Differential replication of two porcine parvovirus strains in bovine cell lines ensues from initial DNA processing and NS1 expression

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Porcine parvovirus (PPV) is a small DNA virus with restricted coding capacity. The 5 kb genome expresses three major non-structural proteins (NS1, NS2 and SAT), and two structural proteins (VP1 and VP2). These few viral proteins are pleiotropic and interact with cellular components throughout viral replication. In this regard, very few cell lines have been shown to replicate the virus efficiently. Cell lines were established from a primary culture of bovine cells that allowed allotropic variants of PPV to be distinguished. Three cell lines were differentially sensitive to infection by two prototype PPV strains, NADL-2 and Kresse. In the first cell line (D10), infection was restricted early in the infectious cycle and was not productive. Infection of the second cell line (G11) was 1000 times less efficient with the NADL-2 strain compared with porcine cells, while production of infectious virus of the Kresse strain was barely detectable. Restriction points in these cells were the initial generation of DNA replication intermediates and NS1 production. Infection with chimeras between NADL-2 and Kresse showed that residues outside the previously described allotropic determinant were also partially responsible for the restriction to Kresse replication in G11 cells. F4 cells were permissive to both strains, although genome replication and infectious virus production were lower than in the porcine cells used for comparison. These results highlight the dependent nature of parvovirus tropism on host factors and suggest that cells from a non-host origin can fully support a productive infection by both strains.

INTRODUCTION

Porcine parvovirus (PPV), of the subfamily Parvovirinae of the family Parvoviridae has a non-enveloped, icosahedral capsid, 25 nm in diameter (Simpson et al., 2002), and contains a non-segmented single-stranded, linear 5 kb DNA genome. PPV and other members of the genus Parvovirus are autonomous and most package only the negative DNA strand. Their genome is characterized by distinct terminal palindromic sequences and two main gene cassettes, for non-structural (NS) and structural (VP) proteins (Tattersall, 2006).

Replication of parvoviruses occurs mainly in the nucleus and is initiated by conversion of an incoming single-stranded genome to a double-stranded monomeric replication form (mRF) during early S phase, and requires cellular stranded genome to a double-stranded monomeric replica-and is initiated by conversion of an incoming single-

generation of NS1 protein, which in turn enables genome amplification (Rhode, 1989) and trans-activation of the VP promoter (Rhode, 1985). Further genome replication proceeds by a rolling-circle mechanism (Tattersall & Ward, 1976) by creating genome concatemers of varying lengths, including dimeric replicative forms (dRFs) from which the single-stranded genomes are excised and packaged into preformed capsids (Cotmore & Tattersall, 2005). Alternative splicing directs synthesis of capsid protein VP1 and its smaller version VP2 (Bergeron et al., 1993; Mani et al., 2006; Weichert et al., 1998). The unique N-terminal extension of VP1 (150 aa) contains phospholipase A2 (PLA2) activity (Zádori et al., 2001), which is necessary for endosomal pathway escape (Farr et al., 2005). Newly synthesized VP proteins are transported as trimers to the nucleus where further capsid assembly and DNA packaging take place (Lombardo et al., 2000). Finally, full virions are exported from the nucleus in a process involving NS2 (Eichwald et al., 2002; Engelsma et al., 2008; Miller & Pintel, 2002) while the rest of the virus is released following cell lysis induced by cytotoxic activities of the NS proteins (Anouja et al., 1997; Nüesch & Rommelere, 2006; Nüesch et al., 2009).

PPV causes reproductive failure in swine (Rodeffer et al., 1975) and is still a significant burden when vaccination...
programmes are not rigorously followed. Indeed, current vaccines fail to trigger long-term protective immunity in sows, and vaccination must be repeated every 4–6 months (Józwik et al., 2009). Virus replication and shedding still occur in sows where vaccination efficiently prevented reproductive failure (Józwik et al., 2009). Constant virus replication in herds facilitates the emergence of new strains, some of which are not neutralized effectively by serum obtained after vaccination (Zeeuw et al., 2007). This could lead to the emergence of new virulent strains resulting in reproduction failure despite regular vaccination (Streck et al., 2011).

