

## Genome Organization of the Kresse Strain of Porcine Parvovirus: Identification of the Allotropic Determinant and Comparison with Those of NADL-2 and Field Isolates

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**The Kresse strain of porcine parvovirus (PPV) was cloned into pUC19, and independent infectious clones were sequenced. The PPV Kresse and NADL-2 strains, which have different pathogenicities, shared an identical genomic organization and a high degree of sequence identity. Partial genomes (1.5 or 1.6 kb) of 15 field isolates were also amplified by PCR in regions with significant sequence differences between the laboratory strains. Five amino acid differences were consistently present within the VP1/VP2 coding region of the Kresse strain and virulent field isolates. A number of inconsistent point mutations were also found throughout the genomes of field isolates. In addition, among those with the vaccine amino acid profile, all but one isolate (IAF-3) contained a 127-bp noncoding direct repeat downstream of the capsid protein gene. The one exception was also the only vaccine-type PPV obtained from a mummified fetus. In order to identify genetic elements responsible for the distinct tropism (and possibly the pathology) of the Kresse strain, *in vitro* cell systems which differentiated the virulent from the vaccinal strains were established. Subsequently, chimeric infectious clones of the Kresse and NADL-2 strains were used to identify the allotropic determinant located in the VP1/VP2 region. The transfer of the *Bg/III* fragment of the Kresse genome, containing three amino acid differences, into the NADL-2 background, or the opposite construct, caused the phenotype of the target genome to revert to that of the parent strain of the *Bg/III* fragment. Prediction of the localization of amino acid differences on the basis of canine parvovirus capsid structure indicates that each is located on or near the outer surface of the virion. In particular, the position of one mutation (S-436→P) maps by analogy to the threefold spike, the most accessible region of the capsid.**

Porcine parvovirus (PPV) is an autonomously replicating parvovirus with physicochemical properties and genomic sequences which resemble those of minute virus of mice (MVM) and H-1 rodent parvoviruses and which particularly resemble those of canine parvovirus (CPV) and feline panleukopenia virus (FPV) (reviewed in references 9 and 32). PPV is the major causative agent in a syndrome of reproductive failure in swine, which includes stillbirths, mummified fetuses, early embryonic death, and infertility (11, 17). PPV strains can be distinguished by their differential pathogenicity (20). The nonpathogenic NADL-2 strain, which is currently used as an attenuated vaccine, causes only a limited viremia and, in experimental infections, does not cross the placental barrier (24). In contrast, virulent strains isolated from mummified fetuses, such as NADL-8 and IAF-76, cause a viremia and cross the placenta, resulting in fatal infection of nonimmunocompetent fetuses (16). Although NADL-2 is innocuous when administered orally, it is responsible for fetal death when injected directly in utero into extrafetal fluids (10). Strain NADL-8 was both infectious and lethal at much higher dilutions than was strain NADL-2 (>10,000-fold [18]).

A third group of virulent PPV strains, such as the Kresse and IAF-A54 strains, have been associated with dermatitis (15). These dermatitis strains display an increased virulence and, in contrast to the other virulent strains, kill immunocompetent

fetuses (8). Finally, a fourth group of PPVs has recently been recognized by our laboratory after PCR amplification of parvovirus DNA from enteric samples (10a). These enteric strains (IAF-A83), recently involved in large outbreaks, have been characterized only partially (unpublished observations).

The mRNAs of NADL-2 have been isolated, cloned, and sequenced (5). All splicing patterns and the 5' and 3' ends of the mRNAs have been identified. The genome contains two promoters—the P4 promoter leading to the expression of the nonstructural (NS) NS1, NS2, and putative NS3 proteins, which are involved in a number of replicative functions of the virus; and the P40 promoter, which is involved in expression of a nested set of structural protein-coding sequences termed VP1 and VP2 with molecular masses of 80.9 and 64.3 kDa, respectively (reviewed in reference 31). Three NS protein gene transcripts have been identified. The 4.7-kb transcript is not spliced in the NS gene and was predicted to code for a 75.5-kDa protein (NS1). The two differently spliced 3.3-kb NS transcripts code for the NS2 protein (18.1 kDa). A 2.9-kb transcript would code for an NS-3 protein (12.4 kDa), although such a protein has never been described for other parvoviruses.

Our objectives are to obtain a better understanding of the molecular biology of PPV and to identify the mechanisms responsible for differences in the biological activities of the various strains. In the present study, the complete Kresse strain genome was cloned and sequenced. In addition, partial sequences of several infectious clones were determined. The genomic organization of PPV Kresse was found to be almost identical to that of NADL-2, but key differences were observed in the region coding for the capsid proteins. The allotropic determinant has been identified with chimeric constructs and

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was compared with those of several PPV strains and virus isolates which were obtained from a relatively large number of aborted fetuses and normal tissues.

(This work will be submitted in part by B.H. in a thesis to the Centre de Recherche en Virologie, Institut Armand-Frappier, in partial fulfillment of the requirements for the Ph.D. degree.)

## MATERIALS AND METHODS

**Enzymes, reagents, and oligonucleotides.** Enzymes (restriction endonucleases, polymerases, phosphatases, kinases, and ligases) were obtained from Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs, Pharmacia Biotech, or Promega. [ $\alpha$ - $^{35}$ S]dATP (800 Ci/mmol) was purchased from Amersham Corporation. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia). Oligonucleotides used for primer-walking sequences are not indicated because of space limitations, whereas the following were used for PCR amplification: PPV-PR5G (positions 3262 to 3291), 5' GTGGGTACAGAATCA GCAACCTCACCACC; PPV-PR06 (positions 4858 to 4833), 5' GITAATAGT AACACATGAGAGCTTG 3'; and PPV-PR27 (positions 3277 to 3295), 5' GTGGTACCAGAATCAGCAACCTCACCACCA 3'.

