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Sequence of porcine circovirus DNA: affinities with plant circoviruses

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The complete nucleotide sequence (1759 nt) of the ssDNA genome of porcine circovirus (PCV) was determined from a cloned dsDNA replicative form isolated from PCV-infected cells. Sequence analysis detected no significant nucleic acid or protein similarity with another animal circovirus, chicken anaemia virus (CAV) but, surprisingly, the highest protein similarity was obtained between the product of the largest predicted PCV ORF (ORF1; encoding a potential protein of 35·7 kDa) and a putative protein encoded by the plant circovirus banana bunchy top virus (BBTV). High protein similarity was also detected with the other plant circoviruses subterranean clover stunt virus (SCSV) and coconut foliar decay virus (CFDV). This region of protein identity corresponds with the putative plant circovirus replication-associated protein (Rep). The presence of a nonanucleotide sequence at the apex of a potential-stem loop structure, identical to that found in the plant circoviruses CFDV and SCSV and similar (one mismatch) to that found in the plant circovirus BBTV and in the geminiviruses, suggests that rolling-circle replication may operate during PCV DNA replication. These findings show that PCV is unique in that it bridges the gap between animal and plant circoviruses. The taxonomic relationship of PCV with other members of the *Circoviridae* is discussed.

Introduction

Since the first isolation of porcine circovirus (PCV), a small isometric virus 17 nm in diameter with a monopartite circular ssDNA genome (Tischer *et al.*, 1982), similar viruses have been identified in a range of animal (Ritchie *et al.*, 1989; Todd *et al.*, 1990) and plant species (Rohde *et al.*, 1990; Chu *et al.*, 1990; Harding *et al.*, 1993). Their unrelatedness to other ssDNA viruses has led to their classification in a new virus family, the *Circoviridae* (Lukert *et al.*, 1995). Although limited protein similarity exists between plant circoviruses (Harding *et al.*, 1993; Burns *et al.*, 1995) no significant physico-chemical, nucleotide sequence or protein similarity has yet been demonstrated within the animal circoviruses (Allan *et al.*, 1994*b*; Todd *et al.*, 1991*b*, 1993) or between the animal and plant circoviruses. Although tentatively placed within the *Circoviridae* (Lukert *et al.*, 1995) the only characteristics that the multipartite plant circoviruses, banana bunchy top virus (BBTV), subterranean clover stunt virus (SCSV) and coconut foliar decay virus (CFDV), share with the monopartite animal circoviruses, PCV, chicken anaemia virus (CAV) and psittacine beak and feather disease virus (BFDV), are that all have small isometric virions and a circular ssDNA genome. This has led to the proposal that they should either be classified separately or that the plant circoviruses should be placed in a separate genus within the *Circoviridae* (Burns *et al.*, 1995) or more closely associated with the geminiviruses (Boevink *et al.*, 1995).

Previous work in this laboratory on a comparative study of animal circoviruses (Todd *et al.*, 1991*b*) showed that PCV resembled BFDV in both particle and genome size but differed in these characteristics from CAV, and recommended that it would be inadvisable to place CAV in the same family as either or both of the other viruses. Subsequent physico-chemical characterization (Allan *et al.*, 1994*b*) has further described differences between CAV and PCV. Investigation of the genomic organization of both PCV (Mankertz *et al.*, 1990, 1993; Todd *et al.*, 1993) and CAV (Meehan *et al.*, 1992; Todd *et al.*, 1993) highlighted differing genomic organization and gene expression strategies and revealed very little nucleic acid

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or protein similarity between the two viruses (Todd *et al*., 1993). Although a poster presentation describing the cloning and sequencing of the PCV genome has been abstracted (Buhk *et al*., 1985) no published information or database submission pertaining to the nucleotide sequence of PCV is available.

In this paper we report the nucleotide sequence of a cloned PCV replicative form (RF) shown to produce infectious virus following transfection, identify a sequence motif thought to play a role in virus DNA replication and discuss the sequence of the largest potential protein in relation to the taxonomic classification of circoviruses.