Little is known about the mechanisms that control PPV tropism either in vivo or in vitro. Studies have shown that tropism is determined primarily at the cell surface for other members of the genus Parvovirus. The host-range evolution of the feline to canine parvovirus strains (FPV and CPV, respectively) involved interaction between the viral capsid and the transferrin receptor type 1 (TfR) (Hoelzer & Parrish, 2010; Hoelzer et al., 2008; Truyen et al., 1995). For minute virus of mice (MVM), capsid residues were shown to be partially responsible for the phenotypes of the fibrotropic (prototypic) or lymphotropic strains, although they did not involve attachment to the cell surface (Ball-Goodrich & Tattersall, 1992; Gardiner & Tattersall, 1988). Depending on the cell type, restrictions have been identified at delivery of the incoming genome to the nucleus (Previsani et al., 1997), activation of the early promoter (Deleu et al., 1999) and genome replication and post-encapsidation steps (Rubio et al., 2001). For PPV, infection of Madin–Darby canine kidney cells (MDCK) demonstrated that the cellular association of PPV was strong and specific with both swine testis (ST) cells and MDCK cells. However, viral genome replication and transcription are factors limiting permissivity in canine cells (Oraveerakul et al., 1992). Others identified a variant of the non-pathogenic attenuated NADL-2 vaccine strain, designated P2, which had gained the ability to replicate in canine cells after only two amino acid substitutions: one in the NS protein and one in the capsid protein (Vasudevacharya & Compans, 1992).

Our previous studies demonstrated that a primary bovine testis cell culture (TV cells) was only sensitive to the NADL-2 vaccine strain of PPV, while infection with the highly pathogenic Kresse strain was non-productive (Bergeron et al., 1996). These strains differ by only 13 nt, six of which alter the VP gene sequence, and by a 127 nt repeated sequence downstream of the VP gene cassette in the NADL-2 strain. Furthermore, the allotropic determinant restricting Kresse replication in TV cells was found to consist mainly of a short BglII restriction fragment encoding three of the non-synonymous substitutions between the strains (Bergeron et al., 1996). To extend analysis of mechanisms involved in this differential tropism, cell lines were established from primary TV cells. Thirteen cell lines were obtained, and their ability to support PPV replication was determined. We observed three categories of cells: cells that could replicate viruses of both strains, cells that could replicate only NADL-2 efficiently and cells that could not replicate either strain. Three bovine cell lines (D10, G11 and F4) were chosen for further studies as they represented the full spectrum of observed phenotypes to viral infection. This report assessed multiple steps of the PPV replication cycle in these bovine cell lines and compared them with those in PT cells, a permissive porcine testis cell line.

**RESULTS**

**Sensitivity of the bovine cell lines to infection with NADL-2 and Kresse viruses**

Initially, sensitivity to infection was assessed by scoring development of cytopathic effects (CPE) with both viral strains in different cell lines. The results in Table 1 represent CPE observed from duplicate wells at different times post-infection (p.i.) until the mock-infected cells reached confluence. For all results shown in this study, experiments were repeated independently at least three times. The F4 bovine cell line was almost as sensitive to both strains as the PT cells. The G11 bovine cell line was sensitive to NADL-2, while Kresse infection showed very limited effects. Finally, the D10 cell line displayed almost no CPE regardless of the PPV strain. Infectious virus production in each cell line was monitored by subsequent titration of supernatants at different times p.i. Residual background virus measured at 12 h p.i. was lower than the detection level in this assay (10² f.f.u. ml⁻¹). Following infection with the NADL-2 strain (Fig. 1a), high, intermediate and a 3 log lower virus production was observed in the PT, F4 and G11 cells, respectively, while D10 cells consistently failed to produce infectious virus. Virus production was 1 log lower in porcine cells when infected with the Kresse strain (Fig. 1b) compared with the NADL-2 strain, F4 cells produced almost equivalent amounts of infectious virus from either strain, and G11 cells produced only a very limited amount of infectious Kresse virus. No infectious particles were detected after infection of D10 cells.