**Cell lines and production of virus.** Kresse parvovirus was kindly supplied by M. L. Frey (National Veterinary Service Laboratory, Ames, Iowa), while the NADL-2 reference strain was obtained from the American Type Culture Collection (ATCC VR-742). Several PPV isolates were obtained from thyroid cells (obtained from a local slaughterhouse), whereas other isolates were obtained from samples (fetuses, mummies, and lungs) submitted to the Service de Diagnostique Virologique (MAPAQ) at our institute.

Two cell lines and one primary cell culture were used throughout the experiments reported. The porcine testis (PT; clone from swine testis [ST] cells) and porcine fallopian tube (PFT [6]) cell lines and a primary culture of bovine testis (TV) cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with penicillin (100 IU/ml), streptomycin (50  $\mu$ g/ml), and 8% fetal calf serum (HyClone).

The characterization of PFT cells has been discussed in a prior publication (5). The susceptibility of PFT cells to PPV was established at many passages (susceptibility varies with passage number), but for virus production, only PFT cells at passages 85 to 90, PT cells at passages 190 to 210, or TV cells at passages 3 to 5 were used. Semiconfluent cells were infected with PPV at a multiplicity of infection of 0.1 to 2.0, and after a 2-h adsorption period at 37°C, the cells were trypsinized and the culture was split at a ratio of 1:3.

Cells with a pronounced cytopathic effect (7 to 10 days postinfection [p.i.] for PFT cells but 4 to 7 days p.i. for PT cells) were frozen and thawed three times, and then the cellular debris was removed by centrifugation for 30 min at 8,000  $\times$  g. For virus purification, one-fifth volume of 20% polyethylene glycol 8000-2.5 M NaCl was added to the supernatant. The virus was collected by centrifugation for 30 min at 30,000  $\times$  g after an overnight incubation at 4°C, and the pellet was dissolved in a small volume of 1 $\times$  TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA).

**Cloning of the Kresse genome and PCR amplicons from field isolates.** Replicative-form DNA from the Kresse strain was extracted from infected PT cells (24 h p.i.) as previously described (5). The DNA was subjected to electrophoresis in agarose (0.8%), and the viral replicative form DNA band at a position corresponding to approximately 5 kbp was excised and electroeluted. Complete genomic replicative form DNA (after being blunt ended with S1 nuclease and Klenow enzyme) was cloned into *Sma*I-digested pUC18. XL1-Blue competent cells were transformed with 10 ng of DNA by standard methods (29). Genomes from other PPV strains were either cloned as for the Kresse strain or partial genomes were amplified by PCR from 5  $\mu$ l of cell culture supernatant with primers PPV-PR5 and PPV-PR6 (yielding fragments from positions 3389 to 4857) followed by cloning in pUC19. These constructs were sequenced from 66 to 91 map units. Another field isolate, IAF-A54, was amplified with primers PPV-PR27 and PPV-PR6, which generated a 1.6-kbp fragment. Semidirectional cloning of this fragment into pBluescript II KS- was performed after blunting of the amplicon followed by digestion with *Hind*III.

**Determination of nucleotide sequence.** The nucleotide sequence of the Kresse strain and field isolates was determined by primer walking. Approximately 30 primers synthesized according to the NADL-2 strain sequence were used to sequence the Kresse strain (5). The sequence was determined either by the conventional dideoxy chain termination method with [ $\alpha$ - $^{35}$ S]dATP (30) or by the dideoxy chain termination method on an A.L.F. automated sequencer with the Autoread kit (Pharmacia Biotech). Terminal hairpins were resolved on conventional sequencing gels containing 30% formamide as previously described (5). Sequence differences in clones obtained after PCR amplification were confirmed with clones derived from independent amplifications to rule out PCR-generated differences.

Sequences were analyzed and aligned with either software package DNAsis v6.00 (Hitachi) or PC/Gene v6.7 (Intelligenetics) or applications from the GCG suite (v7.2; Genetics Computer Group).

**Infection and transfection in different cell lines.** Semiconfluent monolayers of PT or PFT cells were transfected with approximately 10  $\mu$ g of undigested PPV clones per 10<sup>6</sup> cells by the calcium phosphate method described by Merchinsky

et al. (19). The production of virus was monitored by agglutination of guinea pig erythrocytes and confirmed by electron microscopy (2). Infections of PT, PFT, and TV cells were performed as described above, except that cells were not split when the relative infectivity of the chimeric constructs was compared with that of the wild-type virus.

**Allotropic determinants.** Chimeric constructs were generated from either cloned NADL-2 or Kresse. The *Bgl*II fragment was digested, dephosphorylated with calf intestinal phosphatase, and gel purified. Fragments from NADL-2 and Kresse were recloned in the other background strain. Clones were transfected in PT cells as described above, and chimeric viruses were isolated from cultures 5 to 7 days p.i. Virus was isolated from the cells after three freezing-thawing cycles and was regrown only once to avoid culture-induced changes. The recombinant nature of the chimeras was determined by differential PCR and sequencing.

**Nucleotide sequence accession number.** The sequence for PPV Kresse has been deposited with the Genome Sequence Database and was assigned accession number U44978.

## RESULTS

### Sequence analysis of PPV Kresse and genome organization.

The nucleotide sequence of infectious clones of PPV Kresse and its genomic organization were determined (Fig. 1). The sequencing of the genome termini required the addition of 30% formamide to the gel in order to resolve all compressed areas, such as the heavily compressed 3'-terminal palindrome of the positive strand. Like the NADL-2 strain, the Kresse genome contains two large open reading frames (ORFs). The translated left ORF had a similarity of approximately 80% to the NS1 protein of MVMp (3) and the translated right-hand ORF was found to have a homology of about 56% with VP1 of MVMp, while the similarity to the CPV ORFs showed identities of 67 and 75%, respectively.

