Virology 279, 429–438 (2001)
doi:10.1006/viro.2000.0730, available online at http://www.idealibrary.com on IDEAL®

Replication of Porcine Circovirus Type 1 Requires Two Proteins Encoded by the Viral *rep* Gene

Annette Mankertz[1] and Bernd Hillenbrand

P24 (Xenotransplantation), Robert Koch-Institut, Nordufer 20, Berlin D-13353, Germany

Received August 29, 2000; returned to author for revision September 22, 2000; accepted October 30, 2000

The *rep* gene of porcine circovirus type 1 (PCV1) is indispensable for replication of viral DNA. Truncation or introduction of point mutations into four conserved sequence motifs led to inactivation of Rep as replication initiator. Transcription of *rep* starts at nucleotide 767 ± 10 bp. An intron (nucleotides 1176 to 1558) is removed by splicing. This leads to synthesis of a truncated protein, which was termed Rep′ (19.2 kDa). Because of a frameshift, the last 48 amino acids of Rep′ deviate from the C-terminus of the 35.6-kDa full-length Rep protein. The presence of full-length and spliced *rep* transcripts was demonstrated in PCV1-infected cells as well as in cells transfected with a plasmid carrying the *rep* gene by real-time PCR. In contrast to other viruses replicating via a rolling circle, Rep protein alone cannot promote replication: Rep and Rep′ together comprise the functional replication initiator factor of PCV1.

Key Words: Circoviridae; differential splicing; porcine circovirus type 1; real-time PCR; viral DNA replication.

INTRODUCTION

The virus family *Circoviridae* (McNulty *et al.,* 2000) comprises small icosahedral nonenveloped viruses infecting vertebrates. *Circoviridae* are divided into two genera: *Porcine circovirus* type 1 (PCV1) and *Beak and feather disease virus* (BFDV) (Bassami *et al.,* 1998; Niagro *et al.,* 1998) are assigned to the genus *Circovirus*. Inclusion of recently isolated circoviruses *Porcine circovirus* type 2 (PCV2) (Hamel *et al.,* 1998; Meehan *et al.,* 1998; Morozov *et al.,* 1998) and *Columbid circovirus* from pigeons (CoCV) (Mankertz *et al.,* 2000) into this genus was proposed. *Chicken anemia virus* (CAV) (Kato *et al.,* 1995), the only member of the genus *Gyrovirus,* shows similarities to TT virus (TTV) (Biagini *et al.,* 2000; Okamoto *et al.,* 2000) and TTV-like mini virus (TLMV) (Takahashi *et al.,* 2000), two recently isolated human viruses.

PCV1 is an apathogenic contaminant of cell line PK-15 (Tischer *et al.,* 1974), whereas PCV2 was isolated from pigs affected by postweaning multisystemic wasting syndrome (PMWS), a new emerging disease in swine. PMWS-affected animals show lymph node enlargement, lymphocyte depletion, growth retardation, and dyspnea. Experimental infection with PCV2 showed PMWS typical lesions, clinical disease, and death (Allan *et al.,* 1999; Kennedy *et al.,* 2000), demonstrating the role of PCV2 as an etiological agent of PMWS. The genomes of PCV1 and PCV2 are similarly organized and display a homology of 76% identity. The degree of homology varies in different parts of the genome: the replication initiator protein (Rep) is highly conserved (85% amino acid identity), while open reading frame (ORF) C1 on the counterclockwise strand, which encompasses the capsid protein (Nawagitgul *et al.,* 2000), shows only 65% identical residues. The *rep* gene is essential for replication of PCV1 (Mankertz *et al.,* 1998). The origin of DNA replication of PCV1 is located on a 111-bp fragment (nucleotides 728 to 838) (Mankertz *et al.,* 1997), located between the two major ORFs Rep and C1. It is characterized by a stem–loop structure with a conserved nonamer (5′-TAGTATTAC) in the loop. The sequence CGGCAGCGG/TCAG is found twice and may represent the binding site for the replication initiator Rep.

Several similarities can be seen between the genus *Circovirus* from vertebrates and the genus *Nanovirus* and the family *Geminiviridae* from plants: (1) the genome consists of one or several small, single-stranded, circular DNA molecules; (2) because of the small genome size, the viral life cycle relies extensively on host factors; (3) the origin of replication shows a characteristic stem–loop structure with a conserved nonamer motif at the apex and a Rep binding site; and (4) the Reps belong to a family of enzymes initiating replication in the rolling-circle mode (Ilyina and Koonin, 1992; Koonin and Ilyina, 1993). These proteins display three conserved amino acid motifs and a P-loop for dNTP binding. According to phylogenetic analyses, PCV may represent a link between mammalian and plant viruses (Mankertz *et al.,* 1997; Meehan *et al.,* 1997). Recently, it was proposed that circoviruses evolved by recombination between a nanovirus and an RNA virus, e.g., a calicivirus (Gibbs and Weiller, 1999).

[1] To whom correspondence and reprint requests should be addressed. Fax: +4930-45472598. E-mail: mankertza@rki.de.