As paroviruses require actively dividing cells for their replication (Tattersall, 1972), cell-cycle progression through the S phase was monitored in the bovine cell lines. All cell lines grew efficiently (Fig. 1c), with about 40 % of cells in the S phase at any time point and nearly 80 % of the cells having passed the S phase within 24 h. Thus, low virus replication observed in the bovine cell lines was not due to the cells being in a resting or growth-arrested state. Finally, cellular binding and uptake of both viral strains by the cell lines was monitored by quantitative PCR (qPCR) after 2 h of infection. We demonstrated previously that no significant amount of virus binds to the plates, and thus DNA detection corresponded only to bound and internalized virus (Boisvert et al., 2010). Results are shown in Fig. 1(d) for NADL-2 and Fig. 1(e) for Kresse; all cell lines captured viruses of both strains at comparable levels. Generally, no major restriction level was found in the replication of either strain in the F4 cell line. Moreover, replication was restricted at very early
steps in D10 cells (no detectable viral DNA processing; data not shown), for both strains. We thus decided to focus on G11 cells, because they displayed interesting features, allowed lower but detectable replication compared with porcine cells, and displayed significant differences between the NADL-2 and Kresse strains.

**Generation of genomic replication intermediates**

Conversion of incoming single-stranded parvovirus genomes to dsDNA (mRF) is a primordial step in the replication cycle, as it precedes viral transcription and depends entirely on cellular factors (Bashir et al., 2000; Cossons et al., 1996). Synthesis of different viral genome replicative forms was monitored in infected cells by Southern blotting. As shown in Fig. 2, high levels of mRF were observed in PT cells regardless of PPV strain. In G11 bovine cells, significantly reduced amounts of mRF were observed, suggesting either lower genome delivery to the nucleus (no detection of incoming DNA at 8 h p.i.) or poor association with cellular factors required to initiate DNA processing. These results reflected the low percentage of cells with virus replication, as seen by the NS1 immunofluorescence (Fig. 3a) and total viral DNA detection by qPCR (Fig. 4). The dRF, generated by the ‘rolling hairpin’ replication of parvoviruses, was below detection level in G11 cells, most likely due to the lower amount of DNA processing.

**NS1 production and localization**

Generation of the mRF allows production of multifunctional NS1 viral protein. NS1 production and localization was thus monitored by immunofluorescence and confocal microscopy. Infection with the Kresse strain led to a delayed production of NS1. At 12 h p.i. with NADL-2 in PT cells (Fig. 3a), NS1 protein was clearly distinguishable throughout the nucleus, while staining was strong only in small regions and very weak in the rest of the nucleus when cells were infected with Kresse. At 16 h p.i., the intensity of the NS1 staining pattern in Kresse-infected cells matched the levels observed at 12 h p.i. with NADL-2. At all time points, NS1 staining was brighter and in greater proportions of the nucleus in PT cells infected with NADL-2 compared with Kresse. In G11 cells, very few cells were positive for NS1, and the staining patterns were observed at later time points compared with PT cells (Fig. 3b). Differences in staining intensity were also observed in G11 cells when comparing NADL-2 and Kresse, i.e. at the same time p.i., NS1 was detected in a greater part of the nucleus with greater intensity when cells were infected with NADL-2 compared with Kresse. The delay in NS1 accumulation in PT cells infected with the Kresse strain was corroborated in Western blotting experiments (Fig. 3c). Again, infection of PT cells with NADL-2 resulted in an earlier accumulation of NS1 and at higher levels, compared with infection with Kresse. This comparison was not feasible for G11 bovine cells, because amounts of NS1 were below detection.

Differences in numbers of cells expressing NS1 were observed when comparing NADL-2 and Kresse infections and, more importantly, very few G11 bovine cells were positive for the NS1 protein. In PT cells (Fig. 3d) the proportion of NS1 expressing cells was nearly double in

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**Table 1. CPE caused by PPV NADL-2 and Kresse strains**

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NADL-2 infected cells (60%) as compared with Kresse-infected cells (30%) at an m.o.i. of 5. In G11 bovine cells these percentages dropped significantly (less than 5%); again, more cells were NS1-positive when infected with NADL-2 compared with Kresse (Fig. 3e). Expression of NS1 viral protein was thus an important difference in