A number of differences between the NADL-2 and Kresse genomes were identified. The noncoding regions of the genomes are nearly identical (two differences). All four of the differences found in the NS region are silent, while six of eight of those located in the structural region genes (VP1/VP2) alter the coding sequence. All of these differences are located in the VP2 coding region except for one in the VP1 unique portion (A-92 $\rightarrow$ R).

In contrast to NADL-2, in which a repetition of 127 nucleotides was found directly after the right-hand ORF, the Kresse strain did not contain such a repetition. Furthermore, a nucleotide difference at the end of what would be the first repeat (C-4660 $\rightarrow$ A) was observed. The presence or absence of this repeat was confirmed by PCR (primers PPV-PR6 and PPV-PR7) and by direct sequencing of the viral genome (primer PPV-PR7).

**Comparison of different PPV isolates.** Analysis for the presence of the 127-bp repeat downstream of the ORF of the structural proteins in the genomes of several field isolates obtained from mummified embryos and thyroid cells (e.g., isolate IAF-22) since 1986 (15 independent samples) showed that all of the genomes lacked this repeat.

As mentioned previously, the Kresse and NADL-2 genomes shared a high degree of sequence identity. Among the 529 amino acids of VP2, five changes were consistent by being present in field isolates (I-215 $\rightarrow$ T, D-378 $\rightarrow$ G, H-383 $\rightarrow$ Q, S-436 $\rightarrow$ P, and R-565 $\rightarrow$ K) (Fig. 2). Among those with the NADL-2 amino acid pattern in these five key locations, only one strain that lacked the 127-bp repeat (IAF-3) was isolated from a mummified fetus.

**Tissue tropism and production of virus.** Levels of viral replication on PT and PFT cells were not identical. The NADL-2 strain replicated at higher titers than Kresse in both PT and PFT cells. Although the NADL-2 strain yielded similar titers on both cell lines, the Kresse strain replicated better on PT cells than on PFT cells. TV cells supported the replication of PPV NADL-2, but not that of PPV Kresse. TV cells were