0042-6822/01 $35.00

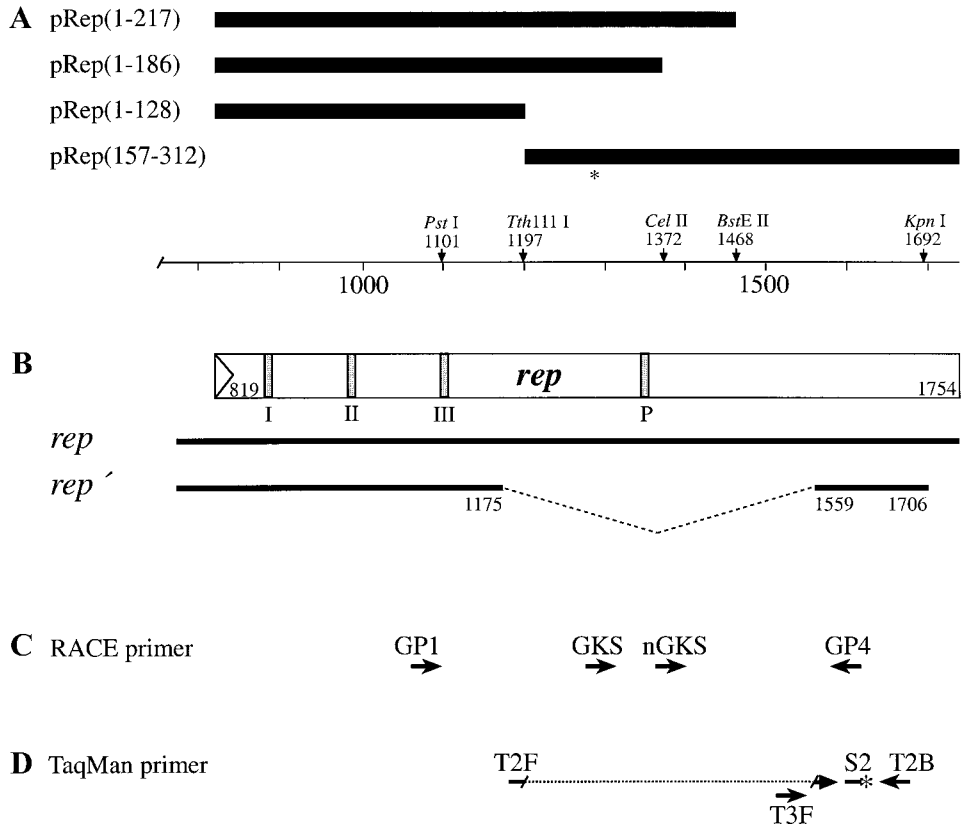

FIG. 1. Map of the *rep* gene. (A) 5′- and 3′-terminal deletion derivatives of the *rep* gene are indicated by black bars. The internal start codon at nt 1287 is marked by an asterisk. (B) Map of the *rep* gene, which shows the location of the two *rep* transcripts and positions of restriction enzymes used in this study. Position of the start and stop codons of *rep* as well as the splice donor and acceptor of the *rep′* transcript are shown. Gray boxes mark the conserved domains within the *rep* reading frame. Direction of transcription is indicated by an open triangle. (C) Positions of primers utilized for RACE analysis. (D) Positions of primers used in TaqMan PCR. Note that TaqMan primer T2F spans the junction of the two exons of the *rep* transcript, thus amplifying only the *rep′* transcript.

To understand the mechanisms of pathogenicity of PCV and to develop therapeutics and vaccines against PMWS, we have to enlarge our understanding of the molecular biology of PCV1 and PCV2, which will enable us to analyze pathogenic determinants and provide a model for the disease. Porcine viruses are also in the focus of attention with respect to xenotransplantation, since they must be regarded as potential agents of new zoonoses in human recipients. In this report, we describe transcriptional analyses of the *rep* gene of PCV1 and the capability of the encoded gene products to promote replication. It could be demonstrated for the first time that two differentially spliced products from the *rep* gene together comprise the initiator of viral DNA replication of PCV1.

RESULTS

Mutants of Rep and their activity in the replication assay

The N-terminus of Rep includes three amino acid motifs (I, II, and III) that are conserved in rolling-circle replication initiator proteins. To determine the contribution of these motifs to replication of PCV1, a set of deletion mutants of the *rep* gene was constructed (Fig. 1A). Plasmids pRep(1–217), pRep(1–186), and pRep(1–128) carry a *rep* gene shortened progressively from the 3′-terminus. Only pRep(1–217) and pRep(1–186) retain all four motifs, whereas the P-loop is deleted in pRep(1–128). Plasmid pRep(157–312) is deprived of the N-terminus with motifs I to III. The ability of truncated Rep to promote replication was tested in the *Dpn*I assay, in which each of the four plasmids was cotransfected with plasmid pOP11 containing the replication origin of PCV1 into PS cells and subsequently subjected to a *Dpn*I assay. None of the truncated Rep proteins could initiate replication at the PCV1 origin (Fig. 2, lanes 1–6). To perform a more detailed analysis, the four conserved motifs were altered by site-directed mutagenesis (motif changes are indicated in bold). In pRep-mutI, motif I FTLNN is changed to **L**TL**K**N. HLQGF was replaced by HL**P**GF in pRep-mutII. **F**CS**I** was introduced instead of the YCSK motif in pRep-mutIII. In plasmid pRep-mutP, the dNTP-binding core motif was altered from GKS to G**R**I.

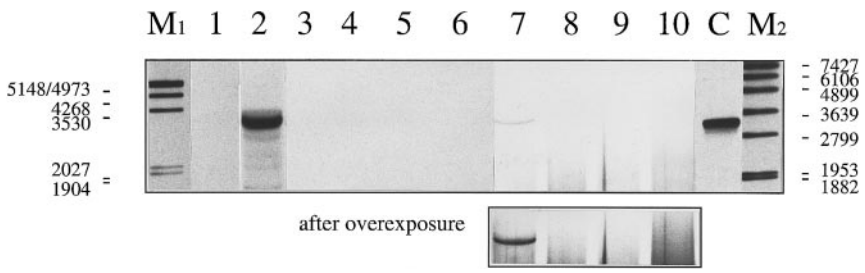

FIG. 2. Activity of Rep mutant plasmids in replication. Activity of truncated and mutagenized variants of Rep protein to initiate replication of PCV-derived plasmid pOP11 was analyzed using a *Dpn*I assay. Lane M_1, DIG-labeled size marker III (Roche Molecular Biochemicals, Mannheim, Germany); lane 1, mock-transfected PS cells (negative control); lane 2, PS cells cotransfected with pORF4A and pOP11 (positive control); lane 3, PS/pRep(1–217) and pOP11; lane 4, PS/pRep(1–186) and pOP11; lane 5, PS/pRep(1–128) and pOP11; lane 6, PS/pRep(157–312) and pOP11; lane 7, PS/pRep-mutI and pOP11; lane 8, PS/pRep-mutII and pOP11; lane 9, PS/pRep-mutIII and pOP11; lane 10, PS/pRep-mutP and pOP11; lane C, 10 pg pOP11 (2950 bp) restricted with *Bam*HI; lane M_2, DIG-labeled size marker VII (Roche). Lanes 7 to 10 of the autoradiograph are shown after overexposure.

When the replication assay was performed, mutII and mutIII were inactive (Fig. 2, lanes 8 and 9). Activity of plasmid pRep-mutI was strongly decreased (Fig. 2, lane 7). pRep-mutP was almost inactive, in that only a faint band was seen after overexposure (Fig. 2, lane 10). Combined, these results indicate that the four conserved motifs as well as the C-terminus of Rep contribute to replication of PCV1.

Nuclease S1 mapping

To obtain more information about the expression of the *rep* gene, the starting point of the *rep* transcript was determined using the nuclease S1 technique (Berk and Sharp, 1977). RNA isolated from PCV1-infected PSM cells was hybridized with 3′-labeled probe 1 (nts) and subsequently digested with nuclease S1. A signal of 290 ± 10 bp in size was detected (Fig. 3B). This signal was absent when RNA isolated from PCV1-free PS cells was used. This demonstrates the presence of a PCV1 plus-strand-encoded transcript starting at nt 766 (±10 nt). To investigate the extent of the *rep* transcript, probes 2 and 3 were used. When RNA from PSM cells was hybridized with 3′-labeled probe 2 (nts 796–1100), the full-length probe was protected against nuclease S1 digestion (Fig. 3C), suggesting extension of the transcript up to nt 1100. With 3′-labeled probe 3 (nts 728–1424), no nuclease S1-resistant signal was visible in RNA from PCV1-infected cells, a result that indicates termination or discontinuity of the *rep* transcript between nts 1101 and 1424.

