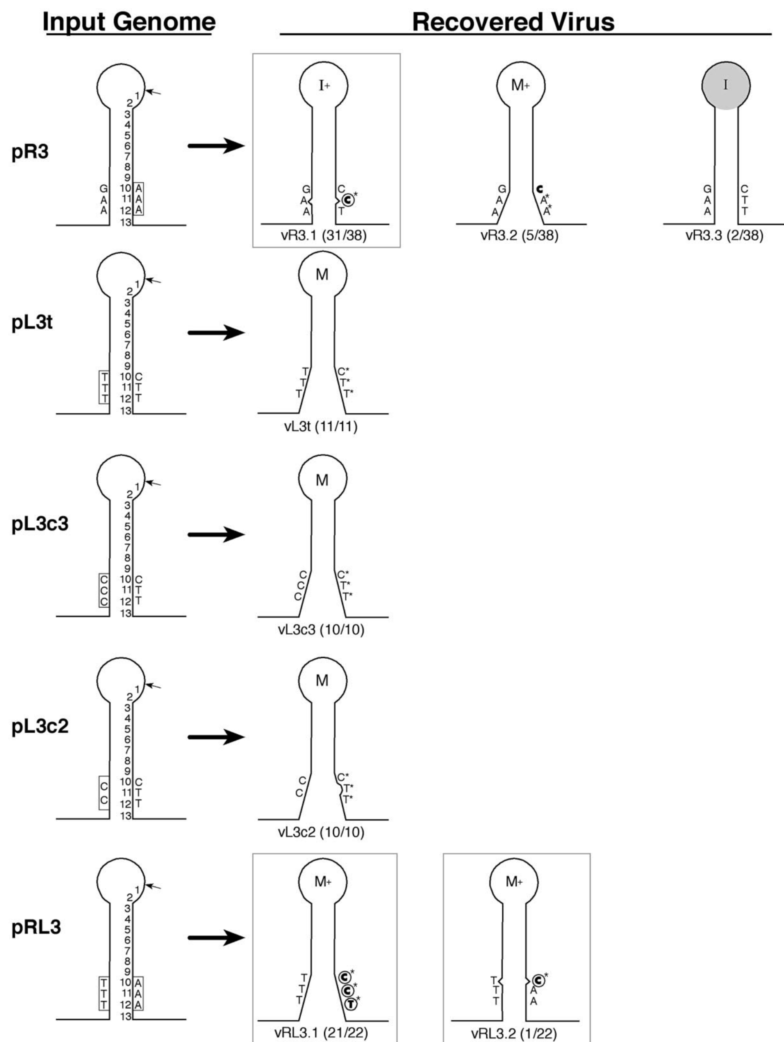


FIG. 3—*Continued*.

(i) Mutations in the upper regions (Fig. 3A). Plasmid R1 (L1:R1 = CTG:TTT) contained 3 T's in region R1, and five viruses, wild-type virus vR1.1 (group I) and four variants, were recovered. Virus vR1.2 (group M+) could be generated by using the minus-strand genome DNA as a template plus a mutation at position 3; alternatively, the C nucleotide at position 3 could be acquired through TS. The four group I and I+ viruses all involved TS during initiation of DNA replication, and viruses vR1.3, vR1.4, and vR1.5 (all in group I+) each acquired a point mutation. It is possible that the point mutations in vR1.3 and vR1.5 were results of reverse TS (i.e., from an inverted-repeat DNA template back to a minus-strand genome DNA template); therefore, only the noncomplementary A nucleotide in virus vR1.4 is considered an illegitimate nucleotide. Excluding the wild-type virus vR1.1, the other four viruses contained at least one noncomplementary nucleotide in the new palindrome. Due to the non-base-pairing nucleotide in vR1.2 and vR1.5, these two viruses may exhibit a shortened palindrome and an extended loop.

Plasmid L1 (L1:R1 = TTT:CAG) contained 3 T's in region L1. Two experiments (both with passage-3 virus) were conducted, and identical results were obtained. No evidence of TS was observed with virus vL1 (group M). This virus has an expanded loop (18 instead of 12 nt), a shortened palindrome (8 instead of 11 nt), and noncomplementary L1 and R1 regions.