<> 20 a 40 60 80 b 100  
AATCTTTAAACTGACCAACTGTCTTTGCGTATGGTGACGTGATGACGCGGCTACGCGCGCTGCCTTCGGCAGTCACACGTCACCATCAGCAAAGACAGT  
c <>120 140 d 160 e 180 f  
TGGTCAGTTTAAAGATTAATAAGACATTCATTGGCTGAAAGAGGCGGAAATTCAAAAAAGAGGCGGAAAAAAGAGGTGGAGCCTAACACTATAA  
220 240 260 280 *Pst*I g 300  
ATACAGTTGCTTACTTCAGTTAGTTCCTTTCTGCTTCAGACTGCACTTCGCTCCAGAGACACAGCTACAACTACTCTCAGCTACTGCAGCATGGCAGCG  
M A A  
320 340 360 380 400  
GGAAACACTTACTCGGAAGAGGTAATAAAGCTACCAACTGGCTTCAAGATAATGCTCAAAAAAGAGCATTCTCTTATGTATTTAAAAACAAAAAGTCA  
G N T Y S E E V L K A T N W L Q D N A Q K E A F S Y V F K T Q K V N  
A 420 440 460 480 500  
ATCTGAATGGAAAAGAAATGCTTGAATAACTACAACAAAGATACAACAGATGCGGAAATGATAAACCTACAAAGAGGAGCAGAAACATCATGGGACCA  
L N G K E I A W N N Y N K D T T D A E M I N L Q R G A E T S W D Q  
520 *Taq*I C540 ▼h *Sca*I560 580 600  
GGCAACAGACATGGAAATGGGAATCAGAAATCGACAGTCTCAAAAACGGCAAGTACTGATTTTTGACTCTCTTGTTAAAAAATGTCTCTTTGAAGGTATA  
A T D M E W E S E I D S L T K R Q V L I F D S L V K K C L F E G I  
620 640 660 680 700  
TTGCAAAGAACCTAAGTCAAGTACTGCTACTGGTTCATACAGCATGAACATGGTCAAGATACTGGCTACTGCCATGTACTACTAGGTGGAAAAG  
L Q K N L S P S D C Y W F I Q H E H G Q D T [G Y H C H V L L G] G K G  
720 740 760 780 800  
GCTTACAACAAGCAATGGGAAAATGGTTCAGAAAACAATTAACAATTTATGGAGTAGATGGTTAATAATGCAATGCAAAGTACCTCTAACACCAGTTGA  
L Q Q A M G K W F R K Q L N N L W S R W L I M Q C K V P L T P V E  
p 820 840 860 880 900  
AAGAATAAAATTAAGGGAATTAGCAGAGGATGGTGGTATCGCTACTAACCTACACTCAAAACAAACTAAAAACAATATACAAAAATGACTCAT  
R I K L R E L A E D G E W V S [L L T Y T H K Q T K] K Q Y T K M T H  
920 p 940 960 980 1000  
TTTGAAATATGATTGCTTACTACTCTAAATAAAAAAGAAACAACCTGAAAGAGAGCATGGATATTATCTCAGCTCAGATTCTGGCTTCATGACAA  
F G N M I A Y Y F L N K K R K T T E R E H G Y Y L S S D S G F M T N  
1020 1040 p 1060 1080 1100  
ATTTCTTAAAAAGGCGAGAGACTTAGTCAGTCACCTATTTACTGAAGCAATAAACTGAAACTGTGGAACAACGGTACTACAGCTCAGGAAGC  
F L K E G E R H L V S H L F T E A N K P E T V E T T V T T A Q E A  
1120 1140 1160 p 1180 1200  
CAAAAGAGGCAGAATACAAACAAAAAAGAAAGTAAAGCATAAAATGCACAATAAGAGACTTGGTTATAAAAAGATGTACTAGCATAGAAGACTGGATGATG  
K R G R I Q T K K E V S I K C T I R D L V N K R C T S I E D W M M  
1220 1240 1260 1280 1300  
ACAGATCCAGACAGTTATATAGAATGATGGCTCAAACCGGAGGAGAAAATTTAATCAAAAATACACTAGAAAATAACAACCTTACTCTAGCAAGAACAA  
T D P D S Y I E M M A Q T G G E N L I K N T L E I T T L T L A R T K  
1320 1340 *Sph*I 1360 1380 *Sph*I 1400  
AAACAGCATATGACTTAATACTTGAAGGCAAAACCAAGCATGCTACCAACATTTAATATTAGCAATACAAGAACATGTAATAATTAGCATGCACAA  
T A Y D L I L E K A K P S M L P T F N I S N T R T C K I F S M H N  
1420 1440 1460 1480 1500  
TTGGAACTACATTAAGTCTGCCATGCTATAACTTGTTACTAAACAGACAAGGAGGAAAAAAGAAATACAATTTCTATTTTCTGGCCAGCATCAACAGGA  
W N Y I K V C H A I T C V L N R Q G G K R N T [I L F H G P A S T G]  
1520 A 1540 1560 1580 p 1600  
AAAAGTATAATTGCTCAACACATTGCAAACCTGGTTGGTAAATGTTGGTGTACAATGCAGCCAATGTGAACCTTCCATTTAATGACTGTACAATAAAA  
K S I I A Q H I A N L] V G N V G C Y N A A N V N F P F N D C T N K [N  
1620 1640 1660 1680 1700  
ACTTAATATGGATTGAAGAAGCAGGAAACTTCTCTAACCAAGTAAACCAATCAAAGCCATATGTTCAGGTCAACAATTAGAATTGACCAAAAAGGTAA  
L I W I E E A G N] F S N Q V N Q F K A I C S G Q T I R I D Q K G K  
1720 1740 1760 1780 1800  
AGGAAGCAAACAAATGAACCAACTCTGTAATAATGACTACAATGAAGACATAACTAAAGTTAGAATAGGATGCGAGGAAAGACCAGAACATACACAA  
G S K Q I E P [T P V I M T T N E] D I T K V R I G C E E R P E H T Q  
1820 1840 1860 *Ba*I 1900  
CCAATAAGAGACAGAAATGTTAAACATAAACCTAACCGAAAACTGCCAGGTGATTTGGACTTTTAGAAGAAACTGAATGGCCACTAATATGTGCTTGGT  
P I R D R M L N I N L T R K L P G D F G L L E E T E W P L I C A W L  
*Bst*EII1920 *Nhe*I } 1940 i 1960 k A 1980 j 2000  
TGTTAAAGAAAGGTTACCAAGCAAACTGGCTAGCTATATGCATCATTGGGAAATGTACTGGTCCGAAAAATGGGAGGAGCCAAAAATGCAAAAC  
V K K G Y Q A T M A S Y M H H W G N V P D W S E K W E E P K M Q T  
p l ▼m 2040 2060 2080 2100  
CCCAATAAATACACCAACAGACTCTCAGATTTCCACATCAGTGAACCTCGCCAGCGGACAACAACCTACGCAGCAACTCCAATACAGGAGGACCTGGAT  
P I N T P T D S Q I S T S V K T S P A D N N Y A A T P I Q E D L D  
L S D F H I S E N F A S G Q Q L R S N S N S N T G G P G F

FIG. 1. Complete nucleotide sequence of the Kresse strain of PPV (positive strand) and restriction sites of major endonucleases. The sequence of viral transcripts and translation products was predicted from NADL-2 transcription mapping (5). a and b, ATF sites; c, CAAT box; d and e, GC box; f, TATA box of the P4 promoter predicted from the location of NS transcripts; g, initiation codon of NS proteins; ▼h, donor splicing site of NS2 and NS3; i, enabler; j, GC box; k, CAAT box; l, TATA box of the P40 promoter as predicted from VP transcripts; ▼m, acceptor splicing site of NS2; ▼o, donor splicing site of VP2; q, initiation codon of VP-1; ▼r, donor splicing site of VP1; ▼s, acceptor site of VP1 and NS3; u, initiation codon of VP-2; p, poly(A) site; t, stop codons after translation regions; <>, beginning and end of terminal palindromes; [ ], beginning and end of 127-bp repeats; { }, beginning and end of 14-bp center of putative transactivation region (100% homology and same location with respect to P40 as the transactivation region element of H-1 parvovirus) (14, 25); †, begin/end of repetition in NADL-2. The initiator protein motif is underlined; the superfamily III helicase motif is double underlined.