Methods

■ **Cell and virus growth.** A continuous pig kidney (PK-15) cell line persistently infected with PCV was obtained from the Central Veterinary Laboratory, Weybridge, UK. Virus growth was monitored by immunofluorescence as described by Todd *et al*. (1991*b*) using both a rabbit antiserum to purified PCV (kindly provided by I. Tischer, Robert Koch Institute, Berlin, Germany) and monoclonal antibodies to PCV (Allan *et al*., 1994*a*). A PK-15 cell line shown to be free of PCV infection was used as a control. For the purposes of virus and viral DNA purification the yield was improved by treating the cells with glucosamine (Tischer *et al*., 1987). PCV virions were partially purified by differential centrifugation and equilibrium sucrose density centrifugation as described by Todd *et al*. (1991*b*).

■ **Cloning of PCV-specific DNA.** PCV RF DNA was recovered from persistently infected PK-15 cells (10×75 cm^2 tissue culture flasks) harvested 72–76 h after glucosamine treatment essentially as described for the cloning of the circovirus CAV RF (Todd *et al*., 1991*a*) employing a modification of the Hirt procedure (Hirt, 1967) as described by Molitor *et al*. (1984). Briefly, DNA (1–5 µg) extracted by the Hirt procedure was treated with S1 nuclease (Amersham) in accordance with the manufacturer's instructions, digested with *Pst*I and fractionated by electrophoresis in 1% (w/v) agarose gels in the presence of ethidium bromide as described previously (Todd *et al*., 1990). A gel slice corresponding to the predicted position of the linearized 1·7 kbp dsDNA RF of PCV (Tischer *et al*., 1982; Tischer & Buhk, 1988) was excised and the DNAs were recovered by electroelution (Sambrook *et al*., 1989). PCV-specific RF DNA which had been digested with *Pst*I was ligated as a single fragment into the vector pGEM-1 (Promega) that had been cut with *Pst*I and alkaline phosphatase treated. *Escherichia coli* ED8767 transformed by recombinant plasmids were identified by the colony hybridization technique using a randomly primed radiolabelled DNA probe (Feinberg & Vogelstein, 1984) generated using purified virus genomic ssDNA as described by Todd *et al*. (1991*b*).

■ **Transfection capabilities of cloned PCV DNA.** Transfection was carried out essentially as described by Todd *et al*. (1991*a*) using the DEAE-dextran method of Sompayrac & Danna (1981). Briefly, 0·1–0·5 µg of *Pst*I digested PCV cloned DNA in pGEM-1 was added to 350 µl RPMI containing 200 µg/ml DEAE-dextran, applied to a semi-confluent monolayer of PK-15 cells and incubated for 4–6 h before washing the cell surface with fresh medium. Virus infection was detected 48 h post-transfection and following passage by indirect immunofluorescence using acetone-fixed cells dried on multispot slides as described previously (Todd *et al*., 1991*b*). For the generation of virus pools the transfected monolayer was incubated with fresh medium for 72 h prior to subculturing.

■ **Nucleic acid sequence determination.** The nucleic acid sequence of a selected cloned PCV RF (pPCV1) was determined by dideoxy chemistry using a dye terminator ready reaction cycle sequencing kit utilizing AmpliTaq DNA polymerase FS (Perkin Elmer) on an Applied Biosystems ABI 373A automated DNA sequencer in accordance with the manufacturer's instructions. Initial sequencing reactions were carried out using the T7 and SP6 primers of pGEM-1. The remaining sequence data was generated using a primer walking strategy. The nucleotide sequence of a PCV clone (pPCV1) was determined in each direction in at least duplicate. Nucleic acid sequence data were assembled and edited using a combination of the Applied Biosystems 373A DNA Sequencer Data Analysis Program and Sequence Navigator software.

■ **Genomic organization of PCV and relationship to other circoviruses.** The nucleotide sequence of the PCV genome was analysed using the MacDNASIS sequence analysis software (Hitachi). Putative PCV ORFs were further analysed using the Blast algorithm (Altschul *et al*., 1990) using the non-redundant nucleic acid sequence database at the National Centre for Biotechnology Information (NCBI, USA).

