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Virology 343 (2005) 36 – 46
VIROLOGY
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Analysis of the subcellular localization of the proteins Rep, Rep′ and Cap of porcine circovirus type 1

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Received 12 April 2005; returned to author for revision 26 May 2005; accepted 9 August 2005
Available online 15 September 2005

Abstract

Porcine circovirus type 1 (PCV1) encodes two major ORFs. The *cap* gene comprises the major structural protein of PCV, the *rep* gene specifies Rep and Rep′, which are both essential for initiating the replication of the viral DNA. Rep corresponds to the full-length protein, whereas Rep′ is a truncated splice product that is frame-shifted in its C-terminal sequence. In this study, the cellular localization of PCV1-encoded proteins was investigated by immune fluorescence techniques using antibodies against Rep, Rep′ and Cap and by expression of viral proteins fused to green and red fluorescence proteins. Rep and Rep′ protein co-localized in the nucleus of infected cells as well as in cells transfected with plasmids expressing Rep and Rep′ fused to fluorescence proteins, but no signal was seen in the nucleoli. Rep and Rep′ carry three potential nuclear localization signals in their identical N-termini, and the contribution of these motifs to nuclear import was experimentally dissected. In contrast to the *rep* gene products, the localization of the Cap protein varied. While the Cap protein was restricted to the nucleoli in plasmid-transfected cells and was also localized in the nucleoli at an early stage of PCV1 infection, it was seen in the nucleoplasm and the cytoplasm later in infection, suggesting that a shuttling between distinct cellular compartments occurs.
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Keywords: Circoviridae; *Porcine circovirus* type 1 (PCV1); Nuclear localization signal (NLS); Rep protein; Cap protein

Introduction

Porcine circoviruses (PCV) are members of the family *Circoviridae*. This family comprises small icosahedral non-enveloped viruses with a small circular single-stranded (ss) DNA genome. Two strains of porcine circoviruses, PCV type 1 (PCV1) and PCV type 2 (PCV2), have been characterized. PCV1 was first identified in 1974 as a persistent infection of the porcine kidney cell line PK-15 (Tischer et al., 1974). PCV1 does not induce a cytopathogenic effect in cell culture or disease symptoms in experimentally infected animals (Tischer et al., 1986). In contrast, PCV2 is the etiological agent of a new emerging disease in swine, the so-called postweaning multisystemic wasting syndrome (PMWS) (Ellis et al., 1999). The genome of PCV1 consists of 1759 nucleotides (nt), while that of PCV2 is 9 nt larger; the genomes of these two viruses display a homology of approximately 70% (Hamel et al., 1998). Both PCV variants display two major open reading frames (ORF), of which the smaller ORF *cap* is located on the minus strand. The Cap protein (234 amino acids, aa) is the only structural component of the viral capsid and the dominant immunological agent of PCV (Nawagitgul et al., 2002). The capsid shows $T = 1$ symmetry rules and is built from 12 pentamer units containing 60 subunits (Crowther et al., 2003). The N-terminal part of Cap displays nuclear and nucleolar localization signals, mutation of which alters the localization of Cap (Cheung and Bolin, 2002; Liu et al., 2001). The larger ORF *rep* is located on the viral plus strand and encodes the replication proteins Rep and Rep′. In contrast to other viruses, characterized by small ssDNA genomes, e.g. *Adeno-associated virus* type 2 (AAV2) and members of the genus *Mastrevirus* of the family *Geminiviridae*, the replication of PCV cannot be promoted by a single Rep protein. The full-length Rep protein (312 aa) and the isoform Rep′ (168 aa) are both indispensable for viral DNA

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doi:10.1016/j.virol.2005.08.021

replication (Mankertz and Hillenbrand, 2001; Mankertz et al., 2003). The sequence of the N-terminal 119 aa of Rep and Rep′ is identical. This region contains three conserved amino acid motifs typical for proteins mediating replication via a rolling-circle mechanism (Koonin and Ilyina, 1992). Additionally, the full-length Rep protein comprises a P-loop for dNTP-binding, which is removed from the spliced Rep′ protein. Introduction of point mutations into these four conserved sequence motifs inhibits PCV1 replication (Mankertz and Hillenbrand, 2001). Both replication proteins bind to double-stranded (ds) DNA fragments comprising the origin of replication in vitro (Steinfeldt et al., 2001).

Due to the small genome size and the limited coding capacity, it is generally acknowledged that the viral life cycle of PCV must rely extensively on host factors. The synthesis of viral DNA is performed during the S-phase (Tischer et al., 1987), and replication of viral DNA is thought to occur in the nucleus. In this report, the localization of the PCV1-encoded proteins Rep, Rep′ and Cap with and without concurrent infection was investigated. We analyzed whether PCV-encoded proteins mutually influence their localization and accumulate in distinct compartments during the infection cycle. Additionally, peptide regions were identified which mediate nuclear translocation of Rep-EGFP fusion proteins.

Results

Generation of antisera specific for either Rep or Rep′

To generate Rep- and Rep′-specific antisera, the non-homologous C-terminal sequences of Rep and Rep′ were expressed as GST fusion proteins in *E. coli* from plasmid pGEX:Rep120–312 and pGEX:Rep′120–168 (Fig. 1). Fusion proteins were purified from the insoluble fraction by SDS-PAGE and used for immunization of rabbits. The resulting antisera α-Rep120–312 and α-Rep′120–168 detected the full-length His-tagged Rep protein (40 kDa) as well as the Rep′ protein (26 kDa) expressed in *E. coli* (Fig. 2). Cross-reaction of Rep- and Rep′-specific antisera between the two protein isoforms was not observed in Western blot or immune fluorescence assays (Figs. 2, 3Ai and j).

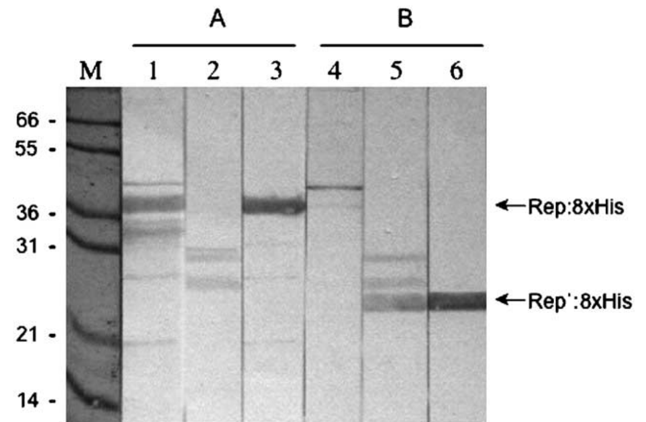

Fig. 2. Immunological staining of Rep:8xHis and Rep′:8xHis. Immunoblot of Rep:8xHis and Rep′:8xHis with α-Rep120–312 and α-Rep′120–168 antisera. (A) Crude extracts from *E. coli* expressing Rep:8xHis were resolved by SDS-PAGE and analyzed by immunoblotting. Blots were incubated with α-Rep120–312 antiserum (lane 1), α-Rep′120–168 antiserum (lane 2) and monoclonal α-HisTag antibody (lane 3). (B) Rep′:8xHis was probed with α-Rep120–312 antiserum (lane 4), α-Rep′120–168 antiserum (lane 5) and monoclonal α-HisTag antibody (lane 6). Mark 12™ Wide Range Protein Standard (lane M; molecular weight in kDa) was stained with amido black solution.