RACE analysis of *rep* transcripts

A RACE analysis was performed for further characterization of the *rep* transcript. After 3′-RACE with the adapter primer AP1 and the *rep* gene-specific primer GP1 (nts 1049–1068; primer locations are shown in Fig. 1C), a PCR product of about 400 bp was detected in subsequent gel electrophoresis. The fragment was cloned and sequenced, from which 31 clones were obtained, showing a sequence corresponding to PCV1 (with the exception of some mismatches acquired during amplification). The RACE product started at nt 1049 and was discontinuous, i.e., nucleotides 1175 and 1559 were joined (Fig. 1B). The transcript proceeded to the stop codon TGA at nt 1706 and passed the arbitrarily selected point, where nt 1759 is followed by nt 1. Polyadenylation took place further downstream at positions 9 and 16. For 5′-RACE, *rep*-specific primer GP4 (nts 1605–1584) and adapter primer AP1 were used. A PCR product of approximately 500 bp was cloned into vector pCR2.1. A sequence analysis of all 19 clones obtained again revealed the transition between nts 1559 and 1175, indicating that the *rep* transcript is subjected to splicing. The 5′-ends of the amplified transcript were located between nts 786 and 817. The 3′- and 5′-RACE products were combined using the internal *Pst*I site (nt 1101) and cloned into vector pUC19. A construct expressing the Rep′ protein, under the control of the SV40 late promoter, was built by cloning a *Pst*I–*Kpn*I fragment (nts 1001–1175:1559–1692) into plasmid pORF4A. Amplification of an unspliced *rep* transcript failed. A RACE product was neither seen with *rep*-specific primer GKS (nts 1288–1309) and the adapter primer AP1, nor when these products were reamplified in a nested PCR using primers AP2 and nGKS (nts 1362–85) for the second round of amplification.

Real-time PCR detecting *rep* and *rep′* transcript

Since the full-length *rep* transcript was not detected by nuclease S1 mapping and RACE analysis, real-time PCR was applied. TaqMan probe S2 (nts 1585–1609) hybridizes to both the full-length and the spliced *rep* transcripts. Differentiation was achieved with two primer pairs: a 111-bp fragment was amplified from the spliced transcript with primer pair T2F (nts 1157–1175: 1559–1560) and T2B (nts 1650–1625), spanning the junction between the two exons. Primers T3F (nts 1537–1558) and T2B were designed to detect full-length *rep* transcript by amplification of a 114-bp fragment. Specificity of

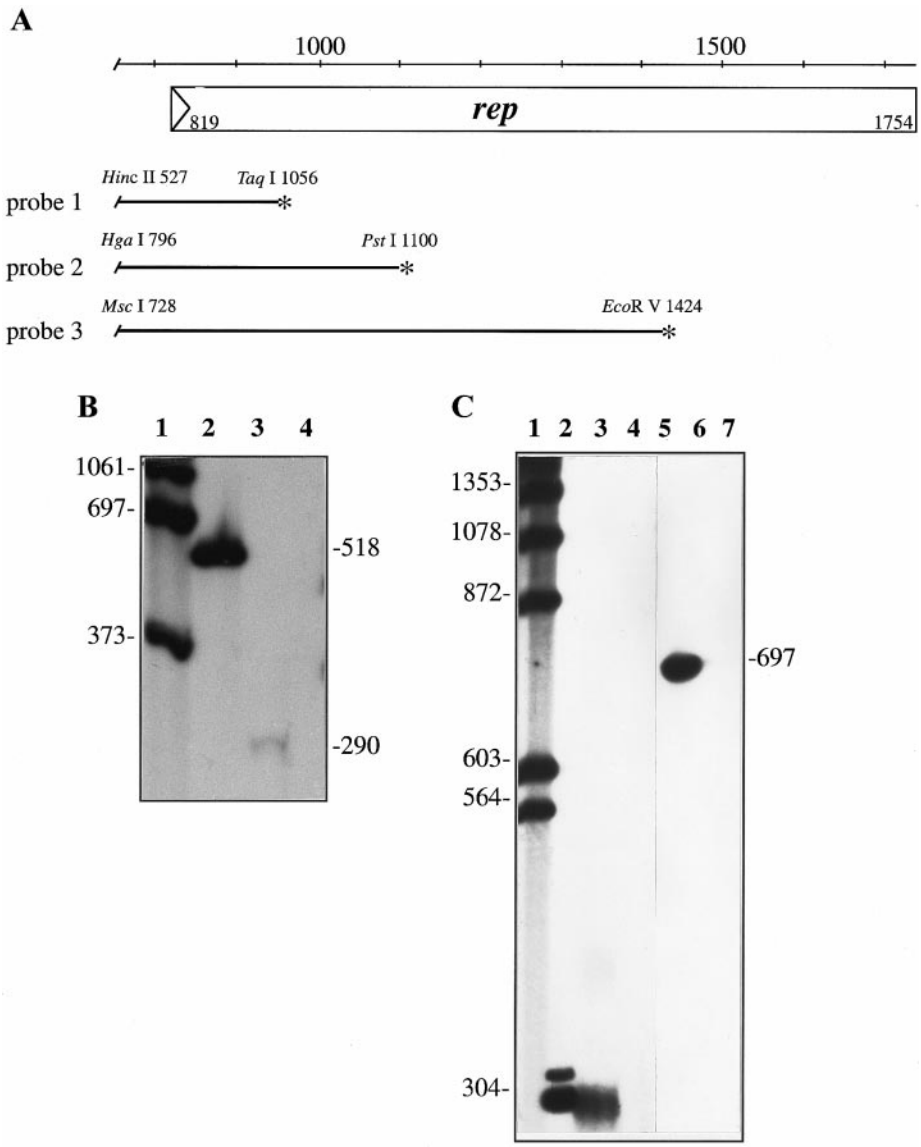

FIG. 3. Nuclease S1 mapping of *rep* gene products. (A) Map of the *rep* gene and the probes used for nuclease S1 mapping. Restriction enzymes used for generation of the nuclease S1 probes are indicated. (B) Starting point of the *rep* transcript. RNA from PSM and PS cells was hybridized with probe 1, a 531-bp *Hinc*II–*Taq*I fragment (nts 527–1056), 5′-^{32}P-labeled at the *Taq*I site. Lane 1, size marker; lane 2, probe 1 not treated with nuclease S1; lane 3, probe 1 hybridized with RNA isolated from PCV1-infected PSM cells; lane 4, probe 1 hybridized with RNA from virus-free PS cells. (C) Discontinuity of the *rep* transcript. The result of nuclease S1 mapping using probes 2 and 3 is shown (probe 2: 305-bp *Hga*I–*Pst*I fragment, nts 796–1100, 5′-^{32}P-labeled at the *Pst*I site; probe 3: 697-bp *Msc*I–*Eco*RV fragment, nts 728–1424, 5′-^{32}P-labeled at the *Eco*RV site). Lane 1, ϕx174/*Hae*III size markers; lane 2, probe 2 not treated with nuclease S1; lane 3, probe 2 hybridized with RNA from PSM cells; lane 4, probe 2 hybridized with PS RNA; lane 5, probe 3; lane 6, probe 3 hybridized with PSM RNA; lane 7, probe 3 hybridized with PS RNA.

both primer pairs was demonstrated with DNA from plasmids pORF4A and pAM4, instead of cDNA; pORF4A was amplified only with primer pair T3F/T2B, whereas primer pair T2F/T2B resulted in specific amplification of pAM4, but not of pORF4A (data not shown).