Fig. 1. Cell lines sensibility to PPV infection. (a, b) Viral titres in supernatants at different times p.i. with NADL-2 (a) and Kresse (b) expressed as f.f.u. ml⁻¹ (mean ± SD) from duplicate wells (in PT cells only for the first 24 h because of rapid CPE). (c) Transit of cell lines through S phase as measured by 5-ethynyl-2'-deoxyuridine (EdU) incorporation. Results are expressed as the percentage of cells in S phase relative to the total cell number (determined by Hoechst staining) from triplicate wells (cells in S phase: open bars, 1 h pulse labelling; cells having passed through S phase: shaded bars, 24 h labelling). (d, e) Viral uptake of bovine (F4, G11 and D10) and porcine (PT) cell lines after infection by PPV strains NADL-2 (d) and Kresse (e) scored by qPCR. Results are expressed in genome copy equivalent (GCE) ml⁻¹ from triplicate samples. All results are representative of at least three independent experiments.

Fig. 2. Synthesis of viral genome replication intermediates. Southern blotting after infection of porcine (PT) and bovine (G11) cells, with either viral strain at the indicated times p.i. showed three viral genomic forms. Results are representative of at least three independent experiments. ss, Single-stranded genome; mRF, monomer replicative form (transcription template); dRF, dimer replicative form.
Fig. 3. NS1 production. (a, b) Kinetics of NS1 production and accumulation in the nucleus of porcine (PT) (a) and bovine (G11) (b) cells. Cells were infected with either NADL-2 or Kresse strain at an m.o.i. of 5 and fixed at the indicated times p.i. Immunofluorescence was performed using anti-NS1 mAb and Alexa Fluor 488-conjugated secondary antibody and imaging was done by confocal microscopy. (c) Cell lysates from PT-infected cells were harvested at the indicated times p.i. with either strain and Western blotting was performed using anti-NS1 mAb and anti-actin mAb as a loading control. n.i., Not infected. (d, e) Percentage of NS1-positive PT (d) and G11 (e) cells after infection with either strain at an m.o.i. of 5 for 24 h (PT cells) or 48 h (G11 cells). Cells were analysed by immunofluorescence performed as in (a). Results in (d) and (e) were mean ± SD of at least 300 analysed cells in triplicate, and all are representative of at least three independent experiments (a–e).
NADL-2 versus Kresse infection in both cells, and was substantially restricted in G11 bovine cells.

**Virus genome replication**

Quantitative analysis of PPV genome amplification in the porcine and bovine cells was performed by qPCR. Genome replication in PT cells was readily detected from 10 h p.i. and reached a peak at 20 h p.i. (Fig. 4). In these cells, newly produced virus was already present in the supernatant at 16 h p.i., and thus DNA replication was not monitored after 24 h to avoid detection from the second round of replication. Kresse genome replication was detected later (12 vs 10 h) and reached similar levels as NADL-2 at 16 h p.i. Genome replication from the NADL-2 strain in G11 bovine cells was delayed compared with in PT cells, as it started at 16–20 h and reached a peak only at 40 h p.i. Kresse genome replication in G11 cells was only slightly above the detection limit after 48 h.

**Capsid protein production**

After their synthesis, capsid proteins are transported to the nucleus where they are assembled into capsids before DNA packaging. Production and localization of capsid proteins was monitored by immunofluorescence and confocal microscopy at different times p.i. with VP2- and capsid-specific antibodies. In PT cells, almost all VP2 staining was located in the nucleus together with capsid staining, suggesting very efficient transport of capsid proteins to the nucleus. Few G11 cells displayed capsid proteins when infected with Kresse (≤0.1%) compared with NADL-2 (≤1%; data not shown). However, an interesting pattern was observed for NADL-2 infection. While 80% of infected G11 cells displayed a normal phenotype, a portion of NADL-2-infected cells displayed an alternative localization of the structural proteins. Co-localization of capsid and VP2 staining in the nucleus was weak, while cytoplasmic accumulation of capsids was evident (Fig. 5). This pattern was readily observable when infecting at low m.o.i., where entering virus could not be detected at earlier time points. It was also not detectable when transcription was inhibited by treatment of cells with actinomycin D, and thus represented newly synthesized capsid (data not shown).