Plasmid RL1 was engineered so that sequence complementarity was restored to the upper regions (L1:R1 = AAA:TTT). The recovered virus vRL1 (group M+) showed no evidence of TS but had incorporated an illegitimate C nucleotide at position 3. This virus may exhibit an extended loop (13 nt) and a slightly shortened palindrome (10 nt).

This collection of progeny viruses showed that position 3 was

D Consecutive Regions

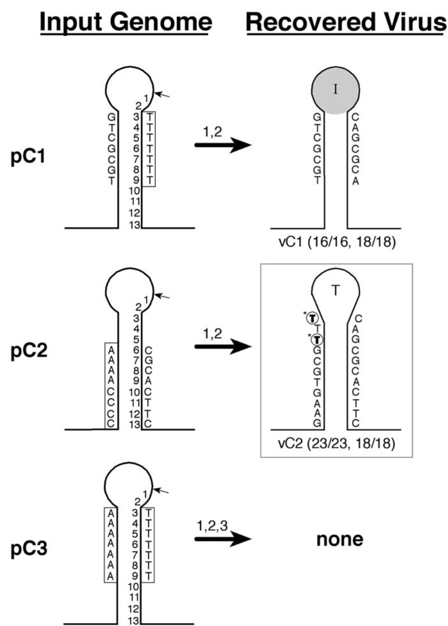


FIG. 3—*Continued*.

occupied exclusively by a C nucleotide and that positions 4 and 5 were quite flexible. Since region L1 could also accommodate several unrelated sequences, the left and right upper regions did not have to be complementary to yield infectious viruses (e.g., vL1). The incorporation of an illegitimate C nucleotide at position 3 of vRL1 was a reversion from an engineered mutation to a wild-type nucleotide, and it did not appear to be an error or a random event. It was a violation of the semiconservative DNA replication mechanism in that it did not maintain base-pairing complementarity between the growing leading strand and the available templates at this position. The absence of a C nucleotide at position 3 in the input genome did not affect protein synthesis, and the engineered mutation was readily converted to wild type in the absence of an identifiable template, which was then maintained in the progeny viruses. To distinguish this type of de novo synthesis from a random illegitimate mutation error (e.g., position 5 of vR1.4, position 11 of vR3.1, and positions 5′ and 3′ of vC2), position 3 was designated a "birthright" position which can be filled only with a C nucleotide irrespective of the DNA templates. Potentially, the birthright of the C nucleotide at position 3 was dictated by and "inherited" from the Rep protein complex, and a C nucleotide at this position is essential for viral DNA replication.

(ii) Mutations in the central regions (Fig. 3B). Plasmid R2a (L2:R2 = TGCG:TTTT) contained 4 T's in region R2. In two separate experiments (passages 1 and 3), three variant viruses were recovered. TS during initiation was detected with the wild-type virus vR2a.2 (group I), and TS during termination was detected with viruses vR2a.1 (group T) and vLP1 (group T). In addition, vLP1 had recruited 7 nt (positions 14 through 20) just outside the right arm into the original palindrome during initiation, and it then used this sequence as a template during termination. Concurrently, 2 bases (CT) at positions 1750 to 1751 and 3 bases (AAA) at positions q, r, and s were deleted. Thus, vLP1 contained a 4-nt loop and a 21-nt palindrome.

Plasmid R2b was further engineered to disrupt the original 11-nt palindrome by duplicating the 9-nt sequence ACCAG TTTT at the presumed nick site. For plasmid R2b, the right arm had 20 nt (CAG-TTTTACCAGTTTT-CTTC), while the left arm had only 13 nt (GAAG-TGCG-CTG). This DNA could assume two different configurations, and it is unlikely that any stable stem-loop structure could be formed during initiation of DNA replication. Two experiments were conducted (passages 1 and 3), and two variant viruses were recovered. TS during initiation was observed in the wild-type virus vR2b.1 (group I), and TS during termination was detected in virus vLP2 (group T). The generation of vLP2 was similar to that of vLP1, in which 7 nt downstream from the right arm of the original 11-nt palindrome were recruited into the new palindrome as templates for the left arm. Several deletions were also observed; they include the duplicated 9-nt sequence, the 3 nt (CTG) at positions 1750 to 1752, and the 3 nt (AAA) at positions q, r, and s. This virus has a 9-nt loop and an 18-nt palindrome.