Results and Discussion

Transfection capabilities of cloned PCV RF

Colony hybridization using a PCV-specific probe successfully identified a number of PCV clones for further characterization. One PCV-specific clone (pPCV1) was shown by restriction endonuclease digestion with *Pst*I to contain an insert of approximately 1·7 kbp (Fig. 1). The 1·7 kbp cloned PCV fragment, when released by *Pst*I digestion, was shown by immunofluorescence to represent a functional PCV RF capable of producing infectious virus following transfection (Fig. 2). These results are in agreement with previous studies involving the selection and cloning of circovirus genomes in this laboratory (Meehan *et al*., 1992; Todd *et al*., 1995).

Nucleotide sequence analysis of the PCV genome

The nucleotide sequence of the 1759 bp cloned PCV RF (pPCV1) contained as a *Pst*I insert in pGEM-1 is given in Fig. 3. By analogy with plant circoviruses and geminiviruses the strand presented is thought to represent the encapsidated virus strand ssDNA. Potential polyadenylation addition signals (AATAAA; Nevins, 1983) are present on both strands of the PCV RF, in the putative virus strand (two possible sites at nt positions 314–319, 973–978) and in the complementary strand (a cluster of three potential sites encompassing positions 730–745, and a single site at nt position 571–576). This arrangement of polyadenylation signals is consistent with the observation that both strands of the PCV genome are transcribed and encode proteins (Mankertz & Buhk, 1990; Mankertz *et al*., 1993). Unpublished Northern blot analysis of PCV-infected cells (Mankertz & Buhk, 1990; Mankertz *et al*., 1993) has indicated that, in contrast to CAV (Kato *et al*., 1995; Noteborn *et al*., 1991; Phenix *et al*., 1993, 1994), transcription takes place on both DNA strands to generate three distinct

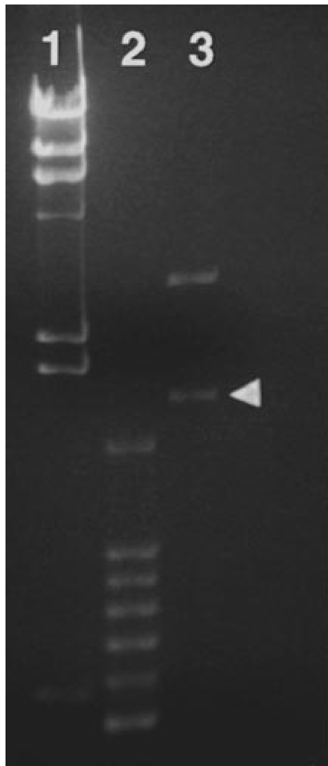

Fig. 1

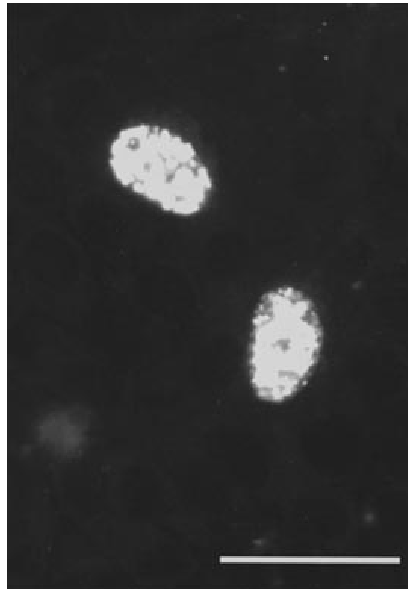

Fig. 2

Fig. 1. Gel showing the cloned 1·7 kbp PCV RF present in pPCV1. Lane 1, size markers (*Hin*dIII digest of λ DNA); lane 2, 100 bp ladder (Promega); lane 3, the 1·7 kbp fragment released from the plasmid pPCV1 following *Pst*I digestion.

Fig. 2. Light micrograph showing typical immunofluoresence in PCV-infected PK-15 cells following transfection and passage. Bar represents 10 μm.