**Viral chimeras**

Kresse replication was not productive in the parental primary TV bovine cells (Bergeron et al., 1996). A short genome fragment (the allotropic determinant) was responsible for the difference of replication between NADL-2 and Kresse strains in TV cells (Fig. 6a). Subsequently, only two of the three amino acid differences in the allotropic determinant were shown to be sufficient to restore Kresse replication in the TV cells (D378G and H383Q; data not shown). Thus, a chimera of Kresse containing the CR2 fragment from NADL-2 having those two coding changes was used (Fernandes et al., 2011) to determine whether the same determinant was responsible for poor Kresse replication in G11 cells. The results in Fig. 6(b) showed that, while the K-CR2-N chimera produced more f.f.u. in supernatants than Kresse, it failed to reach NADL-2 virus production levels. As NS1 was also found to indicate an important difference between the two strains, production of this protein was evaluated using different chimeras of the strains. The K-CR2/3-N chimera was the best in terms of NS1 production in both cell lines (percentage of NS1-expressing cells; Fig. 6c, d), except for chimera K-CR1/2/3-N which contained all NADL-2 coding regions and the non-coding region of Kresse. K-CR2/3-N was also the best chimera in terms of infectious virus production (expressed as f.f.u.) in G11 cells (Fig. 6e), as observed previously with PT cells (Fernandes et al., 2011).
DISCUSSION

Binding and entry

Cell binding and/or viral entry was not a major factor for allotropism in bovine cells, indicating that the first contact between the virus and cells is not an essential tropism determinant, as has been observed previously in a canine (MDCK) cell line (Oraveerakul et al., 1992; Ridpath & Mengeling, 1988). While many parvoviruses utilize classic receptor-mediated endocytosis pathways to enter cells (Hansen et al., 2001; Mani et al., 2006; Parker & Parrish, 2000), fluid-phase endocytosis (macropinocytosis) has also proved significant for PPV uptake for both purified and non-purified virus (Boisvert et al., 2010). Once activated, this mechanism does not rely on specific receptor binding, providing a mechanistic explanation as to how parvoviruses may bind and enter cells, regardless of their capacity to support infection. Similarly, adenovirus type 5 binding to cells can activate macropinocytosis and potentiate virus entry (Meier & Greber, 2004).

DNA processing

Initial processing of the incoming DNA genome was an important restriction point for G11 cells (Fig. 2). It remains unclear whether this resulted from lower nuclear delivery of virus or from reduced conversion of the incoming genome to double-stranded intermediates. However, nuclear targeting of parvovirus virions during entry is known to be an inefficient process even in fully permissive cells, and proceeds by mechanisms that have yet to be fully elucidated (Harbison et al., 2008; Mani et al., 2006; Parrish, 2010; Porwal et al., 2013). Low expression of DNA polymerase δ-associated factors could explain the reduced conversion of incoming virus DNA in bovine cells, as these cellular components are known participants in initial replication of parvovirus genomes. Increased expression of proliferating cell nuclear antigen in certain cervical cancer isolates has been shown to enhance DNA replication of the parvovirus adeno-associated virus (Kang et al., 2009), suggesting that the expression level of these factors can alter parvovirus permissivity.

NS1 production and DNA replication

A critical early step of PPV replication is the production of multifunctional NS1, involved in multiple steps of the replication cycle (Brandenburger et al., 1990; Rhode, 1985, 1989). Early DNA amplification is lower in porcine cells when infected with Kresse than when infected with NADL-2 (Fernandes et al., 2011). Here, we showed that Kresse DNA amplification was delayed compared with NADL-2, but reached the same level at 16 h p.i. As NS1 is implicated in the early step of DNA amplification (Willwand et al., 1998), we hypothesized that NS1 production might be impaired in Kresse infection compared with NADL-2 infection. Indeed, as shown in Fig. 3, NS1 production in Kresse-infected cells was observed in only half the cells and was delayed compared with NADL-2 infection, despite the production of similar amounts of mRF DNA template. The synthesis of NS1 is thus an important replication difference between NADL-2 and Kresse infection in porcine cells. In bovine cells, generation of the mRF was clearly impaired compared with porcine cells. NS1 production was significantly impaired in these cells, for both strains, especially for Kresse. NS1 production is clearly a limiting factor in G11 cells. Genome amplification, which requires NS1, was also a restricted in G11 cells infected with Kresse (Fig. 4b). However, as DNA amplification was normalized to entering DNA at 8 h p.i. and very few G11 cells actually amplified virus, it was difficult to accurately measure low, yet effective viral DNA amplification.