Subcellular localization of Rep, Rep′ and Cap in PS cells

To investigate the subcellular localization of Rep, Rep′ and Cap, PS cells were infected with PCV1, and the presence of immunoreactive proteins was detected 48 h post-infection (pi) with α-Rep120–312, α-Rep′120–168 and α-Cap104–233 (Figs. 3Aa–c). The staining with α-Rep120–312 and α-Rep′120–168 antisera indicated the nuclear localization of both replication proteins. The fluorescence signal of Rep and Rep′ was distributed irregularly within the nucleoplasm, while no fluorescence was observed in the nucleoli. In contrast, two distinct localization patterns of the Cap protein were observed with the α-Cap104–233 antiserum. The signal was localized either throughout the entire nucleus or mainly in the nucleoli (Fig. 3Ac). When the three antisera were applied to mock-infected control cells, no signal was detected (Figs. 3Ae–g). The α-PCV1 antiserum, recognizing PCV1 virus particles, was used as a positive control and showed results comparable to the α-Cap104–233 antiserum (Fig. 3Ad).

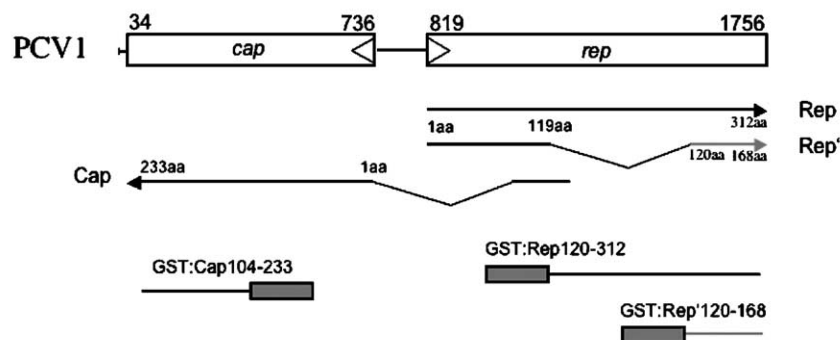

Fig. 1. Genomic organization of PCV1. A linear map of the circular PCV1 genome is shown. The two major ORFs of PCV1 encoding the capsid protein Cap and the replication proteins Rep and Rep′ are divergently transcribed; aa numbers of the N-terminus and the distinct C-termini of Rep and Rep′ are given. Rep, Rep′ and Cap protein fragments fused to GST used for immunization of rabbits are indicated.

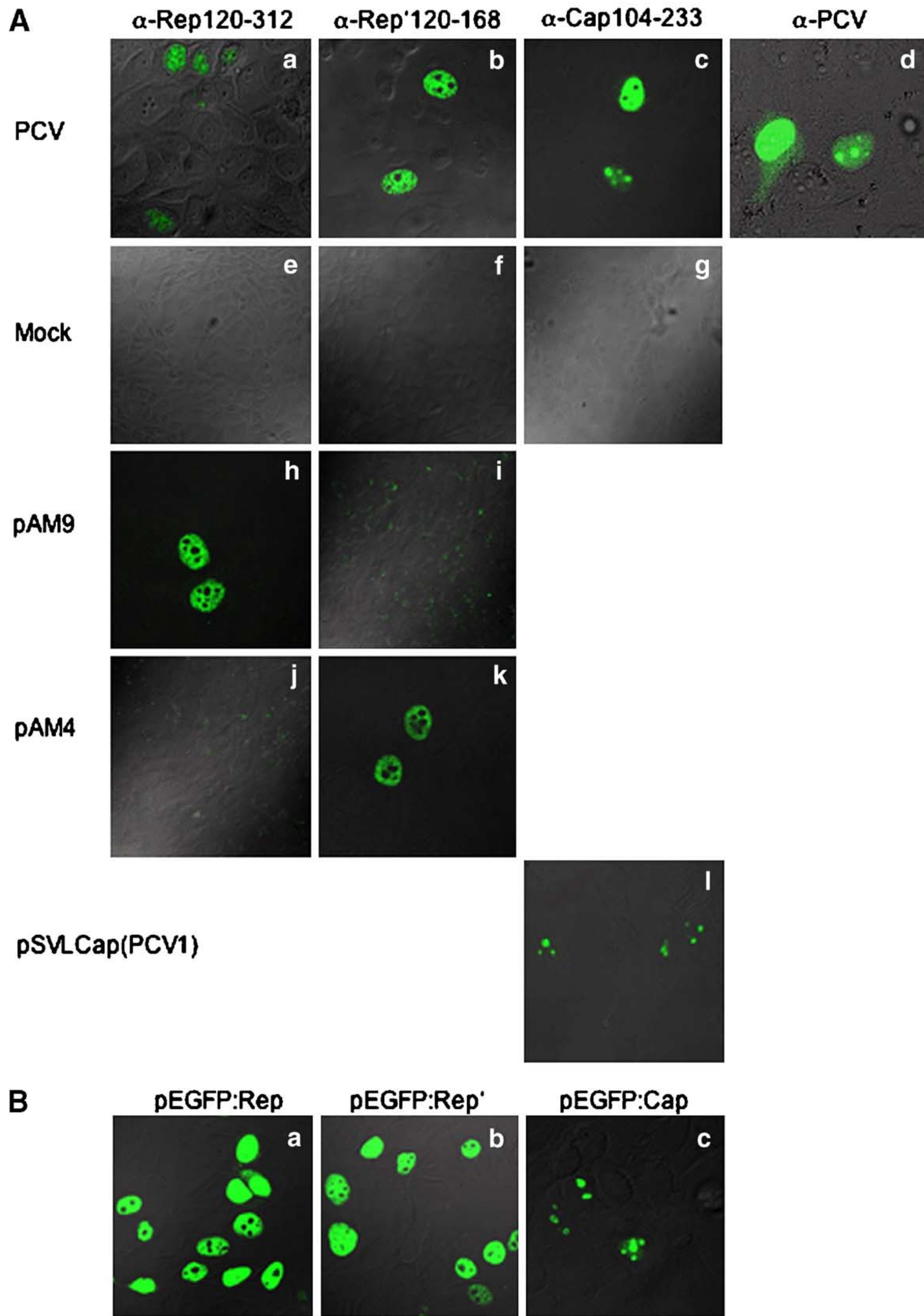

Fig. 3. Subcellular localization of the Rep, Rep′ and Cap protein in PS cells. (A) Immune fluorescence analysis of the subcellular localization of Rep, Rep′ and Cap protein in PCV1-infected and transfected PS cells. Infected cells were stained with α-Rep- (a), α-Rep′- (b), α-Cap- (c) or α-PCV-specific antisera (d). Mock-infected PS cells were used as a negative control (e, f, g). PS cells transfected with plasmids pAM9 (h and i), pAM4 (j and k) or pcDNA-Cap (l) were analyzed by IFA as described previously for infected cells. (B) Plasmids pEGFP:Rep (a), pEGFP:Rep′ (b) and pEGFP:Cap (c) were transfected into PS cells, and subcellular localization of Rep, Rep′ and Cap EGFP fusion proteins was determined by fluorescence microscopy.

