JOURNAL OF VIROLOGY, Mar. 2011, p. 3025–3029 Vol. 85, No. 6
0022-538X/11/$12.00 doi:10.1128/JVI.02215-10
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Genetic Elements in the VP Region of Porcine Parvovirus Are Critical to Replication Efficiency in Cell Culture[▽]

Sandra Fernandes, Maude Boisvert, and Peter Tijssen*

INRS—Institut Armand-Frappier, Université du Québec, 531 boulevard des Prairies, Laval, Québec, Canada H7V 1B7

Received 21 October 2010/Accepted 28 December 2010

Factors controlling porcine parvovirus (PPV) replication efficiency are poorly characterized. Two prototype strains of PPV, NADL-2 and Kresse, differ greatly in pathogenic capacity both *in vivo* and *in vitro*, yet their genomic sequence is nearly identical (13 single-nucleotide substitutions and a 127-nucleotide noncoding repeated sequence). We have created a series of chimeras of these strains to identify the genetic elements involved in replication efficiency in the host porcine cell line. While the capsid proteins ultimately determine viral replication fitness, interaction between the NS1 protein and the *VP* gene occurs and involves interaction with the noncoding repeated sequence.

Porcine parvovirus (PPV) is a nonenveloped icosahedral virus that causes reproductive failure in swine. Like its close relatives from the *Parvovirus* genus (*Parvovirinae*, *Parvoviridae* family), PPV has a linear, single-stranded 5-kb DNA genome of negative polarity, characterized by distinct hairpin termini (23). The large nonstructural (NS) protein NS1 is involved in genome replication (15, 18), transcription regulation (11, 19), and cytotoxicity (1, 16), while NS2 participates in capsid assembly (6) and nuclear export (9, 14). The structural proteins VP1 and VP2 assemble in a 1:10 ratio to form the 25-nm-diameter capsid (3, 12, 25). Little is known about the mechanisms that control parvovirus tropism. For PPV, permissivity is not determined at the cell surface (17, 20), since a high proportion of virus has been shown to enter cells by nonspecific pathways similar to that of macropinocytosis (4). The genomes of two PPV strains, NADL-2 and Kresse, differ by only 13 nucleotides (nt) and by a 127-nt repeated sequence near the right-end hairpin in the NADL-2 genome, yet they replicate with different efficiencies in both porcine and bovine cells (Fig. 1A) (2, 24). Some strains of the parvoviruses minute virus of mice (MVM), canine parvovirus (CPV), and H1 also have variable tandem repeats (65, 60, and 55 nt, respectively) (8). The benefits of these repeats for replication remain controversial (5, 21).

Interestingly, a sequencing analysis of PPV contaminants in pancrelipase extract pools suggested that the variability of North American isolates is very low compared to those of European, Brazilian, and Chinese isolates (Table 1) (13, 29, 30). While no substitutions from the Kresse strain were observed in the C-terminal portion of the VP proteins (nt 3869 to 4546) (2), nearly full-length sequences of the *NS* gene revealed that the contaminants contained substitutions from both stains (NADL-2, c537 and a1971; Kresse, a405, g1533, and c1668). A single nonsynonymous substitution in the NS1 region was observed to occur in two lots from 2009 (a875g or R195K; NS1 numbering). The significance of these changes is unknown at present.

To determine which genetic elements of NADL-2 and Kresse are involved in the difference in replication efficiency in the host porcine testis (PT) cell line, chimeras were constructed from the infectious clones (10). A seamless cloning system was used to swap segments in the right-hand terminus, including the hairpin, the 254-nt or 127-nt repeated sequence from the NADL-2 (Rn) or Kresse strain (Rk), respectively, and the terminal 524 nt of the *VP* gene (coding region 3 [CR3]) (Fig. 1A). Two other segments were swapped in the rest of the genome using classical methods. The first fragment, CR1, encompassed the entire *NS* gene and the 5′ region of the *VP* gene. The second segment (CR2), corresponding to the central part of the VP region, contained two of the three nonsynonymous substitutions (D378G and H383Q from NADL-2 to Kresse; VP2 numbering) previously identified as part of the allotropic determinant for primary bovine testis cells (2). The third residue from this determinant (S436P), within CR3, was recently shown not to be involved in tropism (S. Fernandes, M. Boisvert, J. Szelei, and P. Tijssen, submitted for publication). In total, 14 different chimeras were constructed (Fig. 1B; the chimera naming scheme is X-YnRxy, with X being the backbone, Yn the inset from the other strain, and Rxy the repeat) and transfected in PT cells (28). Infection of fresh cells with the transfection supernatant produced small virus stocks that were titrated by immunofluorescence (IF) (4) and verified by sequencing of PCR fragments overlapping the entire genome. These stocks were used to infect PT cells at a multiplicity of infection (MOI) of 2 fluorescent focus-forming units (FFU) to compare replication efficiencies in single-round infection assays.