Capsid protein production and localization

Infection of G11 cells with NADL-2 resulted in 20% of infected cells displaying aberrant cytoplasmic capsid...
Fig. 6. Chimeras of NADL-2 and Kresse strains. (a) Synonymous and non-synonymous substitutions between both strains (see Fernandes et al., 2011, for details). Viral chimeras from both strains were used to identify critical genome differences between the NADL-2 and Kresse genomes. The first letter in the name indicates the background strain and the last letter indicates the inserted coding region(s) (CR) from the other strain. (b) The switch of the allotropic determinant in primary TV cells (CR2) was first used. Virus released in the supernatant after infection with the designated strain or chimeras was measured by titration and is expressed as f.f.u. ml\(^{-1}\) (mean ± SD) from triplicate samples. (c, d) Chimeras of the different coding regions were used to identify important genome regions for good NS1 expression in porcine (c) and bovine (d) cells. The percentage of NS1-positive cells was determined using DNA staining for the total cell number, in triplicate wells. (e) The same chimeras were used to infect bovine G11 cells, and virus released in supernatant at 48 h p.i. was titrated and expressed as f.f.u. ml\(^{-1}\) (mean ± SD) from duplicate wells. All experiments are representative of three independent experiments (**)\(P<0.005\), (***)\(P<0.0001\).
localization (Fig. 5). These capsids were not entering virus, as this pattern was also observed at a low m.o.i. where entering virus could not be detected. They could represent either aberrant capsid formation prior to nuclear transport or complete virus not properly exported through the cytoplasm. Capsid formation in the cytoplasm has also been observed after B19 transfection in High Five insect cells (C. Belabani, unpublished observations). In either case, accumulation of capsids in the cytoplasm reduced the efficiency of viral infection, leading to lower amounts of virus released in the supernatant.

**Viral chimeras**

Differences between NADL-2 and Kresse replication in G11 cells were partially explained by using chimeras of both strains and assessing their capacity to express NS1. Except for the chimera containing all the NADL-2 coding regions (K-CR1/2/3-N), the best Kresse NS1 producing chimera (K-CR2/3-N; Fig. 6c, d) generated most infectious virus in CR1/2/3-N), the best Kresse NS1 producing chimera (K-CR2/3-N; Fig. 6c, d) generated most infectious virus in porcine cells, as demonstrated in our previous study (Fernandes et al., 2011). This chimera also produced most infectious virus in G11 cells (Fig. 6e). This emphasized the critical role of NS1 for efficient viral replication. Interestingly, the fragment CR2/3 is located in the VP gene and does not involve changes in the NS1 coding sequence. However, the CR2/3 sequences are also present in the 3′-UTR of NS1 mRNA. These nucleotide changes may alter the mRNA stability or might contain an enhancer element that would be responsible for the observed replication differences between the strains (Barrett et al., 2012).

Overall, PPV was shown to be strongly dependent on cellular factors throughout the course of its replication cycle. In porcine cells, NS1 production greatly depends on cellular processing of incoming viral DNA, and this step was impaired following Kresse infection. To our knowledge, this is the first report showing differences in NS1 expression according to the parvovirus strain. In G11 cells, multiple restriction points were observed. Low amounts of DNA processing clearly hindered the rest of the replication cycle. Moreover, even with equivalent amounts of mRF, Kresse replication still led to lower NS1 expression levels than observed with NADL-2. Kresse DNA amplification was also greatly impaired and barely above the detection limits of the assays. This report also demonstrates that non-host species cells may fully support productive replication of both pathogenic and non-pathogenic PPV strains (F4 bovine cell line, Fig. 1). Finally, further analysis of the non-permissive cells may provide some interesting findings pertaining to the cellular factors involved in the different restriction levels observed in these different host cells.