The observed localization patterns of the Cap protein might reflect a redistribution of Cap during different phases of viral infection. To challenge this hypothesis, the location of PCV1-encoded proteins was tested after expression of cloned Cap, Rep and Rep′, that is, proteins were expressed not in the context of a PCV1 infection. Plasmids pAM4 (Rep′ protein), pAM9 (Rep protein) and pSVLCap(PCV1) (Cap protein) were transfected into PS cells, and localization was analyzed by IFA using the corresponding antiserum. In addition, plasmids expressing Rep, Rep′ and Cap fused to EGFP or RFP were used in parallel experiments. At 48 h post-transfection (pt), cells were fixed and analyzed by confocal laser scanning microscopy. Rep and Rep′ (Figs. 3Ah and k) as well as the EGFP variants EGFP:Rep and EGFP:Rep′ (Figs. 3Ba and b) were localized exclusively in the nucleoplasm. In contrast to the results observed after PCV1 infection, the isolated Cap protein and the EGFP:Cap fusion protein were seen only in the nucleoli of transfected cells, but not in the nucleoplasm (Figs. 3Al and Bc).

The nucleolar localization of Cap was further investigated by immunostaining of the nucleoli using a mouse α-human nucleoli monoclonal antibody (Chemicon). This human antibody did not detect the nucleoli in porcine PS cells (data not shown). Therefore, the human 293 cell line was transfected with plasmid pEGFP:Cap. Cells were fixed 48 h pt and analyzed by confocal laser scanning microscopy after staining with the nucleoli-specific antibody (Figs. 4a–c). The observed localization pattern of EGFP:Cap in transfected 293 cells was comparable to the results obtained in PS cells. The merged signals showed that Cap was co-located with the nucleolar staining of the α-nucleoli monoclonal antibody (Fig. 4c).

To investigate the localization of viral proteins with respect to each other, double staining was performed. Analysis of Rep plus Rep′ in PCV1-infected PS cells showed nuclear localization with the exception of the nucleoli for both proteins (Figs. 5a and b), the merge of both signals indicated co-localization of Rep and Rep′ (Fig. 5c). Similar results were obtained after co-expression of EGFP:Rep and RFP:Rep′ (Figs. 5l–n).

Analysis of Cap plus Rep in infected cells showed variable results. The Cap protein was either localized spatially separated from the Rep protein in the nucleoli (Figs. 5d–f) or co-localized with the Rep protein in the nucleoplasm (Figs. 5g–i). This effect was not observed in PS cells co-expressing RFP:Cap with EGFP:Rep and EGFP:Rep′ from plasmids pDSRed:*cap* and pEGFP:*rep*. The RFP:Cap fusion protein was localized in the nucleoli, while Rep and Rep′ fusion proteins were located separately in the nucleoplasm (Figs. 5o–q). Two negative controls have been performed to demonstrate the specificity of the double staining reaction. When rabbit α-Cap and α-Rep antisera were saturated with an excess of the Fab fragment of goat α-rabbit IgG (Fig. 5j), no signals were obtained with Cy5-conjugated donkey α-rabbit IgG. When the infected cells were incubated with rabbit α-Cap and α-Rep antisera and afterwards probed with Cy3-conjugated donkey α-goat IgG, no fluorescence was seen (Fig. 5k). In summary, these results indicate that the localization of Cap is not influenced by the expression of the Rep and Rep′ fusion proteins.

Localization of Cap at different time points after infection

To define whether the two distinct localization patterns of Cap in infected cells can be correlated to the kinetics of an infection, we analyzed the localization of Cap in PCV1-infected PS cells at different time points after infection by IFA with α-Cap104–233 antiserum (Figs. 6a–f). The protein localization was screened at 4 h, 12 h, 16 h, 24 h and 48 h pi. At 4 h pi, only weak fluorescence signals were detected in the cytoplasm by Cap-specific antiserum (Fig. 6b), representing either newly synthesized or internalized Cap protein as recently described for PCV-like particles (Misinzo et al., 2005). At 12 h and 16 h pi, the Cap protein was located mostly in the nucleoli of infected cells (Figs. 6c and d), while redistribution to the nucleoplasm was seen at 24 h pi (Fig. 6e). At 48 h pi, Cap was localized in the nucleoplasm in the majority of the infected cells but also occasionally in the cytoplasm, whereas almost no Cap-specific signal was observed in the nucleoli (Fig. 6f). Since no alteration of Cap localization was seen after co-transfection of Rep and Cap EGFP fusion proteins, this suggests that the change of subnuclear localization may coincide with the viral DNA replication or virion assembly.

To obtain further information about Cap redistribution, Cap:EGFP fusion protein was expressed by the recombinant PCV1 strain RVC2. Localization of Cap:EGFP in living cells was determined at 24 and 36 h pt. At 24 h pt, the majority of cells showed Cap:EGFP in the nucleoli, whereas redistribution of EGFP:Cap to the nucleoplasm was seen in the very same cells at 32 h pt (Figs. 6g and h).

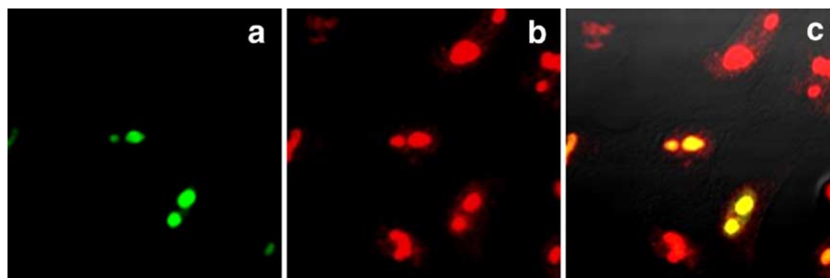

Fig. 4. The EGFP:Cap fusion protein is localized in the nucleoli of transfected 293 cells. 293 cells were fixed 48 h after transfection with pEGFP:Cap, stained with a mouse α-human nucleoli monoclonal antibody and subsequently analyzed by confocal laser scanning microscopy. The signals of EGFP:Cap are given in panel a, while panel b shows the stained nucleoli. The overlay of both channel signals is shown in panel c.