Real-time PCR with cDNA from persistently PCV1-infected PSM cells resulted in detection of the spliced transcript, if T2F/T2B//S2 was used. Amplification of the full-length *rep* transcript was seen as well (T3F/T2B//S2). Both transcripts were also detected in cDNA from PS/pORF4A cells (Fig. 4). In PS cells, serving as a negative control, neither *rep* nor *rep′* transcripts were amplified. This result indicates that both the spliced and the full-length *rep* transcripts are present in PCV1-infected cells, as well as in PS cells transfected with pORF4A.

Sequence analysis of Rep′

The product of the spliced *rep* transcript was termed Rep′. In contrast to the complete Rep protein (nts 819 to 1757) with a predicted size of 312 amino acids (aa) (35.6 kDa), the Rep′ coding sequence extends from nts 819 to

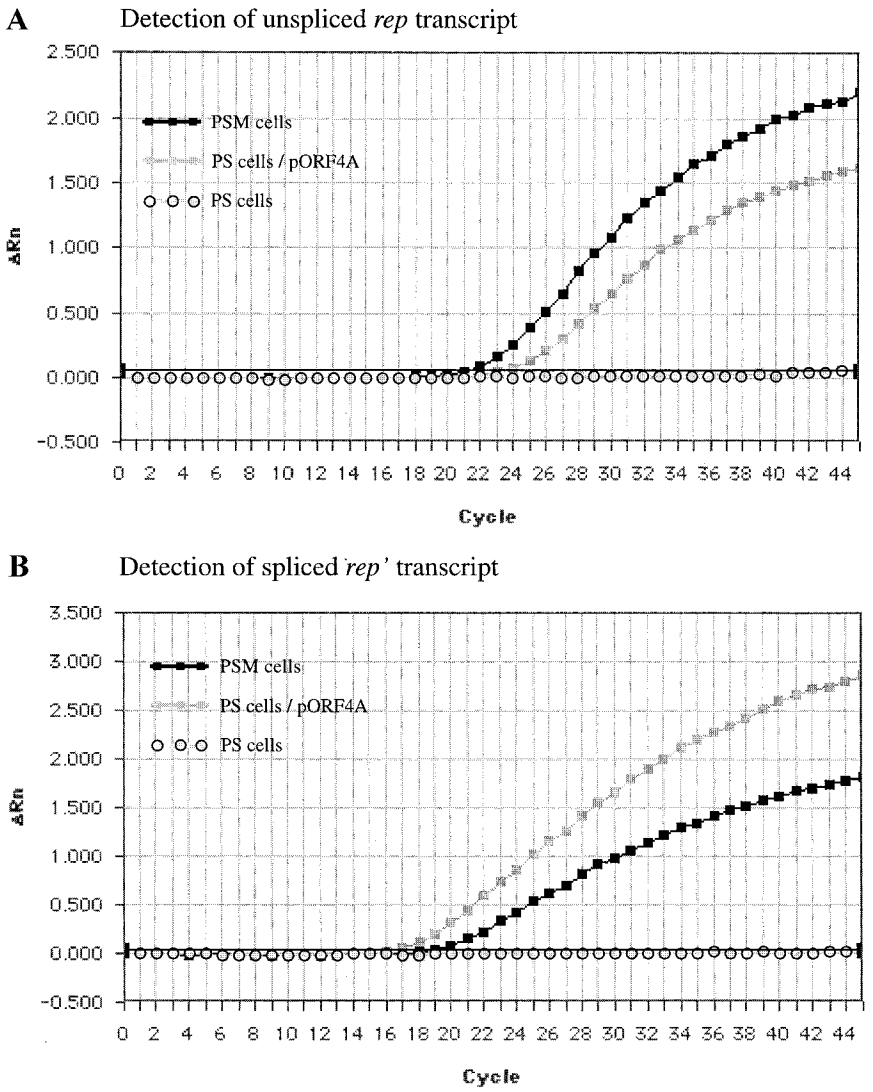

FIG. 4. Real-time PCR of differentially spliced transcripts of the *rep* gene. cDNA was synthesized from RNA isolated from PCV1-free PS cells (gray circles), PS cells transfected with the *rep*-expressing plasmid pORF4A (gray squares), and PSM cells persistently infected with PCV1 (black squares). cDNA was amplified subsequently using real-time PCR either with primer pair TF3F/TF2B and probe S2 to detect the full-length, unspliced *rep* transcript (A), or with primer pair T002F/T002B and probe S2 to amplify a product specific for the spliced *rep′* transcript (B).

1175:1559 to 1708. The Rep′ protein is reduced to 168 aa (19.2 kDa; Fig. 5A). Splicing results in expression of Rep′ in a different reading frame after A119. Therefore, the C-terminal 48 amino acids of Rep′ are different from those of the Rep sequence. Rep′ carries the three domains which are characteristic of rolling-circle-type Rep proteins, but the P-loop for dNTP binding is missing. BLASTP comparison (Altschul *et al.,* 1997) with the C-terminal part of Rep′ revealed limited homology to hypothetical protein AF0007 of *Archaeoglobus fulgidus* (GenBank accession number AE001106, E = 0.0033; E values $< 1 \times 10^{-2}$ are generally assumed to indicate relatedness). Homology (Fig. 5B) was also seen to human, murine, and feline arylsulfatase B precursors (NM_000046, E = 0.0074; D49434, E = 0.010; S48472, E = 0.014).

Activity of Rep and Rep′ in replication

Detection of the full-length transcript of *rep* may be attributed to its function as a precursor molecule for the *rep′* transcript. To test their functional significance, Rep and Rep′ had to be synthesized independently from each other. Plasmid pAM4 carries the cDNA product of *rep′*; Rep′ but not Rep is expressed. Plasmid pAM9 directs the synthesis of Rep but not Rep′, the main splice donor at nts 1176–7 and surrounding GT dinucleotides were altered to prevent splicing; however, silent mutagenesis was not possible: Three amino acid exchanges had to be introduced into Rep in plasmid pAM9. The result of the mutagenesis was examined by RT-PCR. After transfection of plasmid pAM9 into PS cells, the *rep′* transcript was no longer detected (data not shown). The ability of