Plasmid L2 (L2:R2 = AAAA:CGCA) contained 4 A's in region L2. Two experiments were conducted (passages 1 and 3), and only wild-type virus, vL2 (group T), with evidence of TS during termination, was obtained.

Plasmid RL2b (L2:R2 = AAAA:TTTTACCAGTTTT) contained 4 nt in region L2 and 13 nt in region R2. It was engineered to provide partial sequence complementarity to the 4 T's in plasmid R2b. In one configuration, base-pairing could take place at the second duplication and resulted in a bigger loop region. In another configuration, the second duplication formed a loop between regions R2 and R3. Only a variant virus, vRL2b (group M+), that used the minus-strand genome DNA as a template with deletion of the 9-nt duplication, was recovered. However, vRL2b could also be generated via TS during initiation of DNA replication.

The generation of this collection of viruses from the mutant PCV1 DNAs (pR2a and pR2b) indicated that a palindrome was not essential for initiation of DNA replication; however, palindromes that include the central regions consisting of wild-type sequences (vR2a.2, vR2b.1, and vL2) or new sequences (vR2a.1, vLP1, vLP2, and vRL2b), were formed preferentially during termination to generate progeny viruses. For the generation of viruses vLP1 and vLP2, the likely scenario was that the Rep protein complex recognized the distal 6-nt tandem repeat (nt 30 to 41) instead of the proximal tandem repeat (nt 13 to 24) (Fig. 1) and resulted in shoveling 7 additional nucleotides into the palindrome and then using them as a template for synthesis of the left arm during termination. To ensure that both vLP1 and vLP2 were viable, the mutations in these two viruses were engineered into plasmid J1, individually. After transfection of the excised and circularized genomes into PK15 cells, and at cell passage 3, infectious vLP1 (10 of 10 subclones) and vLP2 (13 of 13 subclones) were recovered.

(iii) Mutations in the lower regions (Fig. 3C). Plasmid R3 (L3:R3 = AAG:AAA) contained 3 A's in region R3, and three

variant viruses were recovered. Both viruses vR3.1 (group I+) and vR3.3 (group I) showed evidence of TS during initiation, and vR3.1 had incorporated an illegitimate C nucleotide at position 11. Virus vR3.2 (group M+) did not show convincing evidence of TS and had incorporated a noncomplementary C nucleotide at position 10. Because this C nucleotide could be the result of reverse TS, it was not considered an illegitimate nucleotide. Due to the 2 noncomplementary nucleotides at positions 11 and 12, the palindrome of vR3.2 may be shortened by 4 nt.

Plasmid L3t (L3:R3 = TTT:CTT) contained 3 T's, plasmid L3c3 (L3:R3 = CCC:CTT) contained 3 C's, and plasmid L3c2 (L3:R3 = CC:CTT) contained 2 C's in region L3, and one variant virus was recovered from each mutant genome. All three progeny viruses (group M+) had retained the engineered mutation, and none showed evidence of TS.

Plasmid RL3 was engineered so that nucleotide complementarity was restored to the lower regions. Two variant viruses, vRL3.1 (group M+) and vRL3.2 (group M+), were recovered. Virus vRL3.1 had incorporated 3 illegitimate nucleotides, while vRL3.2 had incorporated 1; TS was not detected with either virus. Virus vRL3.1 may exhibit a palindrome shortened by 4 nt.

This collection of viruses showed that a C nucleotide was exclusively incorporated at position 10 of region R3, while positions 11 and 12 were quite flexible. Region L3 also accommodated several unrelated sequences. In addition, regions L3 and R3 did not have to be complementary in order to generate infectious viruses (vR3.2, vL3t, vL3c3, vL3c2, and vRL3.1). Position 10 qualified as a birthright position, because the absence of a C nucleotide at this position in the modified input genome did not affect protein synthesis, and exclusive incorporation of an illegitimate C nucleotide at position 10 of vRL3.1 and vRL3.2 was a reversion from an engineered mutation to a wild-type nucleotide by de novo synthesis. A C nucleotide at position 10 may also be essential for viral DNA replication.

(iv) Mutations in consecutive regions (Fig. 3D). Plasmid C1 (L1 + L2:R1 + R2 = TGCGCTG:TTTTTTT) contained 7 T's in regions R1 plus R2, and a stable stem-loop structure was unlikely to be formed. Two separate experiments (both at passage 3) were conducted, and only the wild-type virus vC1 (group I) was recovered. Virus vC1 showed evidence of TS during initiation of DNA replication.