PCV-specific RNA transcripts (Mankertz *et al*., 1993; Todd *et al*., 1993). The PCV genome does not, however, have a distinctive non-coding region and shares little nucleotide sequence homology with CAV (Meehan *et al*., 1992; Todd *et al*., 1991*b*, 1993). A sequence with the potential to form a distinctive stem-loop structure is present in the putative virus strand of the PCV genome (nt position 1739–13) and encompasses the nonanucleotide TAGTATTAC (nt position 1753–2). The position of this stem-loop structure in relation to the major ORFs present within the PCV RF is shown in Fig. 3. The convention used for numbering the geminivirus sequences has been adopted, with the 'A' residue immediately downstream of the putative nick site in the nonanucleotide given as nucleotide position 1 (Tan *et al*., 1995). By analogy with geminiviruses (Lazarowitz, 1987; Laufs *et al*., 1995; Stanley, 1995), this stem-loop structure and nonanucleotide motif may represent a putative origin of rolling-circle replication (RCR; Gilbert & Dressler, 1968) in PCV DNA. This hypothesis is further supported by the close similarity demonstrated with the nonanucleotide motifs present in the plant circoviruses BBTV (Harding *et al*., 1993), CFDV (Rohde *et al*., 1995) and SCSV (Boevink *et al*., 1995), which are also thought to replicate using RCR. The position of the PCV nonanucleotide motif on the potential stem-loop structure and its relationship to the putative nonanucleotide motif present in other circoviruses and geminiviruses are given in Fig. 4(*a*, *b*). Although RCR has been investigated in the replication of CAV DNA, no direct evidence for RCR was observed (Todd *et al*., 1996) and the highly conserved nonanucleotide sequence at the apex of a potential stem-loop sequence, typified by that found in the plant circoviruses and geminiviruses, was not present in the genome of CAV. This observation highlights the greater similarity between PCV and the plant circoviruses than between PCV and CAV.

Genomic organization of the PCV genome

The orientation and positions of the seven ORFs with the potential to encode proteins > 5 kDa encoded on either strand of the PCV RF are given in Fig. 5. The molecular mass values of each of the seven predicted proteins in descending order are as follows: ORF1, 35·7 kDa (nt 47–983); ORF2, 27·8 kDa (nt 1723–1024); ORF3, 23·2 kDa (nt 658–40); ORF4, 13·3 kDa (nt 552–207); ORF5, 9·8 kDa (nt 1163–1448); ORF6, 6·7 kDa (nt 1518–1332); ORF7, 6·0 kDa (nt 1670–79). The largest PCV ORF (ORF1) is the only ORF identified in the PCV RF large enough to encode the PCV major structural protein of 36 kDa (Tischer *et al*., 1982) and accordingly ORF1 may represent the major PCV structural protein gene. However, since splicing of a major RNA species thought to encode the PCV structural protein has been observed (Mankertz & Buhk, 1990), conclusive identification of the ORF encoding the PCV major structural protein awaits further transcript mapping and protein sequence investigation.

