Identification of a protein essential for replication of porcine circovirus

Annette Mankertz, Joachim Mankertz, Katarina Wolf and Hans-Joerg Buhk

Fachbereich Gentechnik und Genetik, Robert Koch-Institut, Nordufer 20, 13353 Berlin, Germany

The largest open reading frame of porcine circovirus (ORF 4) encodes a protein of 312 amino acids. The predicted gene product of ORF 4 shows similarities to Rep proteins of other plant circoviruses and geminiviruses. Three motifs have been identified that are characteristic for proteins involved in rolling circle replication and the consensus sequence for a putative dNTP-binding box (GKS) has been found. In this paper, experimental evidence is presented which indicates that ORF 4 encodes the replication protein of porcine circovirus. After cloning of the ORF 4 gene product, it was supplied in trans in a transient replication assay. The ORF 4 gene product promoted the replication of plasmid pOP11, which carries the origin of DNA replication of porcine circovirus. Since pOP11 itself is unable to replicate in virus-free porcine kidney cells, the ORF 4 gene product must be essential for replication of porcine circovirus.

Porcine circovirus (PCV) has been isolated from porcine PK-15 cell line (ATCC CCL31; Tischer et al., 1974). It is a small isometric nonenveloped virus (17 nm in diameter) with a single-stranded (ss) covalently closed DNA genome of 1759 nucleotides (nt) (Tischer et al., 1982). Circoviruses sharing similar features with respect to virion morphology and genome structure have been observed to persist in a wide range of species. Chicken anaemia virus (CAV; Todd et al., 1990) and psittacine beak and feather disease virus (PBFDV; Ritchie et al., 1989) have been isolated from birds, and subterranean clover stunt virus (SCSV; Boevink et al., 1995), banana bunchy top virus (BBTV; Harding et al., 1993) and coconut foliar decay virus (CFDV; Rohde et al., 1990) have been isolated from plants. In contrast to the animal circoviruses, BBTV and SCSV are characterized by a multipartite DNA genome. Another group of plant-invading viruses with small circular ssDNA genomes is the family Geminiviridae. They are named after their twin capsids and are divided into three subgroups with respect to their mono- or bipartite genome and insect vector, respectively (Lazarowitz, 1992). Within the sequences of circoviruses and geminiviruses that are available now, one common feature is found: with the exception of CAV, they all contain a hairpin with a conserved nonamer motif (TAXT-AYTMS) within their replication origin. In CAV, the nonamer is found, but not the hairpin. The hairpin and nonamer motif

![Fig. 1. Nucleotide and amino acid sequence of the PCV Rep protein. The nucleotide sequence with annotated amino acid sequence of the PCV nt 728–143 fragment encoding the ORF 4 gene product is given. The three motifs that are characteristic for proteins involved in rolling circle replication are marked by boxes; the consensus sequence for a putative dNTP binding motif is double underlined.](image-url)
were found to be the start point of viral plus-strand synthesis during rolling circle replication (Stanley et al., 1995; Heyraud et al., 1993). A hairpin with the similar sequence TAGTATTAC was also found in the PCV origin of replication, which was mapped recently (Mankertz et al., 1997). This sequence resembles also the gene A cleavage sites of mapped recently (Mankertz et al., 1997). A hairpin was also found in the PCV origin of replication, which was mapped recently (Mankertz et al., 1997). This sequence resembles also the gene A cleavage sites of X174 and Gram-positive bacterial plasmid families. It is an interesting task to elucidate the replication mechanism of PCV and the interaction of replication factor(s) with the viral and prokaryotic replicons using rolling circle replication. 

The nucleotide sequence of PCV (Meehan et al., 1997) gives rise to six potential open reading frames (ORF) larger than 285 bp (Mankertz et al., 1997), but only a single peptide with a molecular mass of 36000 Da has been detected so far (Tischer et al., 1982). The largest predicted ORF (ORF 4) is thought to encode the replication protein (Rep protein) of PCV. The predicted product of ORF 4 has 312 amino acids (Fig. 1). Four functional motifs have been identified by sequence comparison: a GKS box, which may be involved in nucleotide binding, and three motifs that are characteristic for proteins involved in rolling circle replication (Koonin & Ilyina, 1993). When the putative Rep protein of PCV was compared with the replication-associated proteins of plant circoviruses and geminiviruses, it was found that ORF 4 shows a striking similarity to Rep proteins of BBTV, CFDV and SCSV (Mankertz et al., 1997; Meehan et al., 1997).

PCV seems to represent a link between animal and plant circoviruses and can be related to a large number of different viral and prokaryotic replicons using rolling circle replication. It is an interesting task to elucidate the replication mechanism of PCV and the interaction of replication factor(s) with the viral origin. In this note, we present functional evidence that the Rep protein of PCV is encoded by ORF 4.