Plasmid C2 was engineered to destabilize the palindrome by introducing 4 C's and 4 A's into the left arm of the palindrome from positions 13′ through 6′. Two experiments were conducted (both at passage 3), and identical results were obtained. Virus vC2 (group T) showed evidence of TS during termination of DNA replication and had incorporated 2 noncomplementary T nucleotides into positions 5′ and 3′ of region L1. Although the T nucleotides incorporated at positions 5′ and 3′ were illegitimate, these positions were not considered birthright positions because they have been shown to accommodate other nucleotides. In vC2, 2 mutant T nucleotides were incorporated in place of 2 wild-type nucleotides, and the wild-type nucleotides are not essential for viral DNA replication. This virus may exhibit an expanded loop and a slightly shortened palindrome.

Plasmid C3 (L1 + L2:R1 + R2 = AAAAAAA:TTTTTTT) contained 7 T's in regions R1 plus R2 and 7 A's in regions L1 plus L2. Although the mutant genome of plasmid C3 gave an abundant number of Rep-associated antigen-positive cells, similar to that of the parent viral genome of plasmid J1 (data not shown), no progeny viruses were recovered in three independent experiments.

DISCUSSION

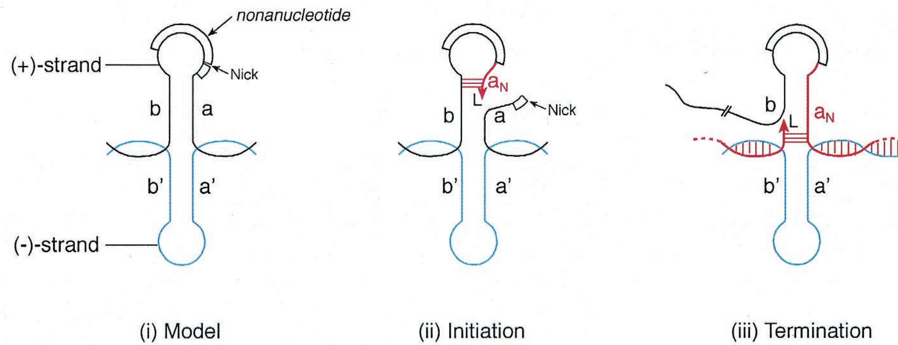
The results demonstrated that the modified PCV1 genomes excised from the 15 mutant plasmids were not impaired in protein synthesis, and 14 of them yielded a total of 26 progeny viruses upon transfection into PK15 cells. Ten viruses (groups M and M+) used the minus-strand genome DNA as a template, while 16 viruses showed evidence of TS (11 in group I or I+ and 5 in group T). Nineteen of the viruses recovered contained a new palindromic sequence, and only seven reverted to the wild-type sequence. Several general concepts pertaining to the initiation and termination of DNA replication at the Ori of PCV1 emerge from this study.

(i) The 11-nt palindrome can accommodate many nucleotide alterations without affecting Rep-associated protein synthesis or progeny virus production. Thus, this palindrome is not sequence specific with respect to the generation of infectious PCV1 progeny. However, whether the modified palindromes have any effect on the kinetics or efficiency of virus replication has yet to be determined. Depending on where the mutation is introduced into the palindrome, either arm of the palindrome can be maintained to form a new palindrome or restored to the wild-type inverted repeats. Previous work with parvovirus (2) and geminivirus (28, 34) has also demonstrated that mutilated inverted repeats engineered at the Ori can be accommodated or rapidly corrected.

(ii) Progeny viruses were recovered from all eight right-arm mutant genomes (pR1, pRL1, pR2a, pR2b, pL2b, pR3, pRL3, and pC1) in this study. Therefore, it is unlikely that Rep recognizes and binds the right arm of the palindrome in a sequence-specific manner in vivo. This finding differs from that of a previous in vitro study that reported binding of the right arm of the 11-nt palindrome by Rep (36). Most likely, Rep binds the nonanucleotide recognition site in a manner similar to that of geminivirus to initiate DNA replication.