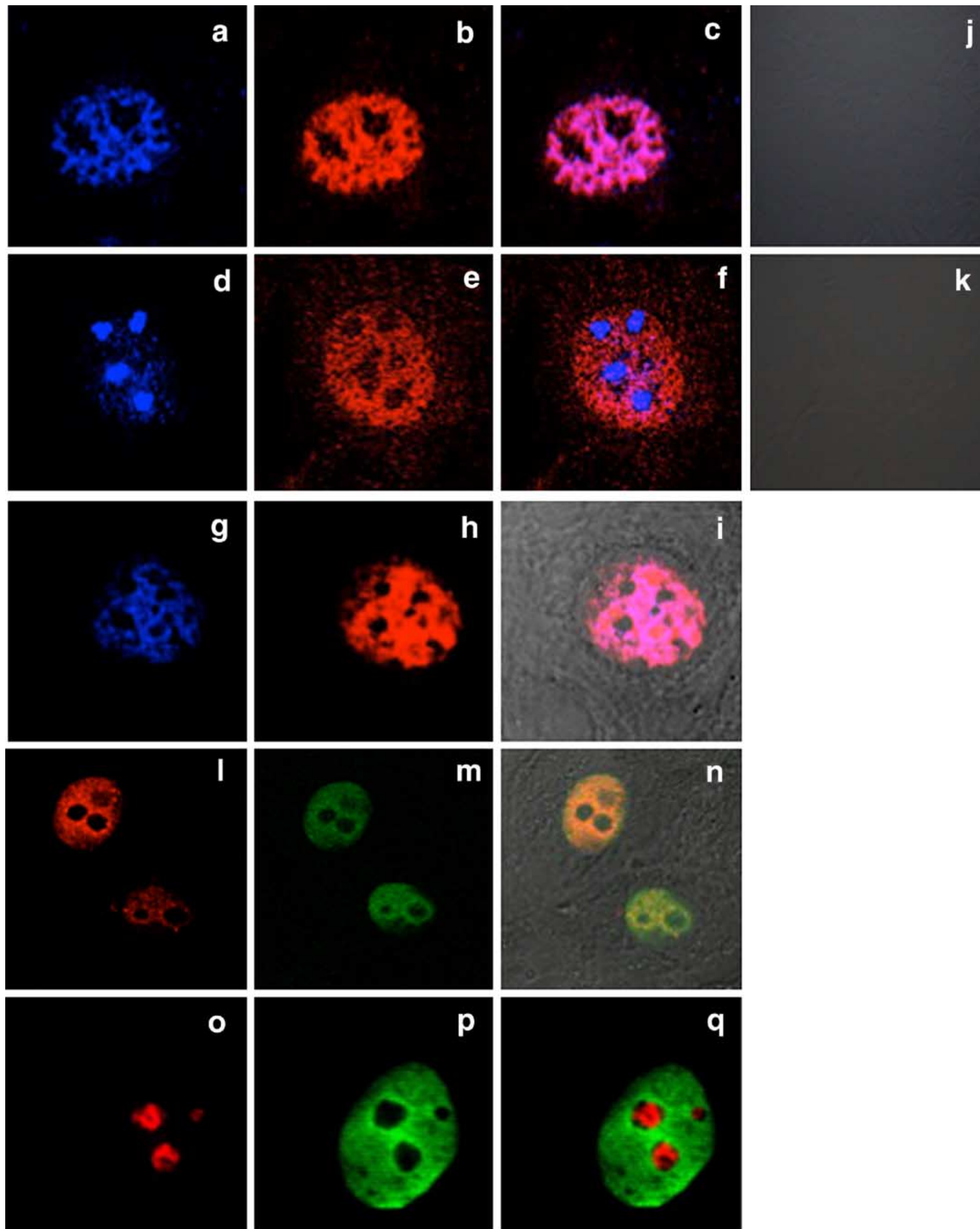

Fig. 5. Co-localization of Rep, Rep′ and Cap in PCV1-infected and non-infected PS cells. Double staining of Rep and Rep′ 48 h pi, using α-Rep- (a) and α-Rep′-specific antisera (b), is shown. The merge of both signals is given in panel c. Double staining of the Cap and Rep protein 48 h pi is shown in two exemplary views. Cap and Rep were detected using α-Cap- (d and g) and α-Rep-specific antisera (e and h). The merged signals are shown in panels f and i. Specificity of the double staining reaction is demonstrated by two negative controls (j and k). Constructs pEGFP:Rep and pDsRed:Rep′, expressing the two *rep* gene products fused to the green or red fluorescent protein, as well as pEGFP:*rep* (expressing EGFP:Rep and EGFP:Rep′) and pDsRed:Cap were co-transfected into PS cells and subsequently analyzed by confocal laser scanning microscopy. For detection of RFP:Rep′ (l) or RFP:Cap (o), excitation at 543 nm was used, for detection of EGFP:Rep (m) or EGFP:Rep/EGFP:Rep′ (p), excitation was performed at 488 nm with the appropriate emission filter sets. The overlay of both channel signals is shown in panels n and q.

Mapping of the NLS of Rep and Rep′

The Rep and Rep′ proteins are localized in the nucleus of PCV1-infected PS cells as well as in non-infected PS cells transfected by cloned Rep and Rep′. This indicates that the nuclear import of the *rep* gene proteins is independent of other viral proteins and that the process might be directed by a nuclear localization signal (NLS). Examination of the aa sequence of Rep and Rep′ revealed that their common N-terminus contains three clusters of basic aa with homology to other NLS, which we have termed NLS1 ($_{12}$**KR**WVFTLNNPSEEE**K**N**K**I**R**-ELP$_{33}$; bold letters indicate basic aa), NLS2 ($_{63}$**KK**QTFN**K**V**K**-WYFGA**R**CHIE**K**A**K**GTD$_{87}$) and NLS3 ($_{107}$P**R**NQG**KR**-SDL$_{116}$). In NLS1, two boxes of basic aa are separated by a spacer sequence of 12 aa, corresponding to the consensus motif of a bipartite NLS (Dingwall and Laskey, 1991). NLS2 is also

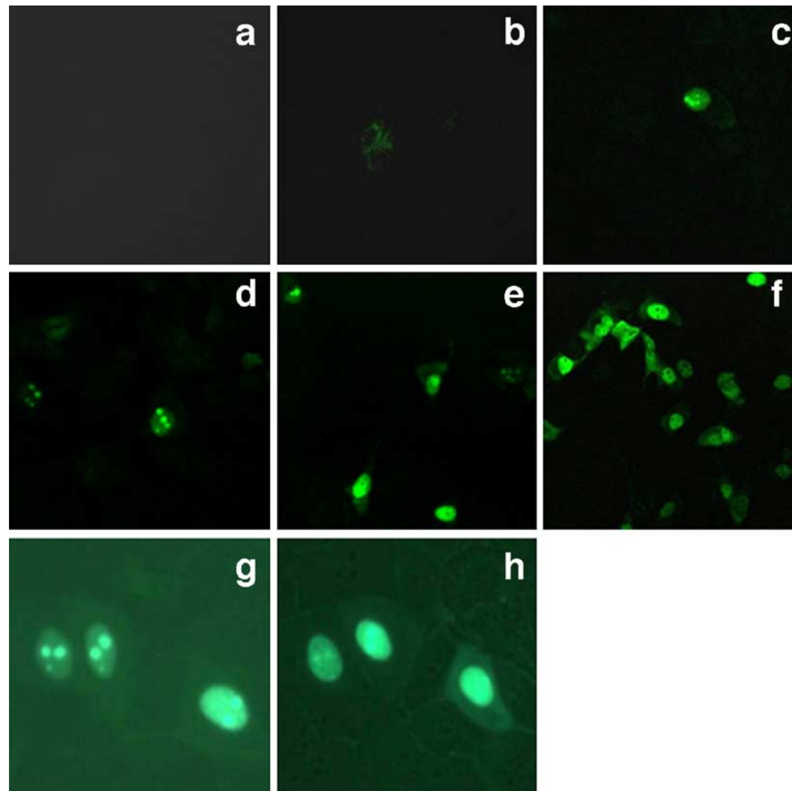

Fig. 6. Subcellular localization of Cap at different time points after infection. PS cells were infected with PCV1 and analyzed by IFA with α-Cap-specific antiserum at 4 h, 12 h, 16 h, 24 h and 48 h pi (b, c, d, e and f). Non-infected PS cells were used as a negative control (a). Living PS cells transfected with RVC2 were analyzed at 24 h and 32 h pt (g and h) by fluorescence microscopy.