To test whether the Rep protein of PCV is encoded by the largest open reading frame, ORF 4 was cloned into the largest open reading frame, ORF 4 was cloned into the expression plasmid pSVL (Pharmacia). For this purpose, plasmid pSK140 (Hpal-linearized PCV genome in pUC8) was restricted with MscI and Stul. The resultant 1175 bp fragment corresponds to nt 728–143 of the PCV genome (EMBL accession number Y09921) and carries ORF 4. It was isolated and subsequently ligated to SmaI-restricted vector pSVL, thereby creating plasmid pORF4A (Fig. 2). In this construct, ORF 4 is placed under the control of the constitutive late promoter of SV40; thus the putative Rep protein of PCV should complement for replication factor(s) needed in trans. Besides pORF4A, a second plasmid was used in this approach, carrying the replication factor(s) needed in cis: pOP11 has a 263 bp insert (nt 722–984) of the PCV genome that includes the origin of viral DNA replication (Fig. 2). pOP11 can replicate in PCV-infected porcine kidney cells (PSM cells) but not in PCV-free porcine kidney cells (PS cells; Mankertz et al., 1997). A 7.5 μg aliquot of each plasmid DNA was mixed in 0.4 cm cuvettes with 5 x 10^5 porcine kidney cells (line PS) and pulsed with 250 μF and 250 V (Bio-Rad electroporation unit). Cells were cultured for 48 h with one change of medium. Plasmid DNA was reisolated subsequently by a standard lysis protocol (Hirt, 1967) and analysed for replication by a Dpml assay (DeLange & McFadden, 1986). A 5 μg aliquot of the extracted DNA was restricted with BamHI and Dpml and subsequently separated on a 1% agarose gel. The DNA was blotted onto a positively charged nylon membrane, UV cross-linked and prehybridized. A PCR-generated digoxigenin (DIG)-labelled 432 bp fragment from the bla gene of vector pUC18 was used as a probe in hybridization. Blots were visualized with CSPD [disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo(3.3.1.1^3,7^)-phenylphosphate]. Plasmid DNA used for transfection of PS cells was isolated from a dam + E. coli strain. Thus, the N6 position of the adenine residues in the sequence GATC is methylated. This methylation makes the DNA susceptible to cleavage with restriction enzyme Dpml. When Dam-methylated DNA is replicated in eukaryotic cells, it undergoes a change in methylation status: the newly synthesized DNA is not Dam-

![Fig. 2. Plasmids used in PS cell transfection. Sketch of pORF4A (a) and pOP11 (b). Plasmid pOP11 carries the origin of replication of PCV. pORF4A expresses the ORF 4 gene product under the control of the constitutive SV40 late promoter. The inserted fragments are indicated by white boxes, the numbers correspond to the PCV genome position. The open reading frame ORF 4 is indicated by a shaded box; the numbers correspond to the location of the start and stop codon.](image-url)
methylated and is therefore resistant to DpnI restriction. However, it can be linearized by any single cutting restriction enzyme not affected by Dam-methylation. For this purpose, we used restriction enzyme BamHI.

When pOP11 and pORF4A were cotransfected into PS cells, a DpnI-resistant DNA band could be seen on the blot (Fig. 3, lane 8). The signal has a size of approximately 3000 bp and comigrates with pOP11 DNA not subjected to the DpnI assay, but linearized directly with BamHI (lane 10). When PS cells were transfected with plasmid pOP11 alone, no DpnI-resistant band was observed on the blot, indicating that pOP11 is unable to replicate in PS cells in the absence of plasmid pORF4A (lane 3). This result shows clearly that pOP11 can replicate in uninfected porcine kidney cells when the gene product of ORF 4 is supplied in trans by plasmid pORF4A. Thus, the product of ORF 4 is essential for PCV DNA replication. As a control, pOP11 was cotransfected with the expression vector pSVL (used for construction of pORF4A) into PS cells. No signal could be detected (lane 6), therefore the possibility that a factor expressed by plasmid pSVL could be responsible for replication of pOP11 is ruled out. Furthermore, expression plasmid pORF4B (see below) was combined with pUC18, which does not carry a PCV origin. pUC18 was not able to replicate in the presence of pORF4B (lane 7). Therefore, replication of pOP11 via a backup mechanism using an alternative origin present within the pUC18 moiety of the construct is excluded. This corroborates previous results (Mankertz et al., 1997), when pUC18 did not replicate after transfection into virus-infected PSM cells. Mock-transfected PS cells did not show any replicational activity (lane 2).

When PS cells were transfected with pORF4A, a faint band approximately 6000 bp in size appeared (lane 4). This signal comigrates with plasmid pORF4A linearized with BamHI which was not subjected to the DpnI assay (lane 11). The appearance of this signal is probably due to the fact that the origin of replication of PCV (nt 728–838 of the PCV genome) partially overlaps with ORF 4. The insert used for construction of pORF4A encompasses the complete origin; therefore, this plasmid should promote its own replication. Since the origin is in close proximity to the SV40 late promoter, replication of pORF4A seems to be severely impaired (in comparison to the replication of plasmid pOP11) by transcription directed from this strong viral promoter. When the plasmids pOP11 and pORF4A are cotransfected into PS cells, the replication of pORF4A is even more repressed, since almost no signal can be seen on the blot (lane 8). We suppose that plasmid pOP11 will titrate the ORF 4 gene product, thereby down-regulating replication of pORF4A. To verify our hypothesis, plasmid pORF4 was reconstructed using a shorter Hgal–Stul fragment derived from PCV (nt 796–143). The resultant plasmid, pORF4B, does not contain the intact PCV origin of replication. When pORF4B was tested in the DpnI assay, autonomous replication was no longer observed (lane 5), while pORF4B still promoted the replication of pOP11 (lane 9).

The role of putative replication enzyme has been assigned to the ORF 4 product based on its homology to the Rep proteins of plant circoviruses and geminiviruses. Now that the function of ORF 4 as a replicase has been experimentally assured, it will be necessary to study the interaction between the Rep protein of PCV and the origin of viral replication.

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References

A. Mankertz and others


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