(iii) The palindrome is very flexible and can incorporate illegitimate nucleotides and noncomplementary nucleotides at high frequencies during DNA synthesis. In this study, 14 of the 26 viruses recovered contained at least 1 noncomplementary nucleotide in the new palindrome. The original 11-nt palindrome can also be extended by recruiting additional nucleotides (e.g., vLP1 and vLP2) or shortened by incorporating noncomplementary nucleotides (e.g., vL3t, vL3c3, and vL3c2). In addition, sequence complementarity between the upper (L1:R1) or lower (L3:R3) regions is not essential for the generation of infectious viruses (e.g., vL1, vL3t, and vL3c2).

(iv) A stable palindrome is nonessential during initiation of DNA replication, as evidenced by the fact that the engineered PCV1 genomes with destabilized palindromes in plasmids pR2a, pR2b, pL2, pC1, and pC2 yielded infectious progeny viruses. The fact that progeny viruses with noncomplementary central regions were not recovered suggests that formation of a palindrome during synthesis of the left arm is preferred.



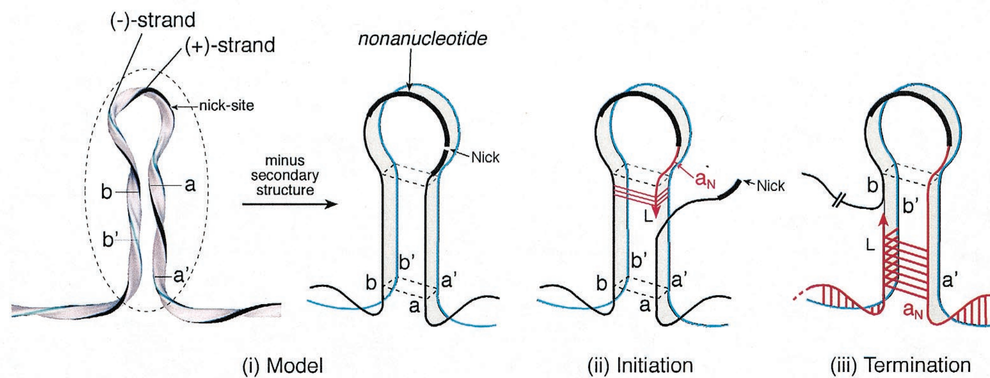


FIG. 4. (A) RCR stem-loop cruciform model. (i) PCV1 Ori after Rep binding and nicking of the nonanucleotide, and separation of the plus (+) and minus (−) strands to form a cruciform structure. (ii) Ori configuration during initiation of DNA replication. The leading-strand (L) displaces strand a and uses strand b as a template. (iii) Ori configuration during termination of DNA replication. The leading strand displaces strand b and uses the newly synthesized strand a_N as a template. (B) RCR melting-pot model. (i) PCV1 Ori after Rep binding to the nonanucleotide (prior to nicking), with the plus- and minus-strand genomes in close proximity to each other. The destabilized environment (i.e., the melting pot) is enclosed by a dashed oval. (ii) Schematic representation of the DNA templates available during initiation of DNA replication after removal of the secondary structure in the model. The leading strand displaces strand a and uses strand a′ or strand b as a template. (iii) Schematic representation of the DNA templates available during termination of DNA replication after removal of the secondary structure in the model. The leading strand displaces strand b and uses the newly synthesized strand a_N or strand b′ as a template. Black, plus-strand genome; blue, minus-strand genome; red, potential base-pairing opportunities available for the current round of DNA replication.

Potentially, the formation of a palindrome is the signal for termination of DNA replication. This finding is in contrast to reports that a flanking palindrome is essential for initiation of geminivirus DNA replication (16, 28, 32) but agrees with a report that the palindrome in wheat dwarf virus, a geminivirus, plays a role in termination but not in initiation of DNA replication (15).