composed of basic aa but displays only limited similarity to the consensus motifs. NLS3 shows homology to the c-Myc NLS with only three basic residues in a cluster of nine aa (Dang and Lee, 1989; Makkerh et al., 1996). To determine whether these putative NLS are involved in nuclear import of Rep and Rep′, truncated or mutated fragments of Rep were fused to EGFP (Fig. 7). The resultant constructs were transfected into PS cells, and the localization of the fusion proteins was analyzed. Nuclear localization of the fusion protein EGFP:Rep1–119 reveals that the N-terminal fragment containing NLS1, NLS2 and NLS3 is sufficient to mediate the nuclear import of Rep (Fig. 8h). When this fragment was truncated at the C-terminus, a predominant nuclear localization was seen for the EGF-P:Rep1–88 and EGFP:Rep1–106 fusion proteins (Figs. 8f and g), while distribution throughout the entire cell was observed for the EGFP:Rep1–50 and EGFP:Rep1–73 fusion proteins (Figs. 8d and e), comparable to EGFP (Fig. 8a). This indicates that NLS2 and 3 are required for nuclear localization of Rep and Rep′.

Next, the Rep protein was truncated at the N-terminus. Deletion of the first two aa of NLS1 (pEGFP:Rep14–312, Fig. 8b) did not change nuclear accumulation compared to wt-Rep fused to EGFP. In contrast, complete removal of NLS1 (pEGFP:Rep30–312, Fig. 8c) directed the fusion protein to the cytoplasm. This demonstrates that aa 14 to 29 of NLS1 are essential for nuclear localization of Rep and that NLS2 plus NLS3 are unable to traffic the truncated protein to the nucleus.

To define the contribution of each NLS for nuclear localization, we mutated NLS1, 2 and 3. Mutagenesis of two basic aa in NLS1 (pEGFP:Rep-mutNLS1, Fig. 8i) or of six basic aa in NLS2 (pEGFP:Rep-mutNLS2, Fig. 8j) abolished nuclear localization of the fusion proteins, while exchange of two basic aa in NLS3 (pEGFP:Rep-mutNLS3, Fig. 8k) did not interfere with nuclear accumulation of the protein. These experiments signify that NLS1 and NLS2 are required to mediate the nuclear import, while NLS3 merely enhances the nuclear import of Rep. Similar experiments have been performed with the Rep′ protein and gave analogous results (data not shown).

Discussion

The nucleus of mammalian cells is organized in several subcompartments including the nucleoli, nuclear speckles, transcription and replication foci and chromosome territories (Lamond and Earnshaw, 1998). Since knowledge about the site of replication and maturation of PCV is rather scant, investigation of the subnuclear localization of PCV-encoded proteins will provide additional information to understand these processes.

In this study, we have investigated the localization of the PCV1-encoded proteins Rep, Rep′ and Cap. Visualization was either achieved by antisera detecting Rep, Rep′ and Cap or with fluorescence tags fused to the viral proteins. Using IFA, expression of the full-length Rep protein and the spliced Rep′

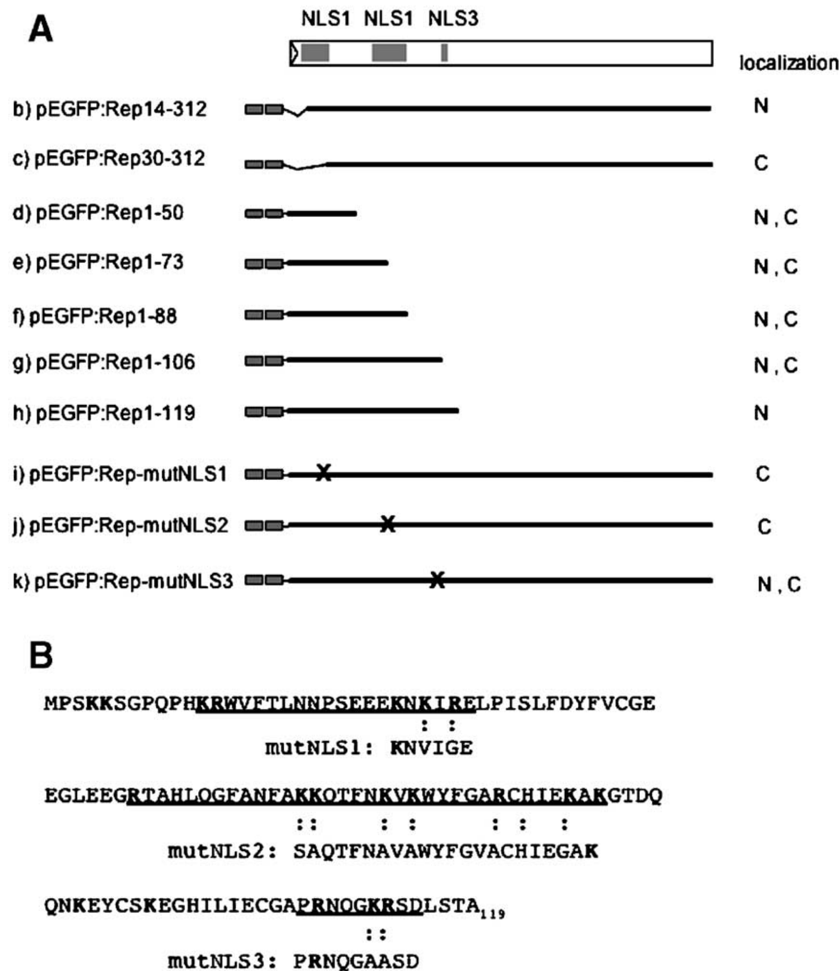

Fig. 7. Map of the EGFP:Rep constructs. (A) A sketch of the *rep* gene indicating putative NLS motifs by shaded boxes is given. Below, the truncated or mutated fragments of the *rep* gene used to produce EGFP fusion proteins are shown. C or N indicates cytoplasmatic or nuclear localization of the resultant EGFP fusion proteins. (B) The aa sequence of the N-terminal part of the Rep protein (1–119 aa) (basic aa in bold) and the aa sequence of the mutated NLS motifs are listed.

protein was detected in the nucleus of PCV1-infected PS cells. Rep and Rep′ were co-localized in the nucleus, but never in the nucleoli, regardless whether the expression was due to infection or cloned proteins. Alteration in localization during the infection cycle was not seen. Accumulation of Rep and Rep′ in defined subnuclear regions as described for other DNA viruses, e.g. the replication compartments of herpes viruses (Quinlan et al., 1984), has not been observed.

Nuclear localization of Cap in infected cells and NLS sequences in the N-terminal part of Cap of PCV1 and PCV2 has been described earlier (Cheung and Bolin, 2002; Liu et al., 2001). We analyzed the localization of Cap with respect to the infection status of the cells and the presence of the replication proteins Rep and Rep′. EGFP- or RFP-tagged Cap protein was seen in the nucleoli, while IFA of PCV1-infected cells revealed two distinct distribution patterns of the Cap protein. During infection, Cap was either found to reside in the nucleoli or to co-localize with the replication proteins in the nucleoplasm of PCV1-infected cells. Kinetic analysis of the subcellular localization of Cap demonstrated that localization to the nucleoli during early infection was followed by redistribution to the nucleoplasm.