2120 2140 2160 2180 2200  
 TTAGCTTTAGCCTTGGAGCGGTGGAGCGGACCAACAACCAACTTTACCAACCTGCACCTAACTCCAACACCGCCAGATTGAGCAATACGGACACCAA  
 L A L A L E P W S E P T T P T F T N L H L T P T P P D S A I R T P S  
 S F S L G A V E R A N N T N F H Q P A L N S N T A R F S N T D T K  
*XcmI* 2220 2240 2260 t ▼o q *NarI* 2300  
 GTCCAACCTGGTCGAAATAGAAACCGACATAAGAGCCTGCTTTGGTAAAACTGTGCACCCACAACAACTTGAATAAGGTAGGATGGCGCCTCTGC  
 P T W S E I E T D I R A C F G E N C A P T T N L E - M A P P A  
 S N L V G N R N R H K S L L W -  
 r▼ 2320 2340 2360 2380 ▼s 2400  
 AAAAAGAGCAAGAGGTAAGGGTAGTTTTAAGGGGGTGGTGGGCATACATATAAACTAACTGCAAATAATTTTTTATATATTACAGGACTAACTTACC  
 K R A R G T N S T  
 L T L P  
 2420 2440 t 2460 2480 2500  
 AGGATACAAATACCTTGGTCCAGGAACTCACTAGACCAAGGAGAACCAACTAAATCCATCAGACGCCGAGCAAAAGAACACGACGAAGCCTACGACAAA  
 R I Q I P W S R K L T R P R T N -  
 G Y K Y L G P G N S L D Q G E P T N P S D A A A K E H D E A Y D K  
 2520 2540 *PvuII* 2560 2580 2600  
 TACATAAAATCTGGAAAAATCCATACTTCTACTTCTCAGCAGCTGATGAAAAATTCATAAAAGAACTGAACACGCAAAAGACTACGGAGGTAAAAATTG  
 Y I K S G K N P Y F Y F S A A D E K F I K E T E H A K D Y G G K I G  
 2620 2640 2660 2680 2700  
 GACATTACTTCTCAGAGCAAAGCGTGCCTTTGCTCCAAAATCTCAGAAACAGACTCACCAACTACATCTCAACAACAGAGGTGAAGAAGATCGCCGAG  
 H Y F F R A K R A F A P K L S E T D S P T T S Q Q P E V R R S P R  
 2720 2740 2760 2780 2800  
 AAAACACCCAGGGTCTAAACACCAGGAAAAAGACCTGCTCCAAGACATATTTTTATAAACTTAGCTAAAAAAAAGCTAAAGGGACATCTAATACAAAC  
 K H P G S K P P G K R P A P R H I F I N L A K K K A K G T S N T N  
 u 2820 2840 2860 2880 2900  
 TCTAACTCAATGAGTGAATGTGGAACAACACAACCTATTAATGCAGGCCTGAATTGTCTGCAACAGGAAATGAATCTGGGGGTGGGGCGCGGGT  
 S N S M S E N V E Q H N P I N A G T E L S A T G N E S G G G G G G G  
 2920 2940 C 2960 2980 3000  
 GCGGGGTAGGGGTGCTGGGGGGTGGTGTGTCTACAGGTAGTTTCAATAATCAACAGAAATTTCAATACTTGGGGGAGGGCTTGGTTAGAATCACTGC  
 G G R G A G G V G V S T G S(T) F N N Q T E F Q Y L G E G L V R I T A  
 3020 3040 3060 3080 3100  
 ACACGCATCAAGACTCATACTCTAAATATGCCAGAACACGAAACATACAAAAGAATACATGTACTAAATTCAGAATCAGGGGTGGCGGGACAAATGGTA  
 H A S R L I H L N M P E H E T Y K R I H V L N S E S G V A G A Q M V  
 3120 3140 3160 A 3180 *BstXI* 3200  
 CAAGACGATGCACACACAAAATGGTAACACCTTGGTCACTAATAGATGCTAACGCATGGGGGTGTGGTTCAATCCAGCGGACTGGCAGTTAATATCCA  
 Q D D A H T Q M V T P W S L I D A N A W G V W F N P A D W Q L I S N  
 p 3220 3240 *SspI* *ScaI* 3260 3280 3300  
 ACAACATGACAGAAATAAATCTAGTTAGTTTTGAACAAGAAATTTCAATGTAGTACTTAAACAATTACAGAATCAGCAACCTCACCACCAACCAAAAT  
 N M T E I N L V S F E Q E I F N V V L K T I T E S A T S P P T K I  
 3320 *HindIII* 3340 3360 3380 3400  
 ATATAAATGATCTAACTGCAAGCTTAATGGTGCAGCTAGACACCAATAACACACTCCATACACACCAGCAGCAGCTAGAAGTGAACACTTGGTTTT  
 Y N N D L T A S L M V A L D T N N T L P Y T P A A P R S E T L G F  
 T *NcoI* *BstEII* 3420 3440 T 3460 3480 3500  
 TACCCATGGTTACCTACAAAACCAACTCAATACAGATATTACCTATCATGCACCAGAAACCTAAATCCACCAACATCACTGGACAATCACAACAAATAA  
 Y P W L P T K P T Q Y R Y Y L S C T(I) R N L N P P T Y T G Q S Q Q I T  
 3520 3540 3560 3580 *EcoRI* 3600  
 CAGACTCAATACAAACAGGACTACACAGTGACATTATGTTCTACACAATAGAAAATGCAGTACCAATTCTCTTCTAAGAACAGGAGATGAATTTCTCCAC  
 D S I Q T G L H S D I M F Y T I E N A V P I H L L R T G D E F S T  
 3620 3640 *BglII* 3660 3680 3700  
 AGGAATATACCTTTGACACAAAACCACTAAAATTAACCTACTCATGGCAAACAAACAGATCTCTAGGACTGCCTCCAAAACCTAACTGAACCTACC  
 G I Y H F D T K P L K L T H S W Q T N R S L G L P P K L L T E P T  
 3720 3740 3760 3780 3800  
 ACAGAAGGAGACCAACCCAGGAACACTACCAGCAGCTAACACAAGAAAAGGTTATCACCAAAACAATTAATAATAGCTACACAGAAGCAACAGCAATTA  
 T E G D Q H P G T L P A A N T R K G Y H Q T I N N S Y T E A T A I R  
 3820 3840 3860 3880 3900  
 GGCCAGCTCAGGTAGGATATAATACACCATACATGAATTTGAATACTCCAATGGTGGACCTTTCTAACTCCTATAGTACCAACAGCAGACACACAATA  
 P A Q V G Y N T P Y M N F E Y S N G G P F L T P I V P T A D T Q Y  
 3920 3940 A C 3980 4000  
 TAATGATGATGAACCAATGGTGTATAAGATTTACAATGGGTTACCAACATGGACAATTAACCACATCTTCAACAAGAGCTAGAAGATACACATTCAT  
 N D D E P N G A I R F T M G(D) Y Q H G Q(H) L T T S S Q E L E R Y T F N  
 4020 *SstI* 4040 4060 4080 4100  
 CCACAAAGTAAATGTGGAAGAGCTCCAAGCAACAATTTAATCAACAGGCACCACTAAACCTAGAAAATACAAATAATGGAACACTTTTACCTTCAGATC  
 P Q S K C G R A P K Q Q F N Q Q A P L N L E N T N N G T L L P S D P  
 T 4120 4140 4160 4180 4200  
 CAATAGGAGGAAACCTAACATGCATTTTCATGAATACACTCAATACATATGGACCATTAACAGCACTAAACAATACTGCACCTGTATTTCCAATGGTCA  
 I G G K P(S) N M H F M N T L N T Y G P L T A L N N T A P V F P N G Q