It is possible to account for the progeny viruses that exhibit TS in this study by the current RCR stem-loop cruciform model (Fig. 4A) proposed for geminiviruses (10, 12, 29). Rep binds the nonanucleotide sequence, destabilizes the double-stranded DNA, and allows base pairing of the 11-nt palindrome to form a cruciform structure at the Ori. Rep nicks the nonanucleotide, initiates plus-strand DNA synthesis by dis-

placing the old DNA strand a (Fig. 4Aii), and terminates DNA replication by displacing the old DNA strand b (Fig. 4Aiii). In the presence of a very stable cruciform structure, DNA synthesis proceeds through the right-arm regions (R1, R2, and then R3) during initiation with only strand b available as a template, and DNA synthesis proceeds through the left-arm regions (L3, L2, and then L1) during termination with only the newly synthesized strand a_N available as a template. Under these conditions, two TS events occur, and only group I viruses (7 of 26 recovered viruses) (Fig. 3) will be generated. Therefore, formation of a very stable cruciform structure during initiation will preclude any opportunities to (i) correct the mutilated sequences engineered into the left arm back to the wild-type palindrome (for this study, none of the group T viruses would be generated) or (ii) adopt the engineered mutilated right-arm sequences to form new palindromes in the progeny viruses (for this study, a selected group of M and M+ viruses [vR1.2, vL1, vR3.2, vL3t, vL3c3, vL3c2, and vRL3.1] would not be generated). In a transient environment, formation of a cruciform structure during initiation would generate both group I and group I+ viruses, and formation of a cruciform structure during termination would generate group T viruses. Finally, to account for the group M and M+ viruses, the double-stranded replication intermediates must not engage in any stable cruciform conformation at any time during replication. Therefore, with the RCR stem-loop cruciform model, the replicating DNA molecules must assume a specific conformation at the appropriate time to generate a single species of progeny viruses or assume multiple conformations to generate a combination of progeny viruses with different palindromic sequences.

Here, I propose an alternative RCR "melting-pot" model (Fig. 4B) that can account for the generation of all 26 viruses obtained in this study in a more flexible environment. In this model, Rep binds the nonanucleotide and destabilizes the loop and the palindromic sequences, but there is no formation of a cruciform structure. Instead, the Rep protein-complex induces a sphere of instability—the melting-pot, which encompasses the 12-nt loop and the 11-nt palindrome (Fig. 4Bi). Within this destabilized environment, all four strands of the inverted repeats (strands a, a′, b, and b′) are in a melted state. There is a lack of hydrogen bonding between the plus- and minus-strand genomes to maintain any stable double-helix conformation, but the 4 inverted-repeat strands remain in close proximity to each other and are juxtaposed in a four-stranded tertiary structure. In fact, physical conformational changes induced by DNA replication initiator proteins have been reported in other RCR systems (13, 37). Although the exact configuration of the proposed melting pot remains to be elucidated, it is clear that both the minus-strand genome DNA and a palindromic strand DNA are available, simultaneously, as templates during PCV1 Ori replication to account for the TS events observed in the recovered viruses. The DNA strands available as templates during initiation and termination of DNA replication according to the melting-pot model (without secondary structure) are presented in Fig. 4Bii and -iii, respectively. After Rep nicks the nonanucleotide, DNA replication proceeds with the leading strand descending into the palindromic portion of the melting pot through the right arm and displacing the old strand a. Both the complementary strand a′ and the palindromic strand b are available as templates. For DNA termination, the leading strand ascends into the melting pot through the left arm to displace the old strand b. Both the complementary strand b′ and the newly synthesized palindromic strand a_N are available as templates.

Formation of the melting pot begins when the Rep protein complex binds the nonanucleotide, TAGTATT ↓ AC. The palindromic portion of the normal melting pot probably includes positions 3 through 13, since base pairing was observed between positions 3′ and 3 (vR1 variants) and between positions 13′ and 13 (vC2). With this model, the leading-strand DNA has the choice of using the minus-strand genome DNA or a palindrome strand DNA as a template at any time during initiation or termination of DNA replication. Interestingly, the influences of the left and right arms on each other differ. Regions L1 and L3 were used frequently as templates for regions R1 and R3, but not vice versa. However, region R3 did serve as a template for L3 synthesis when long consecutive mutations (7 to 12 nt) were involved (vC2, vLP1, and vLP2). As described above, the 11-nt palindrome can be lengthened or shortened as the circumstances dictate, and the upper and lower regions do not have to be complementary to yield infectious viruses.