VP gene elements and repeats influence replication efficiency. Viral genome replication levels were monitored by quantitative PCR (qPCR) of cell lysates harvested at different times postinfection (p.i.) as described previously (4, 26). Results are expressed as the increase in the log of viral genome copy equivalents (GCE)/cell over initial GCE/cell at 8 h p.i. (Fig. 2A and B) (27). Early genome replication of Kresse was significantly lower than that of NADL-2 (Fig. 2A). Introducing CR2N into Kresse increased its early replication significantly or even to NADL-2 levels (K-N2, K-N2Rn, K-N23, K-N23Rn).

* Corresponding author. Mailing address: INRS—Institut Armand-Frappier, Université du Québec, 531, boul. des Prairies, Laval, Québec, Canada H7V 1B7. Phone: (450) 687-5010. Fax: (450) 686-5627. E-mail: peter.tijssen@iaf.inrs.ca.

▽ Published ahead of print on 5 January 2011.

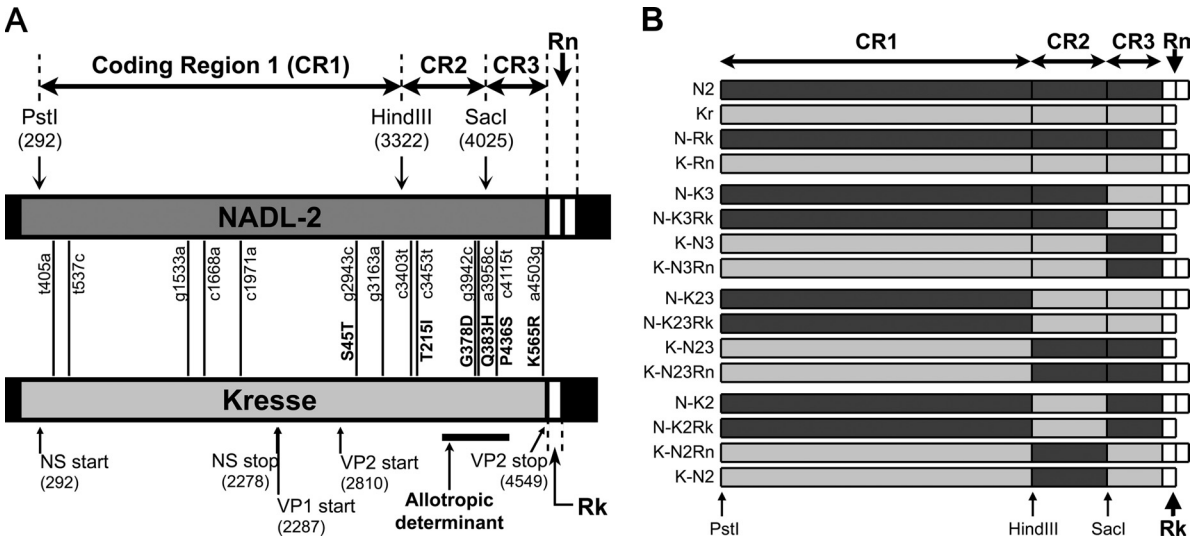

FIG. 1. Construction of chimeras from the NADL-2 and Kresse PPV strains. (A) The genomes of the two wild-type strains differ by 13 nt in their coding regions (CR1 to CR3) (gray boxes) and by the repeated sequence downstream of the *VP* gene (white boxes). No differences are located within the hairpin termini (black boxes). The 127-nt (Rk) or the 254-nt (Rn) repeated sequence and the terminal 524 nt of the coding region (CR3) were amplified by PCR and swapped between backbones by seamless cloning (10). The CR1 and CR2 fragments were swapped in the infectious clones by classical methods. The CR1 fragment (PstI-HindIII, nt 290 to 3322) contained the entire NS coding region, including five synonymous mutations from NADL-2 to Kresse (a405g, c537t, a1533g, a1668c, a1971c) and the first of the nonsynonymous substitutions in the VP coding region (T45S in VP2 and L42V in SAT from NADL-2 to Kresse) (3, 28). The CR2 fragment (HindIII-SacI, nt 3322 to 4025) contained both silent mutations in the VP gene (nt g3163a, c3403t) and three of the nonsynonymous substitutions (VP2 I215T, D378G, H383Q from NADL-2 to Kresse). The C-terminal portion of the VP coding region contained the final two substitutions between the strains (VP2 residues S436P and R565K). Residues D378G and H383Q in CR2 and S436P in CR3 are in the BglII fragment that was previously identified as the allotropic determinant of the PPV strains in primary bovine testis cells (3). (B) A total of 14 chimeras were constructed by swapping CR1, CR2, CR3, and Rn or Rk between the NADL-2 (N2, dark gray) and Kresse (Kr, light gray) strains. The naming scheme is X-YnRxy, with X being the backbone, Yn the inset from the other strain, and Rxy the repeat.