**METHODS**

**Cell lines and viral strains.** PT cells, a clone of ST cells (ATCC CRL-1746), were obtained as described previously (Bergeron et al., 1993) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 6.5% FBS (Wisent) and 2 mM L-glutamine (Invitrogen). The original primary testis TV cells (Bergeron et al., 1996) were stably transfected with plasmid SV3-neo expressing simian virus 40 large T-antigen and selected with neomycin (G418, 0.4 mg ml⁻¹). Individual cell clones were selected twice by limiting dilution under antibiotic selection and maintained in DMEM, supplemented as described for PT cells, with the addition of 0.1 mM non-essential amino acids (Invitrogen) and 1 mM sodium pyruvate (Invitrogen). Three cell lines (F4, G11 and D10) were chosen and stably maintained for at least 50 passages without any changes in morphology or phenotype to viral infection. The NADL-2 PPV strain was purchased from ATCC (VR-742) and the Kresse PPV strain was obtained as described previously (Bergeron et al., 1996). Periodically, NADL-2 and Kresse were obtained from original infectious clones to maintain stock purity. Viral stocks from both strains were grown on PT cells, and supernatants were used directly for infection after a brief centrifugation to remove cellular debris (20 min, 5000 g); titration of the viral stock was carried out with the PT cells as explained below. The absence of cross-contamination of the virus stocks was verified by periodic sequencing.

**Development of CPE after infection.** The degree of CPE developed in each cell line after infection by either strain at different m.o.i. was determined after seeding cells at low density in six-well plates (3.5 × 10⁴ cells per well for PT cells, and 5 × 10⁴ cells per well for F4, G11 and D10 bovine cells). The cultures were visually scored for the appearance of cell rounding or ‘stretching’ and detachment from the bottom of the wells at different times p.i. Only actively growing cells susceptible to parvovirus infection (Tattersall, 1972); therefore, the degree of CPE was scored only until the mock-infected cells reached confluency. Results were compiled from duplicate wells in at least four independent experiments.

**Immunofluorescence and viral titres.** Indirect immunofluorescence was carried out as described previously (Boisvert et al., 2010). Titrations were performed on PT cells in 96-well plates. Briefly, at 20 h p.i., cells were fixed in 3% formaldehyde, permeabilized with 3% Triton X-100 and treated with mAb 3C9-D11-H11 (‘3C9’, ATCC CRL-1745, diluted 1 : 50), which binds to both full and empty capsids, followed by secondary Alexa Fluor 488-conjugated goat anti-mouse antibody (diluted 1 : 2000; Invitrogen). Capsid-positive nuclei were counted in triplicate wells containing 20–200 fluorescent foci and titres of the inoculum were calculated as f.f.u. ml⁻¹. For confocal imaging, cells were seeded on glass cover slips in 24-well plates, infected at an m.o.i. of 5 f.f.u., fixed at different times p.i. and permeabilized as described above. mAb 3C9 was used to detect capsids while anti-VP2 antibody (1 : 1500), generated by an N-terminal peptide immunization of rabbits (Zadóri et al., 2005), was used to detect full capsids and VP1 and VP2 in non-assembled states. Anti-NS1 mAb (Yeung et al., 1991) was kindly provided by Dr David Pintell (University of Missouri, MO, USA). This antibody was generated against MVM NS1 and mapped to aa 656–663 (based on MVM sequence; GenBank accession no. J022751)., which are conserved in PPV (GenBank accession no. NC_001718.1), except for the last one (T663E). Secondary antibodies were goat anti-mouse or goat anti-rabbit conjugated with either Alexa Fluor 568 or Alexa Fluor 488 as indicated (all 1 : 2000; Invitrogen). Coverslips were mounted on glass slides with Fluor Preserve (Calbiochem). Images were collected on a Zeiss LSM780 system equipped with a 30 mW 405 nm diode laser, a 25 mW 458/488/514 argon multiline laser, a 20 mW DPSS 561 nm laser and a 5 mW HeNe 633 nm laser mounted on a Zeiss Axio Observer Z1 and operated with Zen 2011 software (Zeiss). We used a Plan-APochromat ×63 oil DIC 1.4NA objective for our observations.