Since Rep, Rep′ and Cap are all located in the nucleoplasm, this indicates that DNA replication and encapsidation of the circular closed ssDNA occur in the nucleus and not in cytoplasmic compartments. The biological function of the early localization of Cap to the nucleoli remains unclear. Nucleolar localization has been described for proteins of many DNA and RNA viruses (Hiscox, 2002). It is proposed that virus proteins enter the nucleoli to support viral transcription or alter the cell cycle. It will be interesting to learn whether the PCV1-encoded proteins interact with cellular factors regulating transcription and cell cycle. Analysis of the interaction of viral proteins with cellular factors is currently being carried out.

Since co-expression of the viral proteins showed no effect on their mutual localization, the twofold localization pattern of Cap is probably not influenced by the Rep and Rep′ protein. Furthermore, since translocation of both replication proteins to the nucleus is independent of other viral factors, the trafficking of Rep and Rep′ to the nucleus should be regulated by an intramolecular NLS. Three stretches of positively charged aa in the N-terminus of the PCV1 Rep protein show homology to an NLS. NLS1 shows homology to the consensus sequence of the bipartite NLS type (Dingwall and Laskey, 1991), whereas

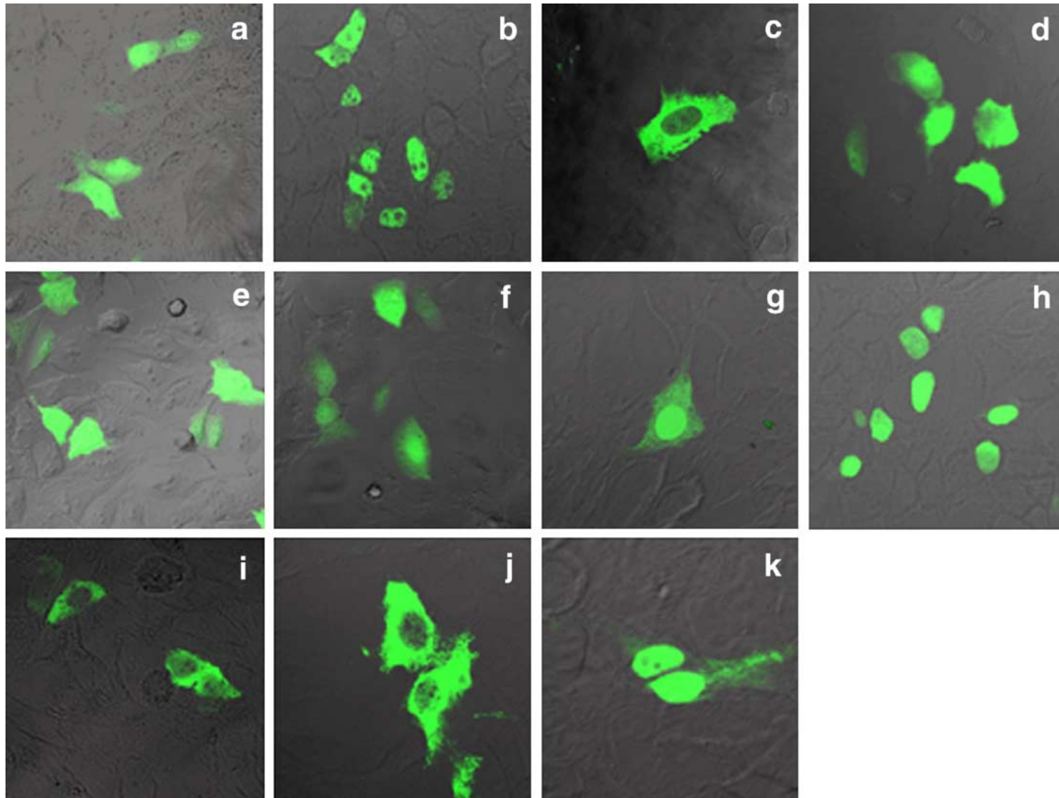

Fig. 8. Mapping of the NLS of Rep and Rep′. PS cells were transfected with plasmids carrying mutated and truncated versions of the *rep* gene to analyze their subcellular location. The following plasmids were used: pEGFP-C2 (a), pEGFP:Rep14–312 (b), pEGFP:Rep30–312 (c), pEGFP:Rep1–50 (d), pEGFP:Rep1–73 (e), pEGFP:Rep1–88 (f), pEGFP:Rep1–106 (g), pEGFP:Rep1–119 (h), pEGFP:Rep-mutNLS1 (i), pEGFP:Rep-mutNLS2 (j) and pEGFP:Rep-mutNLS3 (k). Localization of EGFP fusion proteins was determined by confocal laser scanning fluorescence microscopy 48 h pt.

NLS2 displays only limited homology. NLS3 contains three positively charged aa flanked by Pro and Asp and is thereby similar to the NLS sequences of the c-Myc protein (Dang and Lee, 1989). To characterize the role of these three NLS sequence, truncated and mutated *rep* gene variants were fused to EGFP. The fragment aa 14–119 was found to be sufficient for mediating nuclear import of EGFP. Deletion of the first two aa (K12 and R13) of NLS1 had no impact on Rep localization, signifying that they are not essential for nuclear import, whereas aa 14–29 within NLS1 were shown to be indispensable. Shortening of the fragment from the C-terminus from aa 119 to 106 or 119 to 88 provided evidence that NLS3 enhances nuclear import. The results of three *rep* gene variants mutated in their NLS demonstrated that NLS1 and NLS2 but not NLS3 are essential for nuclear localization. These experiments indicate that the right part of NLS1 and NLS2 are required to mediate the nuclear import, whereas NLS3 enhances the nuclear accumulation of the replication proteins.

The observed subcellular localization patterns of viral proteins led us to propose the following working hypothesis: the Cap protein enters the nucleoli in the beginning of infection, this may be linked to arrest of the cells in the S-phase and modification of host protein production. During the infection cycle, the viral proteins Cap, Rep and Rep′ accumulate in the nucleoplasm, where viral replication, capsid formation and encapsidation take place. Finally, the capsid assembly in the nucleus is a prerequisite for translocation to the cytoplasm and subsequent release of mature virions. This hypothesis is currently under investigation.