FIG. 1—Continued.

4220 *Bg*III 4240 4260 4280 4300  
 AATATGGGATAAAGAAGCTTGATACAGATCTAAAACCTAGACTACATGTTACAGCTCCATTTGTTTGTAAAAACAATCCACCAGGACAACCTATTTGTAAAA  
 I W D K E L D T D L K P R L H V T A P F V C K N N P P G Q L F V K  
 4320 4340 4360 4380 4400  
 ATAGCACCAAACCTAACAGATGATTTCAATGCTGACTCTCCTCAACAACCTAGAATAATAACTTATTCAAACCTTTGGTGGAAAGGAACACTAACATTCA  
 I A P N L T D D F N A D S P Q Q P R I I T Y S N F W W K G T L T F T  
 4420 4440 4460 4480 4500  
 CAGCAAAAATGAGATCCAGTAATATGTGGAACCCTATTCAACAACACACAACAACAGCAGAAAAACATTGGTAACTATATTCCTACAATATTGGTGGCAT  
 A K M R S S N M W N P I Q Q H T T T A E N I G N Y I P T N I G G I  
 G 4520 4540 † t 4560 p 4580 4600  
 AAAAAATGTTCCAGAATATTCACAACCTTATACCAAGAAAATTATACTAGAAATAACTCTGTAAATAAAACTCAGTTACTTGGTTAATCATGTACTACTA  
 K M F P E Y S Q L I P R K L Y -  
 p 4620 p 4640 p c † 4680 p 4700  
 TCATTGTATACTTCAATAAAAAATAAATTTGAAAATCAATAAAACTAAGTTACTTAGTTTATGTATACCAATTATCCCCAAAAACAATAAAATTTTAAAA  
 4720 4740 4760 4780 4800  
 AGAAACAAGCTCTCATGTGTTTACTATTAACCTAAACCAACCACACTTATATGACCTTATGTCTTTAGGGTGGGTGGGAATTACTATGTATTCTTTT  
 4820 4840 4880 4900  
 GAGTTAGTTGGTGCCTTTGGCGGACTAACCAAGCGGCTCTGCCGCTTGTTAGTCGCACGGCGACCAACTAACTCAAAGGAATACATAGTAATTCCAC  
 4920 4940 ◊  
 CCACCCACCCTAAAGACATAAGGTCATATAAGTGTGGTTGGTTAGTT

FIG. 1—Continued.

therefore used as indicator cells in the study of the viral tropism.

The titer of TV cells inoculated with PPV NADL-2 was approximately 10<sup>3</sup>-fold lower than that on PT or PFT cells. No differences in virus yield were observed between the incubations at 37°C and those at 39°C (body temperature of the pig) for either virus or cell line when the cells were permissive.

**Chimeric infectious clones.** In attempts to cause the phenotype of the Kresse strain to revert to that of the NADL-2 strain, we exchanged the *Bg*III fragments among the infectious clones of these strains. This resulted in the exchange of three critical mutations located within the VP1/VP2 capsid gene (Fig. 3). Progeny viruses from these chimeric clones, obtained from either PT or PFT cells, were all infectious in PT and PFT cells. Titers obtained after infection of PT, PFT, and TV cells with