**Cell-cycle (S-phase) analysis.** Cells were seeded on glass coverslips in 24-well plates to obtain 40–50% confluency after a 24 h
incubation. 5-Ethynyl-2’-deoxyuridine (EdU) incorporation was established both for 1 h pulses after 24 h of cell growth and over a 24 h period 24 h post-plating. For pulse-labelling assays, coverslips were inverted on 15 μl complete medium supplemented with 10 μM EdU, and incubated for 1 h at 37 °C in a humidified incubator under 5% CO2. Coverslips were then fixed as described above. For the 24 h experiment, cell medium was supplemented with 4.2 μM EdU at 24 h post-plating; incubation was continued for an additional 24 h and cells were fixed as above. After fixation and permeabilization, cells were labelled with a Click-IT EdU Cell Proliferation Assay (Invitrogen) according to the manufacturer’s recommendations. Total cell DNA was then stained with Hoechst 33342 (2 μg ml-1; Sigma) for 30 min. Cells were mounted on glass slides and counted under a fluorescence microscope. In each assay, the ratio of EdU-Alexa Fluor 488-labelled cells to total cells (Hoechst) was scored from at least 300 total cells in duplicate slides, from at least three independent experiments. Results were expressed as mean percentage of cells in S phase ± SD.

Virus uptake assays. Cells were seeded on glass coverslips at 5 x 10⁴ cells per well. The next day, cells were infected with either NADL-2 or Kresse virus at an m.o.i. of 5. At 2 h p.i., cells were washed extensively with PBS and harvested in STE buffer (1 mM EDTA, 150 mM NaCl, 20 mM Tris/HCl, pH 7.5). DNA was extracted as described previously (Boisvert et al., 2010). Purified DNA samples were diluted 1:10 for PCR purposes. Cell-associated viral DNA was quantified by qPCR.

qPCR. qPCR on PT cells was performed as described previously (Boisvert et al., 2010). For bovine cell normalization, bovine c-myc (GenBank accession no. AF519455) was cloned in pSmart-HC-AmPkr (GenBank accession no. AF399742) using forward primer 5’-CTTGATGGCTACCCCTCT-3’ and reverse primer 5’-CIGTCTAGAGTAGCTGTTCAAGTTTGAGTTTCAAC-3’ between HindIII and XbaI sites. qPCR was performed with forward primer 5’-AGCGCCAGAGGAGAAAACGGAG-3’ and reverse primer 5’-GTTTCAACTGTCTCGCCTCTTCTG-3’ and was linear in the range 0.5 fg to 1 ng. Primer specificity was verified using BLAST analysis and melting-curve analysis from 60 to 95 °C with a temperature increase at each stop of 1 °C for 30 s. Viral DNA was expressed in genome copy equivalents (GCE). Results shown are either GCE or mean fold increase ± SD (log GCE) from at least three independent experiments.

Southern blotting. Cells were seeded in duplicate wells of a six-well plate (2 x 10⁵ cells per well) and infected with either viral strain (m.o.i. of 5). At each time point, cells were harvested by trypsinization. Cell pellets were resuspended in 200 μl STE buffer and frozen and thawed at −20 °C until DNA was extracted as described previously (Boisvert et al., 2010), but DNA was resuspended in a final volume of 40 μl H2O. Total DNA extracted for each sample was loaded on a 0.8% agarose gel and electrophoresis was carried out at 80 V for 2 h. Southern blotting was performed as described in the DIG Application Guide for Filter Hybridization (Roche Applied Science). Briefly, DNA was depurinated, denatured, neutralized and vacuum transferred to positively charged nylon membranes and fixed by UV crosslinking. A PCR fragment of the PPV NS1 coding region was labelled by random priming with DIG-High-Prime and was hybridized to membranes. Detection of the probe was performed with anti-DIG mAb (Roche Applied Science) and alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:1000; Bio-Rad). Colorimetric detection was carried out with nitro blue tetrazolium chloride (NBT) and BCIP (Roche Applied Science) according to the manufacturer’s recommendations. The blots shown are representative of at least three independent experiments.

Western blotting. Western blot experiments were done as described previously (Boisvert et al., 2010). Briefly, after infection with either strain of PPV, PT cells were lysed at the indicated times p.i. An equivalent amount of cell lysate from each sample was loaded on a 10% acrylamide gel. After transfer onto nitrocellulose membrane and blocking, incubation with primary antibody was performed with mouse anti-NS1 mAb (as described above) diluted 1:100, together with mouse anti-actin mAb (1:200; Santa Cruz Biotechnologies) as a loading control. The secondary antibody was goat anti-mouse conjugated with alkaline phosphatase (1:1000; Bio-Rad). Detection was performed with NBT/BCIP colorimetric substrate according to the manufacturer’s instructions.

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