Materials and methods

Plasmids

Plasmids pORF4A, pAM4, pAM9 and pSVLCap(PCV1), expressing genes under control of the SV40 late promoter, are based on vector pSVL (Amersham Biosciences, Freiburg). pORF4A (Mankertz et al., 1998) encodes the wild-type (wt) *rep* gene of PCV1 (GenBank accession number: Y09921), while pAM9 and pAM4 carry only one of the two Rep proteins: pAM9 encodes the *rep* gene mutated in several splice donor sites, thereby disabling synthesis of Rep′, pAM4 carries the cDNA product of the *rep′* transcript (Mankertz and Hillenbrand, 2001). Plasmid pSVLCap(PCV1) was used for expression of Cap (Mankertz and Hillenbrand, 2002). Expression vectors pEGFP-C2, pDsRed1-C1 (BD Biosciences Clontech, Heidelberg), pGEX-6P-1 (Amersham Biosciences) or pTriEx-1.1 (Merck Biosciences, Bad Soden) were used for expression of Rep, Rep′, Cap and truncation mutants fused to EGFP (enhanced green fluorescent protein), RFP (red fluorescent protein), GST (glutathione *S*-transferase) or an 8xHis-tag. DNA fragments were amplified via PCR using the High

Table 1
Oligonucleotides

Name	Oligonucleotide sequence in 5′–3′ direction	Restriction site
F137	GAAGATCTATGGTGAGCAAGGGCGAGGAGCTGT	*Bgl*II
F141	CGGGATCCATTTTTATTTATTTAGAGGGTCTTTTAGGA	*Bam*HI
F209	CCGCTCGAGTTATTTATTTAGAGGGTCTTTTAGGATAAA	*Xho*I
F225	CGGGATCCCCAAGCAAGAAAAGCGGCCCG	*Bam*HI
F245	CGGGATCCAAGCAAGAAAAGCGGC	*Bam*HI
F273	GGAATTCTCCAAGCAAGAAAAGCGGC	*Eco*RI
F312	GGGGATCCGTGAGTACCCTTTTGGAGACGGGG	*Bam*HI
F313	GGGGATCCTATTTTGATTACCAGCAATCAGGC	*Bam*HI
F511	CGGAATTCATGCCAAGCAAGAAAAGC	*Eco*RI
F514	CGGAATTCCGGGAGCTTCCAATCTCCC	*Eco*RI
F518	CGGAATTCTGGGTGTTCACCCTTAATAATCC	*Eco*RI
B519	CGGGATCCACCCTCTTCCAAACCTTCCTC	*Bam*HI
F636	GCAGTTAACGTTATAGGGGAGCTTCCAATCTCCCT	*Acl*I
F638	TTCGCGGGCGCCAAAGGAACCGACCAGCAG	*Nar*I
F640	TCAGACTTTTAACGCGGTGGCTTGGTATTTGGTGCCGCGTGCCACATCGAGGG	
F642	GAGCGGCGCCGCTAGCGACCTGTCTACTGCTG	*Hae*II
B138	GAAGATCTTACTTGTACAGCTCGTCCATGCCG	*Bgl*II
B142	CCGCTCGAGGATGACGTGGCCAAGGAGGC	*Xho*I
B148	TATGACCATGATTACGCCAAGCTT	
B167	CGGGATCCTTACGATGTGATAACAAAAAAGACTCAGT	*Bam*HI
B210	CGGGATCCTATGAATTTTACCCCAGAGACCC	*Bam*HI
B226	GGAATTCGATGTGATAACAAAAAAGACTCAGT	*Eco*RI
B273	GGAATTCTACGTGGCCAAGGAGGCGTTAC	*Eco*RI
B289	GGCGCGCCCAGTAATTTATTTTATATGGG	*Asc*I
B290	GGCGCGCCCAAATCGGCCTTCGGGTACC	*Asc*I
B512	CGGGATCCAGCAGTAGACAGGTCGCTG	*Bam*HI
B520	CGGGATCCATACCACTTCACCTTG	*Bam*HI
B521	CGGGATCCCTGGTCGGTTCCTTTCGC	*Bam*HI
B637	CCAGTTAACGTTTTTCTCCTCCTCGGAAGG	*Acl*I
B639	CGCTATAGCGCTAGCAAAATTAGCAAACCC	*Hae*II
B641	CGCCCTCGATGTGGCACGCGGCACCAAAATACCAAGCCACCGCGTTAAAAGTCTGAGCGC	
B643	ACAAGGGCGCCCTGGTTCCGCGGAGCTC	*Hae*II
B715	GCGGATCCAGCTCCACACTCGATAAG	*Bam*HI
150mut *Bgl*II	TTTTTATTTAGATCTATTTAGAGGGTCTT	*Bgl*II
A147	GGTGGTGGGACTCGGACTGCTTCACTGTAAAACGACGGCCAGTGAATTC	
A147tag	GGTGGTGGGACTCGGACTGCTTCAC	

Fidelity PCR System (Merck Biosciences) and restriction site-tagged primers (Table 1). Cycling parameters were 94 °C, 2 min, 30×{94 °C, 15 s; 60 °C, 30 s; 72 °C, 45 s}, 72 °C, 7 min. PCR products were inserted via the newly introduced restriction sites into suitable plasmids and sequenced to exclude PCR-acquired nucleotide misincorporation. All cloning steps were performed according to standard techniques (Sambrook and Russell, 2001).

(a) Primer pairs and templates for amplification of *rep*-specific PCR products cloned into pEGFP-C2 or pDsRed1-C1 are listed: construct pEGFP:*rep*, primer pair F511-B167, template pORF4A; pEGFP:Rep, F511-B167, pAM9; pEGFP:Rep′, F511-B167, pAM4; pEGFP:Rep14–312, F518-B167, pAM9; pEGFP:Rep30–312, F514-B167, pAM9; pEGFP:Rep1–50, F511-B519, pORF4A; pEGFP:Rep1–73, F511-B520, pORF4A; pEGFP:Rep1–88, F511-B521, pORF4A; pEGFP:Rep1–106, F511-B715; pEGFP:Rep1–119, F511-B512, pORF4A; pEGFP:Rep14–119, F518-B512, pORF4A; pDsRed:Rep′, F273-B167, pAM4. For introducing base alterations within the *rep* gene, template pORF4A and mutagenesis primer sets were used. For construction of pEGFP-mutNLS1, PCR products of F511-B637 and F636-B167 were ligated via the introduced *Acl*I restriction site. For construction of pEGFP-mutNLS2, PCR products of F511-B639 and F638-B167 were ligated with the hybridization product of F640 and B641 using *Hae*II and *Nar*I sites. For construction of pEGFP-mutNLS3, PCR products F511-B643 and F642-B167 were ligated via the introduced *Hae*II restriction sites. All ligation products were inserted into pEGFP-C2 via *Bam*HI and *Eco*RI sites. (b) For cloning of *rep*-specific fragments into pGEX-6P-1, the primer pairs and templates for the PCR reaction are given: pGEX:-Rep120–312, F312-B226, pORF4A; pGEX:Rep′120–168, F313-B226, pORF4A. (c) For construction of pTriEx:Rep primer pair, F245-B289 and template pORF4A were used, and, for construction of pTriEx:Rep′ primer pair, F245-B290 and template pAM4 were used. (d) For cloning the *cap* gene into pEGFP-C2 or pDsRed1-C1, the following primer pairs and templates were used for the PCR reactions: pEGFP:Cap, F141-B142, pSVLCap(PCV1); pDsRed:Cap, F141-B273, pSVLCap(PCV1). (e) For cloning the *cap*-specific fragment into pGEX-6P-1, the primer pair F209-B210 and the template pSVLCap(PCV1) were used for the PCR reaction to produce pGEX:Cap(104–233). (f) Plasmid pSK144 (pUC8 carrying the complete PCV1 genome cloned at the single *Pst*I site) was used

for construction of a recombinant PCV1 strain (RVC2). First, a *Bgl*II site was introduced at the 3′-end of the *cap* gene by site-directed mutagenesis (Stappert et al., 1992). Plasmid pSK144 was used as template with oligonucleotides 150mut *Bgl*II and A147. B148 and A147 tags were used for amplification in the second mutagenesis step. The *egfp* gene was subsequently fused to the *cap* gene via the *Bgl*II restriction site. For amplification of the *egfp* gene, *Bgl*II restriction site containing oligonucleotides F137 and B138 was used with pEGFP-C2 as template. For transfection of PS cells with DNA of the recombinant or wt virus, plasmid pRVC2 or pSK144 was digested with *Pst*I. The linearized genomic fragment was purified by agarose gel electrophoresis and subsequent gel extraction using the QIAquick gel extraction kit (Qiagen, Hilden). The genomic fragment was religated and purified with the QIAquick PCR purification kit (Qiagen) prior to transfection.