Position in NADL-2 seq.	NADL-2	IAF-3	Kresse	IAF-A54	90HS	IAF-22	IAF-28	IAF-76	Position in VP2 seq.	NADL-2	IAF-3	Kresse	IAF-A54	90HS	IAF-22	IAF-28	IAF-76
3358	AGT	---	---	---	---	-C	nd	nd	192	S	-	-	-	-	-	nd	nd
3453	ATC	---	-C-	-C-	-C-	-C-	nd	nd	215	I	-	T	T	T	T	nd	nd
3710	GGA	---T	---	---	---	---	nd	nd	300	G	-	-	-	-	-	nd	nd
3769	ATT	---	---	---	---G	---	---	---	320	I	-	-	-	M	-	-	-
3787	GAA	---	---	---	---	-G	---	---	326	E	-	-	-	-	-	-	-
3835	ATG	---C	---	---	---	---	---	---	342	M	T	-	-	-	-	-	-
3884	ACA	---	---	---	---	G-	---	---	359	T	-	-	-	-	A	-	-
3927	ATA	-A-	---	---	---	---	---	---	373	I	K	-	-	-	-	-	-
3942	GAT	---	-G-	-G-	-G-	-G-	-G-	-G-	378	D	-	G	G	G	G	G	G
3958	CAC	---	--A	--A	--A	--A	--A	--A	383	H	-	Q	Q	Q	Q	Q	Q
3979	GAG	---	---	---	---	---	---	-A	390	E	-	-	-	-	-	-	-
4108	GGA	---	---	---	---	---	---	-G	433	G	-	-	-	-	-	-	-
4112	AAA	---	---	---	---	---	---	G-	435	K	-	-	-	-	-	-	E
4115	TCT	---	C--	C--	C--	C--	C--	C--	436	S	-	P	P	P	P	P	P
4135	AAT	---	---	---	---	---C	---	---	542	N	-	-	-	-	-	-	-
4209	GAT	---	---	---	---	---	---	-T	467	D	-	-	-	-	-	-	V
4263	GGT	---	---	---	---	-C-	---	---	485	V	-	-	-	-	A	-	-
4279	CCA	---G	---	---	---	---	---	---	490	P	-	-	-	-	-	-	-
4498	GGC	---	---	---	---	---	---	-T	563	G	-	-	-	-	-	-	-
4503	AGA	---	-A-	-A-	-A-	-A-	-A-	-A-	565	R	-	K	K	K	K	K	K
4540	AAA	---	---	---	---	---	---	-T	576	K	-	-	-	-	-	-	N
Repeat	+	-	-	-	-	-	-	-									

FIG. 2. Sequences of PPV field isolates in the genome region where coding sequence differences between the Kresse and NADL-2 strains are located. The position noted in the NADL-2 sequence is that of the affected base in the codon coding for the amino acid on the same line. Typical vaccine-like isolates had the sequence shown for NADL-2 and contained a 127-bp repeat. One exception was IAF-3, a vaccine-like isolate from mummified tissues, which lacked the repeat. The 90HS (a virulent strain) sequence is that published by Sakurai et al. (27). IAF-22, IAF-28, and IAF-76 are virulent PPV strains which differ only slightly from 90HS. In this sequence, IAF-28 contained only the consistent point mutations (with respect to NADL-2), whereas others, such as IAF-76, contained additional point mutations that were not consistent among virulent strains. The dermatitis strain, Kresse, had the same sequence in this region as the abortion-causing IAF-28 strain, and the dermatitis determinant should be located elsewhere. nd, not determined.

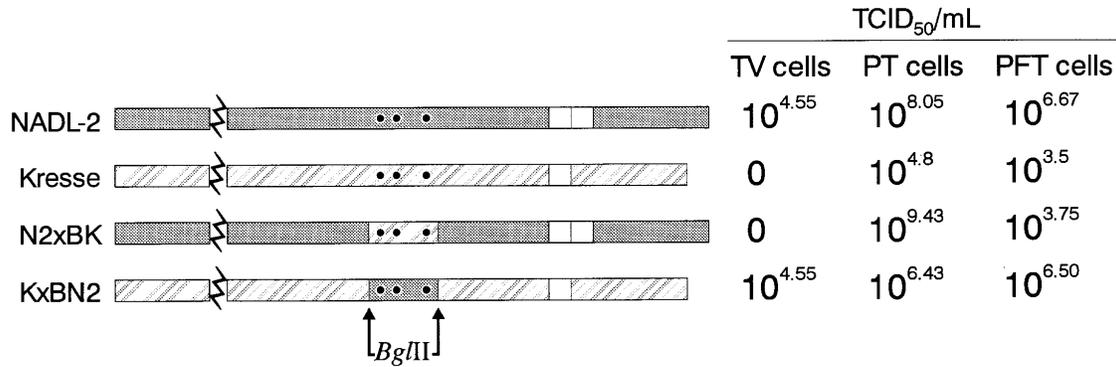


FIG. 3. Construction of chimeric clones of PPV. The *Bgl*III fragments of PPV Kresse and NADL-2 were exchanged in the opposite background. The critical amino acid positions are indicated. These infectious clones were transfected into PT cells, and then their titers (50% tissue culture infective dose [TCID<sub>50</sub>]) in PT, PFT, and TV cells were determined.

either chimeric viruses or native NADL-2 and Kresse virus are shown in Fig. 3. Parallel infection of PT and TV cells with the chimeras resulted in a pattern typical of the wild-type strains from which the *Bgl*III insert originated. Consequently, chimeric virus which harbored the Kresse *Bgl*III insert did not replicate in TV cells.

DISCUSSION

In contrast to previous reports (21), PPV strains replicated at low titers on ST cells and not at all on MDBK cells in our laboratory. This may have been due to the passage number, because PT cells (a clone from ST cells) were permissive and

yielded productive infections (5). Given the many requirements for parvovirus replication in cell culture, cellular systems have been developed to discriminate between virus strains, including, for example, those of MVM (9). We were able to develop a cellular system which enabled us to discriminate virulent from avirulent strains by their cellular tropism.