A

```
pREP-mutI     1                L  K
pREP-mutII    1                                                    P
pREP-mutIII   1                                                                                    F  I
Rep PCV1      1 MPSKKSGPQPHKRWVFTLNNPSEEEKNKIRELPISLFDYFVCGEEGLEEGRTAHLQGFANFAKKQTFNKVKWYFGARCHIEKAKGTDQQNKEYCSKEGHI 100
                *          *          *          *          *          *          *          *          *          *

pREP-mutP   101                                                                                   RI
pAM9        101                     I          I I
Rep PCV1    101 LIECGAPRNQGKRSDLSTAVSTLLETGSLVTVAEQFPVTYVRNFRGLAELLKVSGKMQQRDWKTAVHVIVGPPGCGKSQWARNFAEPSDTYWKPSRNKWW 200
Rep'(pAM4)  101                     YFDYQQSGPPGMVLLNCCPSCRSSLSEDYYFAILEDCWRTINGGTRRPI                              168
                *          *          *          *          *          *          *          *          *          *

Rep PCV1    201 DGYHGEEVVVLDDFYGWLPWDDLLRLCDRYPLTVETKGGTVPFLARSILITSNQAPQEWYSSTAVPAVEALYRRITTLQFWKTAGEQSTEVPEGRFEAVD 300
                *          *          *          *          *          *          *          *          *          *

Rep PCV1    301 PPCALFPYKINY 312
                *
```

B

```
arylsulf.human/cat 107  TGLQHQIIWPCQPSC     SEDYY              VWKTISEGSPSP  388
arylsulf.rat       109  MGLQHYLIMTCQPNC     SEDYY              VWKTISEGSPSP  327
Rep' PCV1          126  SGPPGMVLLNCCPSCRSSLSEDYYFAILEDC-------WRTINGGTRRP   168
AF0007 A.fulgidus  354  SGPPNSLLIGGTPQK----VEEYMKSLLEDCKEGGMMIWPGVDGGISRD  397

                        +G-  +---+c+P+c  .  sEdYy  -ledc      -W.t-+ G+  p
```

FIG. 5. Amino acid sequence of Rep and Rep′. (A) Amino acid sequence variation of mutant Rep proteins. Point mutations were introduced in plasmids pRep-mutI to -mutP into four conserved motifs (underlined). The inactivation of splice donor sites in *rep* led to three amino acid substitutions in pAM9. The different C-termini of Rep and Rep′ (pAM4) are shown. (B) Result of a BLASTP search with the C-terminus of Rep′. Homology to a protein of *A. fulgidus* and arylsulfatase precursors of several species were found. As one example, the sequence of human arylsulfatase B is given. A consensus sequence is indicated when at least three of four residues are conserved; capitals indicate residues conserved in all sequences. Hydrophilic amino acids are shown by "+"; basic, by "."; and hydrophobic, by "-".

Rep and Rep′ to promote replication was tested. Replication of pOP11 was not observed, either when Rep′ was supplied by plasmid pAM4 or when Rep was synthesized by pAM9 (Fig. 6). This implies that neither Rep nor Rep′ alone is sufficient for replication of PCV1. In contrast, replication was seen when PS cells were cotransfected with plasmids pOP11, pAM4 (Rep′), and pAM9 (Rep), thereby demonstrating that replication of PCV1 strictly depends on two *rep* gene products, Rep and Rep′.

DISCUSSION

Four conserved amino acid motifs in the Rep proteins of circo-, nano-, and geminiviruses have been identified. Three, found in the N-terminal part of Rep, are characteristic of proteins initiating rolling-circle replication (Koonin and Ilyina, 1993). A dNTP-binding motif is located in the center of the protein. We tested truncated Rep proteins in a transient replication assay and none was able to promote replication. To analyze the conserved motifs in detail, replication activity was tested after site-directed mutagenesis: No replication was seen after mutagenesis of motif II, potentially involved in metal ion binding (Ilyina and Koonin, 1992), and motif III, corresponding to the catalytic site for DNA cleavage (Laufs *et al.,* 1995). Alteration of the first motif (FTLNN to **L**TL**K**N) resulted in strong reduction of replication activity, while mutagenesis of the dNTP-binding motif GKS to G**RI** resulted in a decrease of activity almost beyond the limits of detection. Assuming that mutagenesis did not result in a misfolding of the Rep protein, this result indicates that both the four conserved domains and the C-terminus of Rep are necessary for efficient replication of PCV1. It further strengthens the hypothesis that PCV replicates via the rolling-circle mode. Our observation is corroborated by results obtained by extensive analysis of the replication initiation proteins of geminiviruses (Eagle *et al.,* 1994; Hanson *et al.,* 1995; Orozco and Hanley-Bowdoin, 1998).

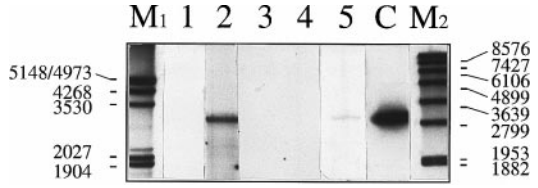

FIG. 6. Function of Rep and Rep′ in replication. The capability of Rep and Rep′ to initiate replication of PCV1-derived plasmid pOP11 is assayed. Lane M_1, DIG-labeled size marker III (Roche); lane 1, mock-transfected PS cells (negative control); lane 2, PS cells cotransfected with pORF4A and pOP11 (positive control); lane 3, PS cells/pAM4 (Rep′) and pOP11; lane 4, PS/pAM9 (Rep) and pOP11; lane 5, PS/pAM9, pAM4 (Rep′ plus Rep) and pOP11; lane C, 10 pg pOP11 (2950 bp) restricted with *Bam*HI; and lane M_2, DIG-labeled size marker VII (Roche).

Transcription of the *rep* gene was analyzed. Using nuclease S1 mapping, the transcript starting point was mapped to the position 766 (±10 bp). This result is consistent with the location of the ATG of the *rep* gene at position 819 and a TATA box at position 739, matching the usual location of −26 nt with respect to transcription start (Penotti, 1990). In RACE analysis, the starting point of the cDNA was mapped between nts 798 and 817, probably because of partial RNA degradation during cDNA synthesis. The transcript was mapped using two probes extending to nts 1100 and 1424, respectively.

Since only hybridization with the probe encompassing nt 1100 yielded a nuclease S1-protected fragment, this result implies termination or a discontinuity of the *rep* transcript between nts 1101 and 1424. RACE analysis revealed that an intron between nts 1176 and 1558 is removed from the *rep* transcript. The intron starts with GTgAGT and ends with CAG, according to the consensus sequences for splice donor and acceptor sites (capitals indicate conformity with the consensus sequence) (Mount, 1982). Since the full-length transcript was not detected, either by nuclease S1 mapping or by RACE, a real-time PCR approach was used. Both full-length and spliced transcripts were detected in PSM cells persistently infected with PCV1 as well as in pORF4A-transfected PS cells. The comparison of *rep* expression in persistently and transiently transfected cells ensures that the *rep′* transcript is not an artifact acquired by PSM cells. Failure of *rep* RNA detection by nuclease S1 mapping and RACE may be attributed to different amounts of both transcripts in the infected cells. Since nothing is yet known about the concentration and stability of the two transcripts, no prediction about Rep and Rep′ protein concentrations can be made.