However, the inverse construct (N-K2) did not decrease NADL-2 levels to that of Kresse unless the repeat was also swapped (N-K2Rk). N-K23 and N-K23Rk appeared normal in early replication but had a diminished level of replication from 10 to 20 h p.i. (Fig. 2B). Replication was usually further impaired by introducing CR3N into Kresse (K-N23 and K-N23Rn versus K-N2 and K-N2Rn; K-N3 versus Kresse) unless the NADL-2 repeat was introduced too (K-N3Rn versus K-N3). Swapping CR3N into Kresse replaces a lysine on the capsid surface with an arginine; this could affect ubiquitination, which was recently shown to be critical for PPV infection (4). At 20 h p.i., most differences in viral DNA replication were no longer apparent (Fig. 2B). Notable exceptions were the above-mentioned N-K23 and N-K23Rk as well as construct N-K3, which all showed surprisingly normal replication between 8 and 10 h p.i. In contrast, N-K3Rk displayed a replication level similar to that of other chimeras. These results suggested that repeat regions (R) interacted with elements in the *VP* gene or the capsid, both early and late in the infection.

CR2 and CR3 from NADL-2 are required for optimal virus production. Infectious virus production was monitored at different times p.i. by titration of supernatants on PT cells by IF (4). Release of virus was apparent by 16 h p.i. (data not shown) and continued to increase until 24 h p.i. (Fig. 2C), by which time cytopathic effects were apparent. As seen with late genome replication, combining CR2K or CR3K with Rn mark-

TABLE 1. Sequence analysis of PPV pools in pancrelipase extracts produced in North America between 2005 and 2009[a]

Strain	Nucleotide(s) at position:										
	403	405	537	875	1533	1668	1971	3942	3958	4115	4503
NADL-2	c	g	c	*a*	a	a	a	**a**	**c**	**t**	g
Kresse	c	a	t	*a*	g	c	c	**g**	**a**	**c**	a
PPVAx2005	ND	ND	c	ND	g	c	a	**g**	**a**	**c**	a
PPVAx2007	tc	ta	c	ND	g	c	a	**g**	**a**	**c**	a
PPVAx2009-1	tc	ta	c	*g*	g	c	a	**g**	**a**	**c**	a
PPVAx2009-2	tc	ta	c	*g*	g	c	a	**g**	**a**	**c**	a