Incorporation of illegitimate nucleotides, addition of extra bases into the palindrome, and deletion of nucleotides from the Ori sequence are signatures reminiscent of "illegitimate-recombination" errors, which occur frequently at the Ori of bacterial phages or plasmids that replicate their DNA via RCR (23, 24). With the RCR melting-pot model, several mutations assigned to group M+ and group I+ viruses (vR1.2, vR1.3, vR1.5, and vR3.2) could also be attributed to reverse TS, and these were not considered illegitimate nucleotides. However, incorporation of illegitimate nucleotides was preferred in viruses vRL1, vR3.1, vRL3.1, and vC2. It is conceivable that the melted state of the inverted repeats, which offers the growing leading strand the freedom to select between two templates or to incorporate illegitimate nucleotides, may have increased the mutation rate at the Ori of PCV1. Although the incorporation of noncomplementary nucleotides may be illegitimate with respect to base pairing, it is possible that some of the incorporation events (e.g., vRL1, vRL3.1, and vRL3.2 but not vR1.4, vR3.1, and vC2) were dictated by the Rep protein complex. As shown in viruses vRL1, vRL3.1, and vRL3.2, the mutant nucleotides engineered at positions 3 and 10 did not affect viral protein synthesis and reverted to the wild-type C nucleotides, regardless of the complementary or palindromic template; moreover, these reversions were maintained in the progeny viruses. Thus, DNA template complementarity is not necessarily the deciding factor for DNA synthesis at the birthright positions of the PCV1 Ori, and the occupants of these strategic positions may have been predetermined or may have inherited their rights to these positions from the Rep protein complex.

Because of the availability of both the complementary and palindromic strands as templates during initiation and termination of DNA replication, the RCR melting-pot model permits "terminal repeat correction" of both the left and the right arm of the PCV1 Ori to regenerate wild-type palindromes (e.g., vR2a.2 and vL2) or to form new palindromes (e.g., vR2a.1 and vLP2) in the progeny viruses. As stated above, formation of a very stable and rigid cruciform structure at the onset of DNA replication will preclude correction of any mu-

tilated sequence engineered into the left arm of the palindrome back to wild-type sequence or adoption of a mutilated right-arm sequence to form a new palindrome. In past studies, DNA replication and terminal repeat correction of the genomes of geminivirus (34), circular adenovirus (9), and circular adeno-associated virus (26) have been attributed to the RCR stem-loop cruciform model coupled with mechanisms of recombination, translocation, and panhandle gene correction (35), respectively. Potentially, the RCR melting-pot model described here for PCV1 is also applicable to other biological agents with circular genomes or circularized linear genomes.

ACKNOWLEDGMENTS

I thank S. Pohl, D. McCullough, D. Alt, K. Halloum, and O. Kohutyuk for technical assistance and M. Marti and S. Ohlendorf for manuscript preparation.