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1     ACCAGCGCACTTCGGCAGCGGCAGCACCTCGGCAGCGTCAGTGAAAATGCCAAGCAAGAA
                                                     M  P  S  K  K
61    AAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAATCCTTCCGAGGAGGA
       S  G  P  Q  P  H  K  R  W  V  F  T  L  N  N  P  S  E  E  E
121   GAAAAACAAAATACGGGAGCTTCCAATCTCCCTTTTTGATTATTTTGTTTGCGGAGAGGA
       K  N  K  I  R  E  L  P  I  S  L  F  D  Y  F  V  C  G  E  E
181   AGGTTTGGAAGAGGGTAGAACTCCTCACCTCCAGGGGTTTGCGAATTTTGCTAAGAAGCA
       G  L  E  E  G  R  T  P  H  L  Q  G  F  A  N  F  A  K  K  Q
241   GACTTTTAACAAGGTGAAGTGGTATTTTGGTGCCCGCTGCCACATCGAGAAAGCGAAAGG
       T  F  N  K  V  K  W  Y  F  G  A  R  C  H  I  E  K  A  K  G
301   AACCGACCAGCAGAATAAAGAATACTGCAGTAAAGAAGGCCACATACTTATCGAGTGTGG
       T  D  Q  Q  N  K  E  Y  C  S  K  E  G  H  I  L  I  E  C  G
361   AGCTCCGCGGAACCAGGGGAAGCGCAGCGACCTGTCTACTGCTGTGAGTACCCTTTTGGA
       A  P  R  N  Q  G  K  R  S  D  L  S  T  A  V  S  T  L  L  E
421   GACGGGGTCTTTGGTGACTGTAGCCGAGCAGTTCCCTGTAACGTATGTGAGAAATTTCCG
       T  G  S  L  V  T  V  A  E  Q  F  P  V  T  Y  V  R  N  F  R
481   CGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAGATGCAGCAGCGTGATTGGAAGACAGC
       G  L  A  E  L  L  K  V  S  G  K  M  Q  Q  R  D  W  K  T  A
541   TGTACACGTCATAGTGGGCCCGCCCGGTTGTGGGAAGAGCCAGTGGGCCCGTAATTTTGC
       V  H  V  I  V  G  P  P  G  C  G  K  S  Q  W  A  R  N  F  A
601   TGAGCCTAGGGACACCTACTGGAAGCCTAGTAGAAATAAGTGGTGGGATGGATATCATGG
       E  P  R  D  T  Y  W  K  P  S  R  N  K  W  W  D  G  Y  H  G
661   AGAAGAAGTTGTTGTTTTGGATGATTTTTATGGCTGGTTACCTTGGGATGATCTACTGAG
       E  E  V  V  V  L  D  D  F  Y  G  W  L  P  W  D  D  L  L  R
721   ACTGTGTGACCGGTATCCATTGACTGTAGAGACTAAAGGGGGTACTGTTCCTTTTTTGGC
       L  C  D  R  Y  P  L  T  V  E  T  K  G  G  T  V  P  F  L  A
781   CCGCAGTATTTTGATTACCAGCAATCAGGCCCCCCAGGAATGGTACTCCTCAACTGCTGT
       R  S  I  L  I  T  S  N  Q  A  P  Q  E  W  Y  S  S  T  A  V
841   CCCAGCTGTAGAAGCTCTCTATCGGAGGATTACTACTTTGCAATTTTGGAAGACTGCTGG
       P  A  V  E  A  L  Y  R  R  I  T  T  L  Q  F  W  K  T  A  G
901   AGAACAATCCACGGAGGTACCCGAAGGCCGATTTGAAGCAGTGGACCCACCCTGTGCCCT
       E  Q  S  T  E  V  P  E  G  R  F  E  A  V  D  P  P  C  A  L
961   TTTCCCATATAAAATAAATTACTGAGTCTTTTTTGTTATCACATCGTAATGGTTTTTATT
       F  P  Y  K  I  N  Y  *
1021  TTTATTTATTTAGAGGGTCTTTTAGGATAAATTCTCTGAATTGTACATAAATAGTCAGCC
1081  TTACCACATAATTTTGGGCTGTGGCTGCATTTTGGAGCGCATAGCCGAGGCCTGTGTGCT
1141  CGACATTGGTGTGGGTATTTAAATGGAGCCACAGCTGGTTTCTTTTATTATTTGGGTGGA
1201  ACCAATCAATTGTTTGGTCCAGCTCAGGTTTGGGGGTGAAGTACCTGGAGTGGTAGGTAA
1261  AGGGCTGCCTTATGGTGTGGCGGGAGGAGTAGTTAATATAGGGGTCATAGGCCAAGTTGG
1321  TGGAGGGGGTTACAAAGTTGGCATCCAAGATAACAACAGTGGACCCAACACCTCTTTGAT
1381  TAGAGGTGATGGGGTCTCTGGGGTAAAATTCATATTTAGCCTTTCTAATACGGTAGTATT
1441  GGAAAGGTAGGGGTAGGGGGTTGGTGCCGCCTGAGGGGGGGAGGAACTGGCCGATGTTGA
1501  ATTTGAGGTAGTTAACATTCCAAGATGGCTGCGAGTATCCTCCTTTTATGGTGAGTACAA
1561  ATTCTGTAGAAAGGCGGGAATTGAAGATACCCGTCTTTCGGCGCCATCTGTAACGGTTTC
1621  TGAAGGCGGGGTGTGCCAAATATGGTCTTCTCCGGAGGATGTTTCCAAGATGGCTGCGGG
1681  GGCGGGTCCTTCTTCTGCGGTAACGCCTCCTTGGCCACGTCATCCTATAAAAGTGAAAGA
1741  AGTGCGCTGCTGTAGTATT
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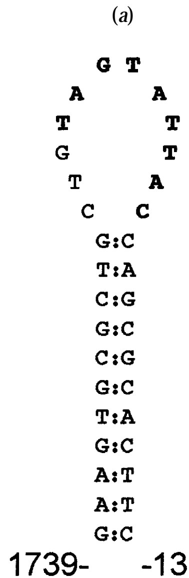
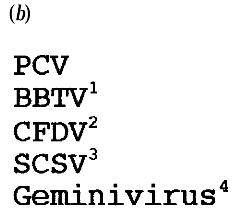
Fig. 3. Nucleotide sequence of the PCV genome. The amino acid sequence of the largest ORF encoding the putative PCV Rep is given. By analogy with plant geminiviruses the ‘A’ residue immediately downstream of the putative nick site in the nonanucleotide motif is given as nucleotide position 1. The potential stem-loop sequence encompassing the putative nonanucleotide sequence is underlined and the potential polyadenylation signals on the putative encapsidated strand are double underlined.