[a] PPV genomes were amplified by overlapping PCR fragments and sequenced by standard methods. Some substitutions were not fully conserved (nt 403 and 405). Sequences covered the following nucleotides in the different samples: PPVAx2005, nt 1279 to 2345 and 3848 to 4679; PPVAx2007, nt 308 to 832, 1018 to 2343, and 3869 to 4675; PPVAx2009-1, nt 303 to 2343 and 3842 to 4677; PPVAx2009-2, nt 303 to 2369 and 3862 to 4546. Sequences were compared to those of NADL-2 (NCBI reference sequence accession no. NC_001718.1) and Kresse (GenBank accession no. U44978.1) strains. Residues in bold are within the BglII fragment (allotropic determinant) identified for bovine cells (2). Residues in italics are different from those in both prototype strains. ND, not determined.

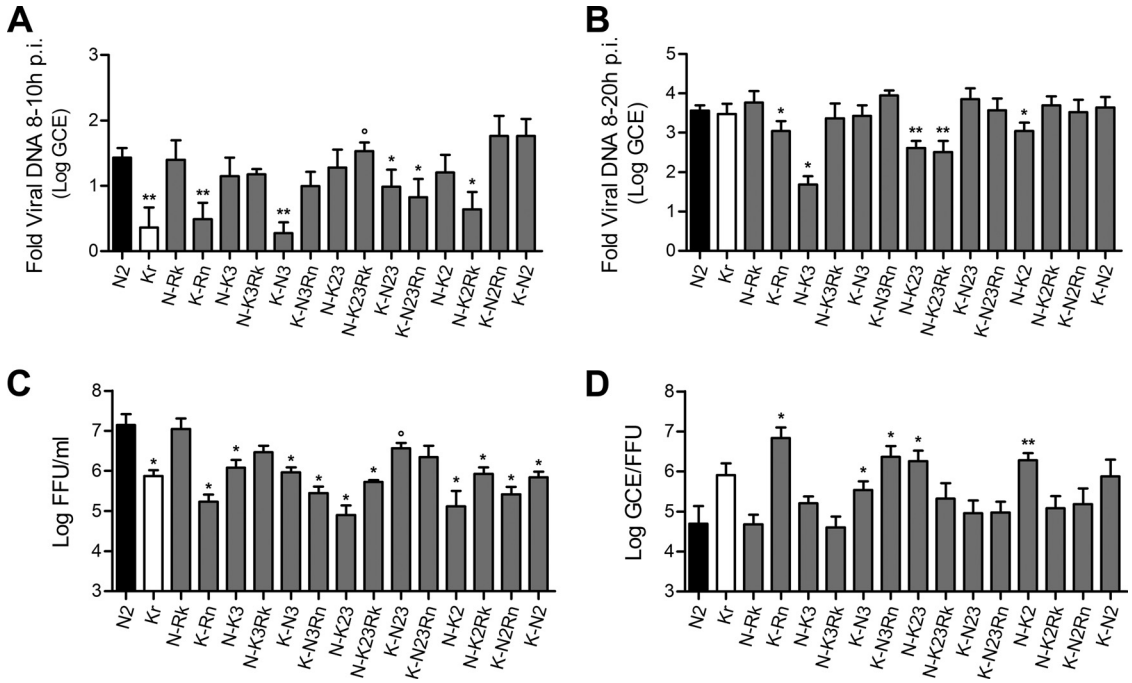

FIG. 2. Replication of the chimeras and wild-type strains in single-round infections assays. Early (A) and late (B) viral genome replication. Viral genome copy numbers were measured by qPCR and normalized to cell numbers using the c-*myc* gene to account for differences in efficiency in DNA extractions and between independent experiments (4, 26). Results are expressed as the increase in the log of genome copy equivalents (GCE) between 8 and 10 h p.i. (A) or between 8 and 20 h p.i. (B) (mean + standard deviation [SD] from at least three representative independent experiments). (C) Infectious virus production at 24 h p.i. Supernatant from cells infected with either chimera was titrated by immunofluorescence (IF) (4) and expressed as log FFU/ml (mean + standard error of the mean [SEM]) from at least three representative independent experiments. (D) Infectivity of the virus released at 20 h p.i. Supernatants from infected cells were titrated by IF, and viral genome copy numbers were determined by qPCR. Results are expressed as log GCE/FFU (mean + SD). Means were compared by unpaired two-tailed *t* test for NADL-2 (*, $P < 0.05$; **, $P < 0.01$) and Kresse (°, $P < 0.01$).