The product of the spliced transcript was termed Rep′. In contrast to Rep with a predicted size of 312 aa, Rep′ is truncated (168 aa) and P-loop-deprived. Splicing results in expression of the last 48 aa in a different reading frame. BLASTX comparison showed homology of the frameshifted C-terminus of Rep′ to bacterial and vertebrate proteins. Although E values indicate probable relatedness, no conclusions with respect to the function of Rep′ could be drawn. Interestingly, the frameshift from Rep to Rep′ coincides with the locus at which recombination between nanovirus Reps and RNA-directed RNA polymerases (2C protein) of caliciviruses was previously proposed (Gibbs and Weiller, 1999). Since Rep′ is the result of a frameshift within the *rep* gene transcript, we searched for homology between Rep′ and either frameshifted 2C protein of Norwalk calicivirus (AF093797) or Rep proteins of gemini- and nanoviruses. No homologies were identified, suggesting that Rep′ was probably not acquired from these viruses, but may have evolved separately. No experimental evidence about the presence of *rep* and *rep′* transcripts in PCV2 is yet available. Nevertheless, the splice donor and acceptor sites involved in processing of the *rep* transcript of PCV1 are conserved in PCV2. Thus, two *rep* variants may also be synthesized in PCV2-infected cells.

To investigate the biological relevance of Rep and Rep′, we determined which product of the *rep* gene is involved in replication. In a transient replication assay, the ability of Rep, Rep′, and both proteins combined to promote replication was tested. Rescue of plasmid pOP11 was not observed either with Rep or with Rep′ expressed alone, but it was restored when replication was tested in the presence of Rep plus Rep′. This result indicates that both proteins are required for replication of PCV1.

Splicing of *rep* transcripts is a well-established feature. Two Rep proteins are expressed via differentially spliced mRNAs in *Maize streak virus* and *Wheat dwarf virus,* geminiviruses in the genus *Mastrevirus*. The spliced form of the transcript allows the production of Rep, a fusion protein from ORF C1 and C2 (Schalk *et al.,* 1989), whereas RepA is encoded by the C1 ORF. Rep protein is absolutely required for replication in all genera of geminiviruses (Palmer and Rybicki, 1998). The unspliced transcript directs the synthesis of the replication-associated protein RepA. RepA interacts with plant homologs of retinoblastoma protein (Rb), which are cell-cycle regulators, and is involved in transactivation of virion-sense gene expression (Gutierrez, 2000). In adeno-associated virus type 2 (AAV-2), the *rep* gene encodes four overlapping proteins which are generated by alternative promoter usage and differential splicing (Redemann *et al.,* 1989). Rep68 and Rep78 possess several biochemical activities in common, including binding to the minimal DNA replication origin, DNA helicase activity, and origin-specific replication of double-stranded linear DNA. In contrast to PCV1, expression of only one protein, either Rep68 or Rep78, is sufficient for replication of AAV-2 (Ni *et al.,* 1994).

The role of Rep and the cooperation between Rep and Rep′ remains to be elucidated. It will be interesting to learn whether both proteins take part in sequence-specific DNA recognition, cleavage/joining function, and interaction with viral and cellular proteins. It is hoped that experiments addressing these questions will enable us to understand the molecular biology of PCV1 and PCV2.

MATERIALS AND METHODS

Plasmid construction

Plasmids pAM4, pORF4A, and pRep-derivatives are based on vector pSVL (Amersham Pharmacia Biotech, Freiburg, Germany), in which genes are expressed by the SV40 late promoter. pORF4A carries the *rep* gene of PCV1 (Mankertz *et al.,* 1998). pAM4 contains the intronless ORF corresponding to the spliced *rep* gene product Rep′. It was constructed by transferring a 209-bp *Pst*I–*Kpn*I (PCV1 nts 1101–1175:1559–1691) fragment from plasmid pAM3 (cDNA of the spliced *rep* transcript cloned into pUC19) into *Kpn*I and *Pst*I restricted plasmid pORF4A. Digestion of pORF4A with *Bst*EII and *Bam*HI (Fig. 1A) and subsequent religation resulted in pRep(1–217), while *Cel*II and *Bam*HI were used for construction of pRep(1–186) and *Tth*111I and *Bam*HI for pRep(1–128), respectively. Restriction and religation of pORF4A with *Xba*I and *Tth*111I led to the deletion of the 5′-terminus of *rep* in plasmid pRep(157–312); translation is initiated using an internal ATG at nt 1287. In pOP11, a 481-bp fragment (nts 619–1100), encompassing the origin of PCV

DNA replication, was cloned into vector pUC19 (Mankertz *et al.,* 1997). All PCV1 coordinates refer to PCV1 isolated from PK-15 cells (accession number Y09921).

Cell lines and maintenance

PS is a PCV-free variant of porcine kidney cell line PK-15 (ATCC CCL33; American Type Culture Collection, Rockville, MD), whereas PSM cells are PS cells persistently infected with PCV1. Cells were grown at 37°C in DMEM with 5% fetal calf serum and 5% CO_2, and passaged weekly.

Site-directed mutagenesis

Mutagenesis of the conserved motifs in Rep was performed according to the recombinant PCR method, amplifying the entire plasmid (Du *et al.,* 1995). Oligonucleotides used for this purpose contain an overlapping region of 24 bp at their 5′-ends. By recombination between these homologous ends, the plasmids are reconstituted after transformation in *E. coli.* Introduction of restriction sites for *Hin*cII and *Afl*II (sites are underlined; mutations are in bold type) with oligonucleotides RPCR7 and RPCR8 (5′-GTGGGTGTT**A**ACC<u>CTTAA**G**</u>AATCCTTCCGAGGAGGAG, 5′-GGATT**C**TTAAGGGT**T**AACACCCACCTCTTATGGGGTTG), led to mutagenesis of F16L and N19K (the first letter and number refer to the amino acid and its position in the protein, the second letter to the modified amino acid) in plasmid pRep-mutI (Fig. 5). The insertion of a restriction site for *Sma*I with oligonucleotides RPCR9 (5′-GAACTGCTCACCT<u>CC**C**GGG</u>GTTTGGCTAATTTTGCTAAG) and RPCR10 (5′-AAAC<u>CCC**G**GG</u>AGGTGAGCAGTTCTACCCTCTTCCAAACCTTCC) resulted in Q56P in pRep-mutII. pRep-mutIII (Y93F, K96I) was constructed by insertion of an *Eco*RI site with oligonucleotides RPCR11 (5′-<u>GAAT**T**C</u>TGCAGTA**T**A**C**AAGGCCACATACTTATCGAG) and RPCR12 (5′-GGCCTT**G**T**A**TACTGCA<u>G**A**ATTC</u>TTTATTCTGCTGGTC), and pRep-mutP (K177R, S178I) with oligonucleotides RPCR13 (5′-CCCGGTTGTGGGA<u>**G**GATC</u>-<u>CAGTGGGCCCGTAATTTTGC</u>) and RPCR14 (5′-CCACT<u>G-GA**T**C**C**</u>TCCCACAACCGGGCGGGCCCACTATGACG; the newly introduced *Bam*HI site is underlined). Recombinant plasmids were screened for existence of the introduced site(s) and subsequently sequenced.