(*a*)



(*b*)



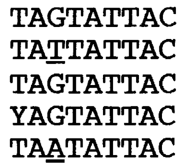


Fig. 4

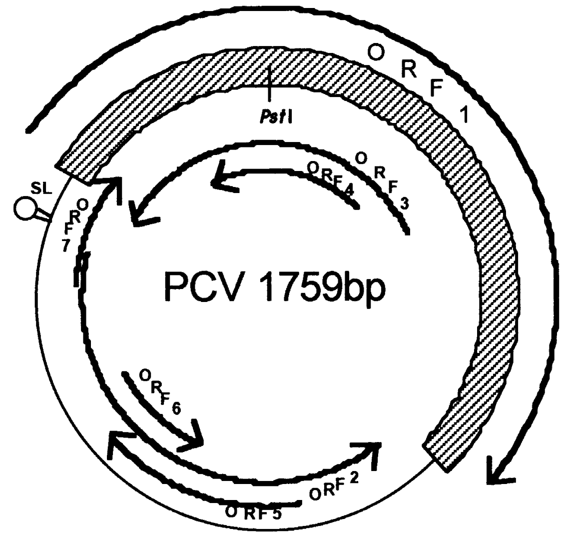


Fig. 5

Fig. 4. (*a*) Stem-loop sequence present in the putative encapsidated strand of the PCV genome; the nonanucleotide motif is in bold. (*b*) Alignment showing the relationship between the PCV nonanucleotide motif and that present in both plant circoviruses and geminiviruses. Bases that differ from the PCV nonanucleotide motif are underlined. 1, Banana bunchy top virus (Harding *et al*., 1993); 2, coconut foliar decay virus (Rohde *et al*., 1995); 3, subterranean clover stunt virus (Boevink *et al*., 1995); 4, geminivirus consensus motif (Lazarowitz, 1987).

Fig. 5. Schematic representation of the PCV genome showing the largest open reading frame ORF1 (hatched) encoding the putative PCV Rep in relation to the *Pst*I site used in the cloning of the PCV RF. The stem-loop region (SL) encompassing the nonanucleotide motif is also shown. The positions of ORFs with the potential to encode proteins > 5 kDa on both DNA strands of the PCV RF are shown by arrows.

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PCV    MPSKKSGPQPHKRWVFTLNNPSEEEKNKIRELPISLFDYFVCGEEGLEEGRTPHLQGFANF
BBTV1           P   +W FTLN  S EE+N +  L       Y V G+E        HLQG+ +
BBTV2              +W FTLN  S  E+             +Y V G+E        HLQG+ +
SCSV3             +R+ FTLN  +E E+     L                       HLQGF +F
BBTV4              +W FTLN  S  E+             +Y V G+E        HLQG+ +
CFDV5             +RW FTLN  +EEE N +R +        Y + G+E        HLQGF +