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

REFERENCES

1. **Allan, G. M., and J. A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Investig. **12:**3–14.
2. **Bohenzky, R. A., and K. I. Berns.** 1989. Interactions between the termini of adeno-associated virus DNA. J. Mol. Biol. **206:**91–100.
3. **Cheung, A. K.** 2003. Comparative analysis of the transcriptional patterns of pathogenic and non-pathogenic circoviruses. Virology **301:**41–49.
4. **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.
5. **Cheung, A. K.** Identification of the essential and non-essential transcriptional units for protein synthesis, DNA replication and infectious virus production of porcine circovirus type 1. Arch. Virol., in press.
6. **Cheung, A. K., and S. R. Bolin.** 2002. Kinetics of porcine circovirus type 2 replication. Arch. Virol. **147:**43–58.
7. **Fenaux, M., P. G. Halbur, M. Gill, T. E. Toth, and X. J. Meng.** 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.
8. **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.
9. **Graham, F. L., J. Rudy, and P. Brinkley.** 1989. Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. EMBO J. **7:**2077–2085.
10. **Gutierrez, C.** 1999. Geminivirus DNA replication. Cell. Mol. Life Sci. **56:** 313–329.
11. **Hamel, A. L., L. L. Lin, and G. P. S. Nayar.** 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. **72:**5262–5267.
12. **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.
13. **Higashitani, A., D. Greenstein, H. Hirokawa, S. Asano, and K. Horiuchi.** 1994. Multiple DNA conformational changes induced by an initiator protein precede the nicking reaction in a rolling circle replication origin. J. Mol. Biol. **237:**388–400.
14. **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.
15. **Kammann, M., H. J. Schalk, V. Matzeit, S. Schaefer, J. Schell, and B. Gronenborn.** 1991. DNA replication of wheat dwarf virus, a geminivirus, requires two *cis*-acting signals. Virology **184:**786–790.
16. **Lazarowitz, S. G., A. J. Pinder, V. D. Damsteegt, and J. S. Rogers.** 1989. Maize streak virus genes essential for systemic and symptom development. EMBO J. **8:**1023–1032.
17. **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.
18. **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:** 381–384.
19. **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.
20. **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.), Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
21. **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.
22. **Meehan, B. M., F. McNeilly, D. Todd, S. Kennedy, A. Jewhurst, J. A. Ellis, L. E. Hassard, E. G. Clark, D. M. Haines, and G. M. Allan.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. **79:**2171–2179.
23. **Michel, B., and S. D. Ehrlich.** 1986. Illegitimate recombination at the replication origin of bacteriophage M13. Proc. Natl. Acad. Sci. USA **83:**3386–3390.
24. **Michel, B., and S. D. Ehrlich.** 1986. Illegitimate recombination occurs between the replication origin of the plasmid pC194 and a progressing replication fork. EMBO J. **5:**3691–3696.
25. **Morosov, I., T. Sirinarumitr, S. Sorden, P. G. Halbur, M. K. Morgan, K.-J. Yoon, and P. S. Paul.** 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **36:**2535–2541.
26. **Musatov, S., J. Roberts, D. Pfaff, and M. Kaplitt.** 2002. A *cis*-acting element that directs circular adeno-associated virus replication and packaging. J. Virol. **76:**12792–12802.
27. **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.
28. **Orozco, B. M., and L. Hanley-Bowdoin.** 1996. A DNA structure is required for geminivirus replication origin function. J. Virol. **70:**148–158.
29. **Palmer, K. E., and E. P. Rybicki.** 1998. The molecular biology of mastreviruses. Adv. Virus Res. **50:**183–234.
30. **Phenix, K. V., J. H. Weston, I. Ypelaar, A. Lavazza, J. A. Smyth, D. Todd, G. E. Wilcox, and S. R. Raidal.** 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus *Circovirus* of the family *Circoviridae*. J. Gen. Virol. **82:**2805–2809.
31. **Pringle, C. R.** 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia. Arch. Virol. **144:**2065–2070.
32. **Revington, G. N., G. Sunter, and D. M. Bisaro.** 1989. DNA sequences essential for replication of the B genome component of tomato golden mosaic virus. Plant Cell **1:**985–992.
33. **Ritchie, B. W., F. D. Niagro, P. D. Lukert, W. L. Steffens III, and K. S. Latimer.** 1989. Characterization of a new virus from cockatoos with psittacine beak and feather disease. Virology **171:**83–88.
34. **Roberts, S., and J. Stanley.** 1994. Lethal mutations within the conserved stem-loop of African cassava mosaic virus DNA are rapidly corrected by genomic recombination. J. Gen. Virol. **75:**3203–3209.
35. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell **33:**135–143.
36. **Steinfeldt, T., T. Finsterbusch, and A. Mankertz.** 2001. Rep and Rep′ protein of porcine circovirus type 1 bind to the origin of replication in vitro. Virology **291:**152–160.
37. **Suarez-Lopez, P., E. Martinez-Salas, P. Hernandez, and C. Gutierrez.** 1995. Bent DNA in the large intergenic region of wheat dwarf geminivirus. Virology **208:**303–311.
38. **Tischer, I., H. Gelderblom, W. Vettermann, and M. A. Koch.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295:**64–66.
39. **Todd, D., F. D. Niagro, B. W. Ritchie, W. Curran, G. M. Allan, P. D. Lukert, K. S. Latimer, W. L. Steffens III, and M. S. McNulty.** 1991. Comparison of three animal viruses with circular single-stranded DNA genomes. Arch. Virol. **117:**129–135.
40. **Todd, D., J. H. Weston, D. Soike, and J. A. Smyth.** 2001. Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. Virology **286:**354–362.
41. **Woods, L. W., K. S. Latimer, B. C. Barr, F. D. Niagro, R. P. Campagnoli, R. W. Nordhausen, and A. E. Castro.** 1993. Circovirus-like infection in a pigeon. J. Vet. Diagn. Investig. **5:**609–612.