Generation of α-Rep(120–312), α-Rep′(120–168) and α-Cap(104–233) antisera

To generate antisera specific for Rep or Rep′, the C-terminal 193 aa of Rep, as well as the C-terminal 49 aa of Rep′ were fused to glutathione *S*-transferase (GST). Resultant plasmids pGEX:Rep120–312 and pGEX:Rep′120–168 were transformed into *E. coli* BL21 (Amersham Biosciences). After expression, the fusion proteins were purified from the insoluble fraction by excision of protein bands after SDS-PAGE. Transformation and protein amplification were done according to the recommendations of the manufacturer (Amersham Biosciences). Rabbits were immunized with 70–90 μg of the purified protein (BioGenes, Berlin) according to standard procedures. Immunization was repeated four times in 3-week intervals. The serum was absorbed with PS cells and *E. coli* according to published protocols (Lane and Harlow, 1982). In a parallel procedure, the C-terminus of the Cap protein was expressed as a GST fusion protein from plasmid pGEX:Cap104–233 and used for generation of an α-Cap104–233 antiserum.

Immunoblot analysis

SDS-PAGE and immunoblotting were performed to test the specificity of the α-Rep120–312 and α-Rep′120–168 antisera. Rep and Rep′ were expressed as 8xHis-tagged proteins from pTriEx:Rep or pTriEx:Rep′ in *E. coli*, strain Tuner™(DE3)*placI* (Merck Biosciences). Bacteria transformed with the expression plasmids were grown at 33 °C in LB to 0.8 OD_{588}, and protein expression was induced with 0.1 mM IPTG for 2 h. *E. coli* cell extracts containing His-tagged Rep and Rep′ proteins were analyzed in Western blots (Sambrook and Russell, 2001). After SDS-PAGE, proteins were blotted onto Immobilon PVDF membranes (Millipore, Schwalbach). The membranes were blocked with 3% BSA in 10 mM Tris–HCl pH 7.5, 100 mM NaCl and 0.05% Tween 20 for 1 h at room temperature and probed with polyclonal rabbit α-Rep120–312 antiserum (1:400), α-Rep′120–168 antiserum (1:400) or α-HisTag antibody (1:5000; Dianova, Hamburg) for 1 h at room temperature. The membranes were washed and treated with a 1:5000 dilution of alkaline-phosphatase-conjugated goat α-rabbit IgG or goat α-mouse IgG antibody. Detection was performed with the multicolor detection set (Merck Biosciences). The molecular weight marker Mark 12™ Wide Range Protein Standard (Invitrogen, Karlsruhe) was visualized with amido black solution (Towbin et al., 1979).

Cells and virus

PS cells free of PCV are derived from PK-15 cells (ATCC CCL33; American Type Culture Collection, Rockville, MD) and were propagated in DMEM with 5% fetal calf serum at 37 °C and 5% CO_2. PCV1 stocks were isolated from PS cells transfected with the religated PCV1 genome. Four days pt, cultures were frozen and thawed three times. Cell debris was removed from infectious supernatants by low-speed centrifugation. Ultracentrifugation followed for 6 h at 66,000 × *g* in a SW28 rotor. The titer of the resultant stock of PCV1 was determined by incubation of PS cells with serial 10-fold dilutions and immunocytochemical staining after three days to be 10^5 $TCID_{50}$ ml^{-1}. This stock was used for all infection experiments.

Fluorescence and indirect immune fluorescence microscopy

PS cells or 293 cells were seeded on glass coverslips in 48-well plates and grown for 24 h in DMEM supplemented with 5% fetal calf serum at 37 °C in 5% CO_2. All transfections were performed with Effectene transfection reagent (Qiagen) using 150 ng of plasmid DNA. For fluorescence microscopy, 70–80% confluent cells were transfected with the recombinant PCV1 strain RCV2, the pEGFP-C2 or the pDsRed1-C1-based constructs described in this study. For analysis in a Zeiss LSM510 confocal microscope, cells were fixed with 4% formaldehyde in PBS and washed. The coverslips were mounted in Immu-Mount (Shandon, Frankfurt). For depiction of EGFP fusion proteins, samples were investigated at 488 nm with the appropriate emission filter sets, while for imaging of DsRed (RFP) fusion proteins, 543 nm was used.

For the indirect immune fluorescence assay (IFA), 70–80% confluent cells on coverslips were transfected with 150 ng of plasmid DNA or infected with PCV1 (10^5 $TCID_{50}$ ml^{-1}) at a multiplicity of infection of 10^{-1} per cell. Higher virus concentration did not improve infection rate of PS cells (Hattermann et al., 2004). Cells were fixed and permeabilized by immersing the coverslips in −20 °C methanol/acetone (1:1) for 10 min and subsequently air-dried. Cells were blocked for 1 h with 1% BSA and 0.05% Tween 20 in PBS and stained with the primary α-Rep120–312 antiserum, 1:300; α-Rep′120–168 antiserum, 1:300; α-Cap104–233 antiserum, 1:300; α-PCV1 antiserum, 1:300 (kindly provided by Dr. I. Tischer, Robert Koch-Institut, Berlin); or mouse anti-human nucleoli monoclonal antibody, 1:30 (Chemicon International), diluted in blocking buffer for 2 h at room temperature. The cells were washed three times with PBS plus 0.05% Tween 20 and

incubated with FITC-conjugated goat α-rabbit IgG (Dianova, Hamburg) or rhodamine red-conjugated goat α-mouse IgG (Dianova). Subsequently, the coverslips were washed, mounted and analyzed by confocal laser scanning microscopy.

For double staining (Negoescu et al., 1994) of Rep and Rep′, Rep was detected with the rabbit α-Rep120–312 antiserum, which was labeled afterwards with excess Fab fragment of goat α-rabbit IgG, 1:100 (Dianova), and visualized with Cy3-conjugated donkey α-goat IgG antibody, 1:400 (Dianova). Cy5-conjugated donkey α-rabbit IgG antibody, 1:400 (Dianova), was used in addition to the rabbit α-Rep′120–168 antiserum to detect Rep′. For double staining assay of Cap and Rep, Cap was detected with the rabbit α-Cap104–233 antiserum in conjunction with the Fab fragment of goat α-rabbit IgG and the Cy3-conjugated donkey α-goat IgG antibody. Detection of Rep was performed with α-Rep120–312 rabbit antiserum and Cy5-conjugated donkey α-rabbit IgG antibody. Cy5 was excited with a He–Ne laser at a wavelength of 633 nm and detected using a 665 nm long-pass filter. Cy3 was excited at 543 nm with an external He–Ne laser and detected using a 575–600 nm band pass filter.

Acknowledgments

This work was supported by the European Union (Project number QLK2-CT-1999-00307) and by the Deutsche Forschungsgemeinschaft (MA 2126/2-1).

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