PCV    AKKQTFNKVKWYFGARCHIEKAKGTDQQNKEYCSKEGHILIECGAPRNQGKRSDLSTAVST
BBTV1   K+      +K  +G+R H E A+GTD++N +YC
BBTV2   K       +K  +G+R H E AKG+D+QN+ YC
SCSV3    K      +K  FG R H E A+G+D QN++YC
BBTV4   K       +K  + +R H E+A+G+D+ N +YC
CFDV5      +    +K   G R H+E  +G+D+QN++YC

       LLETGSLVTVAEQFPVTYVRNFRGLAELLKVSGKMQQRDWKTAVHVIVGPPGCGKSQWARN
       FAEPRDTYWKPSRNKWWDGYHGEEVVVLDDFYGWLPWDDLLRLCDRYPLTVETKGGTVPFL
       ARSILITSNQAPQEWYSSTAVPAVEALYRRITTLQFWKTAGEQSTEVPEGRFEAVDPCALF
       PYKINY*
```

Fig. 6. Protein sequence homology at the N-terminal region of the putative PCV Rep corresponding to the high-scoring pairs detected by BlastX analysis of the nucleotide sequence of the PCV genome. Homologous amino acids are indicated by a '+'. 1, BBTV V1 gene product (accession no. L32166), $P(N)$ $1{\cdot}0\times10^{-6}$; 2, BBTV V2 gene product (accession no. L32167), $P(N)$ $7{\cdot}0\times10^{-6}$; 3, SCSV gene product (accession no. U16713), $P(N)$ $2{\cdot}4\times10^{-5}$; 4, BBTV DNA III ORF VI and ORF CI (accession no. U12586), $P(N)$ $6{\cdot}5\times10^{-5}$; 5, CFDV ORF1 gene product (accession no. A46353, $P(N)$ $1{\cdot}3\times10^{-4}$. [P(N) = smallest sum probability.]

Analysis of protein sequences predicted from the PCV genome

Analysis of the protein sequence encoded by ORF1 revealed some surprising relationships. When the entire PCV genome was analysed for protein similarity with the non-redundant protein database (NCBI, USA) using the Blast algorithm (Altschul *et al*., 1990) the highest levels of homology detected were between the PCV ORF1 and the putative replication-associated proteins (Reps) of the plant circovirus BBTV with high levels of homology also being detected with the other plant circoviruses CFDV and SCSV. The regions of homology between the putative product of PCV ORF1 and the putative plant circovirus Reps are shown in Fig. 6. The putative plant circovirus Reps have been shown to contain dNTP binding motifs (Harding *et al*., 1993; Surin *et al*., 1993) and are thought to play roles in virus DNA replication. The homology between the PCV ORF1 product and the putative plant circovirus Rep genes highlights PCV as being unique in that it bridges the gap between animal and plant circoviruses, possibly indicating a common evolutionary pathway. On the basis of the striking homology between PCV and the plant circoviruses BBTV, CFDV and SCSV, PCV may eventually be assigned to a new genus containing the plant circoviruses. The determination of the nucleotide sequence, genomic organization and sequence relationships of other animal circoviruses such as BFDV will be required before the definitive taxonomic relationship between the animal and plant circoviruses can be established.

The additional similarities between PCV and both plant circoviruses and geminiviruses deserve further comment. The presence of a nonanucleotide sequence at the apex of a potential stem-loop structure, identical to that found in the plant circovirus CFDV (Rohde *et al*., 1995) and homologous to that found in the geminivirus nonanucleotide motif (Laufs *et al*., 1995; Lazarowitz, 1987, 1992; Stanley, 1995) suggests that RCR may operate during circovirus DNA replication. This, in conjunction with the homology to putative plant circovirus Reps (Fig. 6), highlights PCV as the smallest and possibly the most ancient mammalian replicon yet encountered and warrants further investigation into both its replication and gene function. The further characterization of the putative PCV Rep in relation to plant circovirus and geminivirus Reps and to bacterial phage (Baas & Jansz, 1988) and plasmid proteins (Gros *et al*., 1987), may further aid the identification and characterization of conserved sequence motifs (Ilyina & Koonin, 1992) necessary for RCR.

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