To supply only the full-length Rep protein and not Rep′, plasmid pORF4A was engineered. A 113-bp *Sac*II fragment from the *rep* gene was rebuilt using 10 overlapping oligonucleotides, in which seven GC dinucleotides surrounding the junction of exon and intron were altered. This extensive mutagenesis was necessary to avoid usage of alternative GT sequences. Phosphorylated oligonucleotides were mixed, denatured, and renatured. The resultant fragment was ligated with T4-DNA ligase, *Sac*II-restricted, and cloned in *Sac*II-restricted vector pORF4A. This resulted in three amino acid substitutions (V120I, V130I, V132I) in plasmid pAM9 (Fig. 5), verified by sequencing.

Nuclease S1 mapping

Digestion with single-strand-specific nuclease S1 was used to analyze the starting point and the extent of the *rep* transcript. RNA was prepared from PS cells (PCV-free) and PCV1-infected PSM cells using an RNA extraction kit (Pharmacia). Nuclease S1 mapping was performed as previously described (Berk and Sharp, 1977) but with the following modifications: RNA (100 μg) was coprecipitated with 50,000 Cerenkov counts of either probe 1, 2, or 3. The pellet was dissolved in 50 μl hybridization buffer. After denaturation at 85°C for 10 min, the nucleic acids were hybridized for 3 h at 50°C. A 500-μl aliquot of cold nuclease S1 buffer was added and the samples were digested with 100 U nuclease S1 (BRL/Life Technologies, Gaithersburg, MD) at 37°C for 30 min. The reaction was stopped with 0.5 M ammonium acetate, 10 mM EDTA (final concentration). After precipitation, the DNA fragments protected from nuclease S1 digestion were resuspended in 80% formamide, 20% TBE buffer, 0.025% bromphenol blue, and 0.025% xylene cyanol, and resolved on 6% polyacrylamide, 8 M urea gels. Probe 1, a 530-bp *Hin*cII–*Taq*I fragment (nts 527–1056), in which the lower strand was 5′-^{32}P-labeled at the *Taq*I site, was used for starting point determination. Probes 2 and 3 were used to detect transcript sequences (probe 2: 305-bp *Hga*I–*Pst*I fragment, nts 796–1100, 5′-^{32}P-labeled at the *Pst*I site; probe 3: 697-bp *Msc*I–*Eco*RV fragment, nts 728–1424, 5′-^{32}P-labeled at the *Eco*RV site).

RACE analysis

RACE analysis was performed using the Marathon kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions, with RNA isolated from PCV1-infected porcine kidney cells (PSM). cDNA was synthesized using polyadenylated RNA isolated from PSM cells and a $polyT_{30}N_2$-based cDNA synthesis primer carrying restriction sites for *Eco*RI and *Not*I. After second-strand synthesis, adapters carrying restriction sites for *Not*I and *Xma*I were ligated to the double-stranded DNA fragment. Amplification of RACE products was performed with Takara LA PCR kit (Takara Biomedicals, Japan), using a manual hot start and the following PCR parameters: 94°C, 1 min; 35 cycles of 94°C, 30 s; 60°C, 30 s; 68°C, 4 min. 3′-RACE products were amplified with primers GP1 (nts 1049–68; 5′-CTGCCACATCGAGAAAGCGA) and AP1 (5′-CCATCCTAATACGACTCACTATAGGGC, Clontech); 5′-RACE was performed using primer GP4 (nts 1605–1594; 5′-TACAGTCACCAAAGACCCCGTC) and AP1. The first round of nested PCR to detect the full-length *rep* transcript was performed with primer GKS (nts 1288–1309; 5′-TGCAGCAGCGTGATTGGAAGAC) and AP1, while second-round synthesis used primer nGKS (nts 1362–85;

5′-CGTAATTTTGCTGAGCCTAGCGAC) and AP2 (5′-ACTCACTATAGGGCTCGAGCGGC, Clontech). Amplified fragments were cloned into pCR2.1 using the TOPO T/A cloning kit and were then sequenced.

Real-time PCR (TaqMan PCR)

To detect full-length and spliced *rep* transcripts, a real-time fluorescent-probe PCR was used. Unlike a standard PCR reaction, which is characterized by the amount of PCR product after a fixed number of cycles, fluorescence-based PCR reaction is characterized by C_t, the point when amplification is first detected. C_t is defined as the fractional cycle number at which fluorescence generated by separation of reporter and quencher of the probe passes a fixed threshold above baseline. TaqMan probes for detection of *rep* gene transcripts were designed using the Primer Express™ Oligo Design software (Perkin–Elmer/Applied Biosystems, Foster City, CA). The presence of *rep* and *rep′* transcript was analyzed in persistently PCV1-infected PSM cells and in PS cells transfected with plasmid pORF4A. Virus-free PS cells served as a negative control. For this purpose, RNA from PSM cells, PS/pORF4A cells (4 days after transfection), and PS cells (2×10^6 each) was isolated with RNAzol (WAK Chemie, Bad Soden, Germany), following the manufacturer's instructions. After treatment with 50 U DNase I (Pharmacia) for 45 min at room temperature (RT) and subsequent phenol extraction, cDNA was synthesized according to the Qiagen omniscript protocol (Qiagen, Chatsworth, CA) using 1/5 of the isolated RNA. Products were treated with 2 U RNase H for 30 min at RT, and 3-μl aliquots of the RNA preparations were incubated with 1 μl RNase A (1 mg/ml) for 1 h at 37°C. These samples were used as template for subsequent TaqMan PCR, in which no signal was detected (data not shown). This result demonstrated complete removal of DNA traces from RNA preparations. TaqMan PCR was performed in a 50-μl volume with 1/20 of a cDNA preparation. The final concentration was 250 nM of each primer, 100 nM of TaqMan probe S2, 5 mM $MgCl_2$, 1× PCR buffer II (Perkin–Elmer/Applied Biosystems; 0.9 μM ROX, 200 μM dNTPs, 1.25 U AmpliTaq Gold polymerase). Cycling parameters were 95°C, 12 min and 45 cycles of 95°C, 20 s; 68°C, 20 s; 72°C, 20 s, in an ABI prism 7700 thermocycler using optical tubes. TaqMan probe S2 (nts 1585–1609; 6FAM-CCCAGGAATGGTACTCCTCAACTGC-XTPh) was used with two different primer sets to differentiate between the spliced and the unspliced *rep* transcript. Detection of a 114-bp fragment of the unspliced transcript was performed with T3F (nts 1537–1558, 5′-CTGTTCCTTTTTTGGCTCGCAG) and T2B (nts 1650–1625, 5′-AAGTAGTAATCCTCCGATAGAGAGCT), whereas 111 bp were amplified from the spliced *rep* transcript T2F (nts 1157–1175:1559–1560, 5′-CAGCGACCTGTCTACTGCTTA) and T2B.