edly decreased infectious virus production. Furthermore, chimeras containing both CR2N and CR3N released more infectious virus regardless of Rn/Rk or CR1. No differences were observed in levels of VP protein accumulation at 16 h p.i. as assessed by Western blotting, suggesting that lower virus production was not due to reduced VP synthesis (data not shown). Capsid stability may be increased when CR2 and CR3 from the same strain are combined, since VP2 residues 378 and 383 from CR2 are positioned on adjoining sides of loop 3, while residue 565 from CR3 is positioned on the loop immediately adjacent to it, in close contact with the conserved residue L384 (22).

CR2 from Kresse and Rn are incompatible for efficient packaging. The infectivity ratio of the virus released from each chimera was determined by comparing the number of viral genome copies in the supernatant (by qPCR) to the infectious titers measured by IF. At 20 h p.i. (Fig. 2D), concurrent with the bulk release of virus, significant differences in the infectivity ratios were seen, most notably when CR2K and Rn were combined (K-Rn, K-N3Rn, N-K23, N-K2). Although VP residues 215, 378, and 383 are located on or near the capsid surface, we propose that the DNA sequence may also interact with the packaging machinery. Recent studies of MVM have shown that the NS1 protein binds with different affinities to moderately conserved consensus binding sites (TGGT) scattered across the genome and may serve to stabilize the DNA, much like chromatin (7). Interestingly, in the PPV coding regions, there are 21 potential NS1 binding sites in the CR2 fragment, while only 56 more are found in the rest of the protein coding sequence (21 [TGGT] in 703 nt or 1 site every 33 nt in CR2 versus 56 in 3,554 nt or 1 in every 63 nt in CR1 and CR3 combined). Furthermore, the t3453c substitution (VP2 I215T from NADL-2 to Kresse) potentially creates an additional binding site within the Kresse genome. Since NS1 binding sites also occur in the repeated region (three in Rn and two in Rk), negative interactions may occur between the extra binding sites found by combining CR2K and Rn. This may slow the packaging process such that a higher percentage of genomes would be partially encapsidated, therefore increasing the ratio of GCE to FFU.

CR2 and CR3 from NADL-2 ultimately determine viral fitness. To identify the genomic fragment that most significantly contributed to replication efficiency, a fitness competition assay was designed. All possible pairs of chimeras were cotransfected in PT cells. After two subsequent passages, the dominant chimera in the supernatant was identified by comparing the sequence (Fig. 3A) and the size (Fig. 3B) of PCR fragments. Sorting the chimeras according to dominance indicated that the combination of CR2 and CR3 was the most important determinant of fitness in PT cells, while Rn was a secondary factor (Fig. 3C). When all else was equal, clones containing CR3N were dominant over those containing CR3K, suggesting that residues S436 and R565 are crucial to viral fitness. In the prevailing chimeras containing Rn, CR1 invariably came from