Specificity of the primers to differentiate between the two *rep* transcripts was tested using plasmid DNA. Amplification was performed as described above with 1.2×10^7 copies of plasmid pORF4A plus 4.7×10^7 copies of plasmid pAM4.

*Dpn*I assay

*Dpn*I assay was performed as described previously (Mankertz *et al.,* 1997) with restriction of the reisolated DNA with *Dpn*I plus *Bam*HI. Since *Bam*HI activity is not influenced by Dam-methylation, all plasmids are linearized via the *Bam*HI site in the vector moiety of the plasmids tested, regardless of whether they were replicated.

Nucleotide accession numbers

The sequence of Rep′ was submitted to the GenBank database under accession number AF276303. The nucleotide accession number of PCV1 isolated from PSM cells is Y09921.

ACKNOWLEDGMENTS

We thank Dr. Heinz Ellerbrok for his advice concerning TaqMan PCR, and gratefully acknowledge the technical assistance of Ms. Roswitha Lorenz.

REFERENCES

Allan, G. M., Kennedy, S., McNeilly, F., Foster, J. C., Ellis, J. A., Krakowka, S. J., Meehan, B. M., and Adair, B. M. (1999). Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. *J. Comp. Pathol.* **121,** 1–11.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402.

Bassami, M. R., Berryman, D., Wilcox, G. E., and Raidal, S. R. (1998). Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anemia virus. *Virology* **249,** 453–459.

Berk, A. J., and Sharp, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12,** 721–732.

Biagini, P., Attoui, H., Gallian, P., Touinssi, M., Cantaloube, J. F., de Micco, P., and de Lamballerie, X. (2000). Complete sequences of two highly divergent European isolates of TT virus. *Biochem. Biophys. Res. Commun.* **271,** 837–841.

Du, Z., Regier, D. A., and Desrosiers, R. C. (1995). Improved recombinant PCR mutagenesis procedure that uses alkaline-denatured plasmid template. *BioTechniques* **18,** 376–378.

Eagle, P. A., Orozco, B. M., and Hanley-Bowdoin, L. (1994). A DNA sequence required for geminivirus replication also mediates transcriptional regulation. *Plant Cell* **6,** 1157–1170.

Gibbs, M. J., and Weiller, G. F. (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.

Gutierrez, C. (2000). DNA replication, and cell cycle in plants: Learning from geminiviruses. *EMBO J.* **19,** 792–799.

Hamel, A. L., Lin, L. L., and Nayar, G. P. (1998). Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J. Virol.* **72,** 5262–5267.

Hanson, S. F., Hoogstraten, R. A., Ahlquist, P., Gilbertson, R. L., Russell, D. R., and Maxwell, D. P. (1995). Mutational analysis of a putative NTP-binding domain in the replication-associated protein (AC1) of bean golden mosaic geminivirus. *Virology* **211,** 1–9.

Ilyina, T. V., and Koonin, E. V. (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.

Kato, A., Fujino, M., Nakamura, T., Ishihama, A., and Otaki, Y. (1995). Gene organization of chicken anemia virus. *Virology* **209,** 480–488.

Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., and Allan, G. M. (2000). Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. *J. Comp. Pathol.* **122,** 9–24.

Koonin, E. V., and Ilyina, T. V. (1993). Computer-assisted dissection of rolling circle DNA replication. *Biosystems* **30,** 241–268.

Laufs, J., Schumacher, S., Geisler, N., Jupin, I., and Gronenborn, B. (1995). Identification of the nicking tyrosine of geminivirus Rep protein. *FEBS Lett.* **377,** 258–262.

Mankertz, A., Hattermann, K., Ehlers, B., and Soike, D. (2000). Cloning, and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. *Arch. Virol.,* in press.

Mankertz, A., Mankertz, J., Wolf, K., and Buhk, H. J. (1998). Identification of a protein essential for replication of porcine circovirus. *J. Gen. Virol.* **79,** 381–384.

Mankertz, A., Persson, F., Mankertz, J., Blaess, G., and Buhk, H. J. (1997). Mapping and characterization of the origin of DNA replication of porcine circovirus. *J. Virol.* **71,** 2562–2566.

McNulty, M., Dale, J., Lukert, P., Mankertz, A., Randles, J., and Todd, D. (2000). Circoviridae. *In* "Seventh Report of the International Committee on Taxonomy of Viruses" (M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, E. B. C. Bishop, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner, Eds.). Academic Press, San Diego.

Meehan, B. M., Creelan, J. L., McNulty, M. S., and Todd, D. (1997). Sequence of porcine circovirus DNA: Affinities with plant circoviruses. *J. Gen. Virol.* **78,** 221–227.

Meehan, B. M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V. A., Ellis, J. A., Hassard, L. E., Clark, E. G., Haines, D. M., and Allan, G. M. (1998). Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J. Gen. Virol.* **79,** 2171–2179.

Morozov, I., Sirinarumitr, T., Sorden, S. D., Halbur, P. G., Morgan, M. K., Yoon, K. J., and Paul, P. S. (1998). Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J. Clin. Microbiol.* **36,** 2535–2541.

Mount, S. M. (1982). A catalogue of splice junction sequences. *Nucleic Acids Res.* **10,** 459–472.

Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D., and Paul, P. S. (2000). Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J. Gen. Virol.* **81,** 2281–2287.

Ni, T. H., Zhou, X., McCarty, D. M., Zolotukhin, I., and Muzyczka, N. (1994). In vitro replication of adeno-associated virus DNA. *J. Virol.* **68,** 1128–1138.

Niagro, F. D., Forsthoefel, A. N., Lawther, R. P., Kamalanathan, L., Ritchie, B. W., Latimer, K. S., and Lukert, P. D. (1998). Beak and feather disease virus and porcine circovirus genomes: Intermediates between the geminiviruses and plant circoviruses. *Arch. Virol.* **143,** 1723–1744.

Okamoto, H., Ukita, M., Nishizawa, T., Kishimoto, J., Hoshi, Y., Mizuo, H., Tanaka, T., Miyakawa, Y., and Mayumi, M. (2000). Circular double-stranded forms of TT virus DNA in the liver. *J. Virol.* **74,** 5161–5167.

Orozco, B. M., and Hanley-Bowdoin, L. (1998). Conserved sequence and structural motifs contribute to the DNA binding and cleavage activities of a geminivirus replication protein. *J. Biol. Chem.* **273,** 24448–24456.

Schalk, H. J., Matzeit, V., Schiller, B., Schell, J., and Gronenborn, B. (1989). Wheat dwarf virus, a geminivirus of graminaceous plants needs splicing for replication. *EMBO J.* **8,** 359–364.

Takahashi, K., Iwasa, Y., Hijakata, M., and Mishiro, S. (2000). Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus, and chicken anemia virus. *Arch. Virol.* **145,** 979–993.

Tischer, I., Rasch, R., and Tochtermann, G. (1974). Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl. Bakteriol.* [*A*] **226,** 153–167.