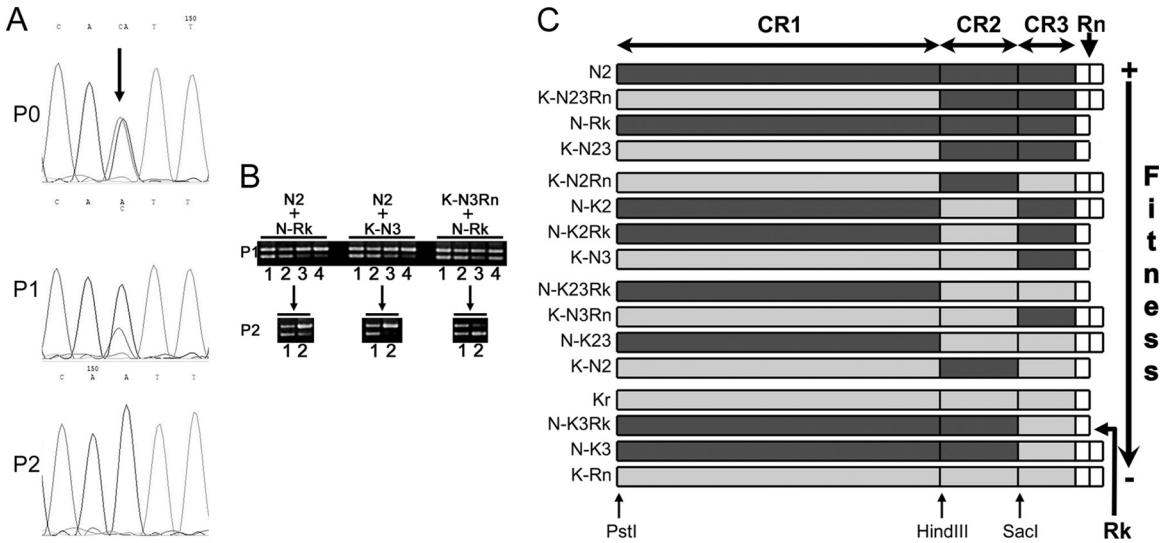

FIG. 3. Relative replication fitness of the chimeras. (A) The chimeras were cotransfected in pairs in PT cells, and the supernatants were used to reinfect cells for one or two viral passages. PCR of final supernatants (Sn) were analyzed by sequencing (A) to determine dominance. As an example, shown is the amplification of CR2 (c3958a) after cotransfection of chimeras N-K3 and N-K2Rk (P0, sequencing of input DNA for transfection; P1, sequencing of Sn from 1st viral passage; P2, sequencing of Sn from 2nd viral passage). (B) Dominance was also verified by comparing the amount of DNA in Sn at different times posttransfection (P1) and after the first viral passage (P2), in cotransfection with chimeras differing in the origin of Rn (top band) and Rk (bottom band). P1, PCR of Sn from cotransfections with N2 and N-Rk, N2 and K-N3, or K-N3Rn and N-Rk. Lane 1, PCR of input DNA; lane 2, PCR of Sn harvested at 24 h; lane 3, PCR of Sn harvested at 48 h; lane 4, PCR of Sn harvested at 72 h. P2, Sn harvested at 24 h from cotransfections was used to infected fresh cells. Lane 1, PCR of input virus (same as P1, lane 2); lane 2, PCR of Sn harvested at 48 h p.i. from 1st viral passage. Based on the results shown, N2 is dominant over N-Rk, N2 is dominant over K-N3, and N-Rk is dominant over K-N3Rn. (C) Relative replication fitness of each chimera was determined from dominance of each clone in cotransfection and reinfection experiments (120 combinations in total, two rounds of reinfection, in three independent experiments) determined as shown in panels A and B.

the same background as CR3, while CR1 matched CR2 when combined with Rk. These results suggest that the NS proteins may interact differently with the viral genome depending on the origin of the repeated sequence, possibly due to the low-affinity binding sites scattered throughout the genome. The fitness assay allowed an improved ranking of all the chimeras compared to the general information given in Fig. 2. As expected, the chimeras with the relatively fittest replication (Fig. 3C) demonstrated high levels of FFU in single-round production (Fig. 2C), whereas clones showing a high GCE-to-FFU ratio (Fig. 2D) displayed a lower level of fitness.

Comparison of NADL-2 and Kresse chimeras highlighted the multiple levels that influence replication efficiency. While the *VP* gene (regions CR2 and CR3) from the NADL-2 strain was the most important for viral fitness, both the proteins and the genome itself could be involved, since there was a low level of late genome replication in NADL-2 chimeras containing either CR2K or CR3K. Furthermore, the NADL-2 capsid may have slightly higher stability than Kresse, while packaging of NADL-2 was more efficient than in hybrid capsids. Finally, we have demonstrated that residues outside the bovine cell allotropic determinant are involved in the replication efficiency of Kresse and NADL-2 strains in porcine cells.

This research was funded in part by a grant from the Natural Sciences and Engineering Council of Canada to P.T. and by scholarships from the Fondation Armand-Frappier to S.F. and M.B.

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