The sequencing of the Kresse strain revealed that minor coding differences located within the VP1/VP2 coding region are responsible for the differential tropism observed in vitro. Five of these differences were also consistently present (with one particular exception [described below]) in virulent field isolates. Differences were not found in the so-called control regions of the virus (promoters and splicing junctions), and

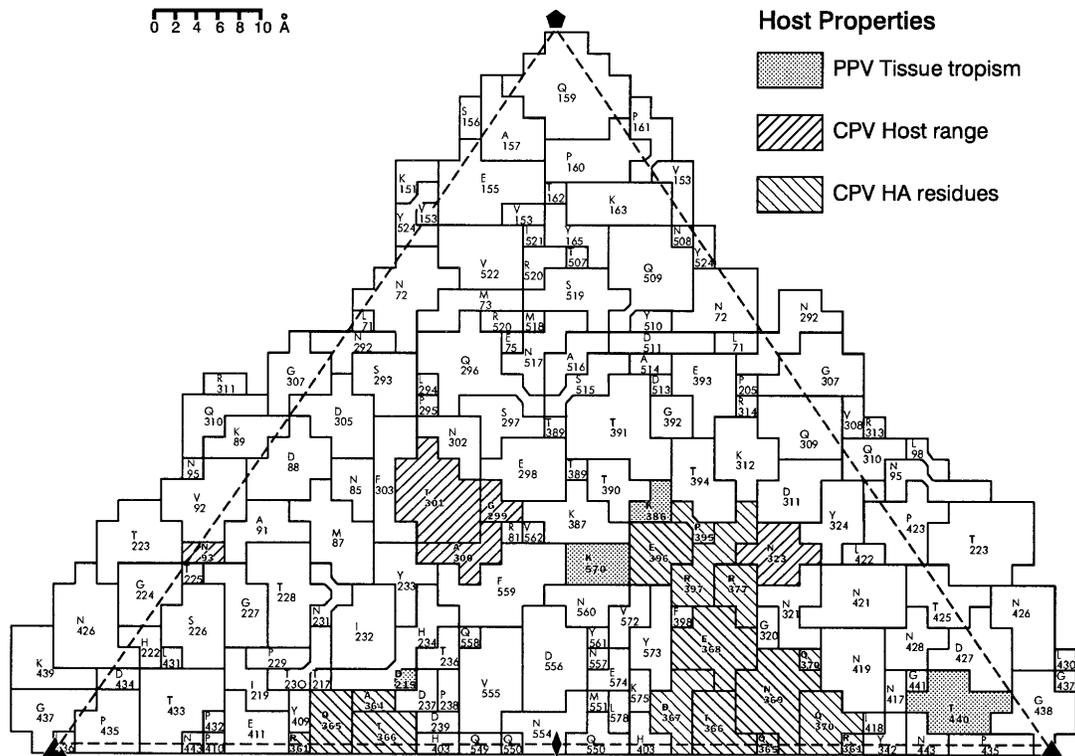


FIG. 4. Modified roadmap of CPV showing a projection of solvent-accessible surface amino acids for one asymmetric unit of the icosahedral particle. CPV residues involved in host range (medium shading) or in hemagglutination (HA [light shading]) are indicated. Residues putatively involved in the host range are also indicated. The CPV-PPV amino acid correspondence is as follows (VP2 numbering): 215-215, 381-378 (below the surface), 386-383, 440-436, and 570-565. The radial distance of the surface residues from the capsid center is shown at the top left (1 Å = 0.1 nm). The figure was produced in part by the software application Roadmap (7).

Kresse transcription mapping and transcript processing are identical to those of NADL-2 (14a).

Previous investigations into the tropism of MVMi/MVMp and CPV/FPV strains have shown that the allotropic determinants are located on the capsid protein (1, 13, 22, 23, 33). For MVM, mutations at amino acid positions 316 and 320 (of VP2) were shown to be sufficient to obtain a different (extended) tropism (4). Residues similarly located on the CPV capsid are responsible for the canine and feline host range, such that residues 80, 564, and 568 differ between CPV and FPV (31). Among the five consistent changes between nonvirulent and virulent PPV strains, none corresponded exactly to the positions which are critical in MVM and CPV, although the inconsistent change I-470→M was at the same position as E-320→G of MVM VP2.

The chimeric constructs (*Bg*/III fragment exchange) carry the differences found at positions 378, 383, and 436 (VP2 numbering). Extrapolation from the three-dimensional structure of the closely-related CPV, determined by X-ray crystallography (33), suggests that D-378→G would be located at or near the base of the threefold spike but not on the surface of the virion. The change S-436→P, on top of the threefold spike, may be particularly critical, because the replacement of this serine by a proline probably results in a considerable modification of the local topology because of the constraints imposed by the imino acid structure of proline. Although this change appears to be dramatic, the presence of localized differences (I-215→T, H-383→Q, and R-565→K) within the twofold depression is potentially as important and meaningful, since both CPV and MVM host range residues and CPV hemagglutination residues are in the vicinity (Fig. 4).

Interestingly, Vasudevacharya and Compans (34) obtained a PPV mutant with NS and capsid protein changes which is adapted to canine A72 cells. Our results for field strains suggest that the capsid proteins are entirely responsible for the tropism of these viruses. Therefore, the *in vivo* relevance of the mutation found within the NS gene of the N2 mutant is not clear. On the other hand, while the 127-bp repeat was not responsible for the difference in tropism found between Kresse and NADL-2, it could be involved in virulence. It must be emphasized that the parental Kresse strain is different from the chimera because of the presence of the repetition within the chimeric clone deriving from NADL-2 (Kresse fragment). Therefore, we cannot assess if only the capsid structure is responsible for *in vivo* differences found between NADL-2 and Kresse, although we may speculate, on the basis of analogies with CPV and MVM, that the initial events are solely capsid dependent. Thus, the correlation between the *in vitro* tropism and the pathogenicity in pigs remains to be established.

The virulent PPV strains and field isolates in our study all lacked the 127-bp repeat. By analogy, MVMi also lacks the 65-bp repeat (3). The tandem repeat may be important for replication, because Salvino et al. (28) observed that removal of one copy of the repeat in MVMp inhibited replication in *cis* by 10- to over 100-fold (depending on the host cell). One virulent PPV isolate (IAF-3) with the vaccine-like amino acid pattern also lacked the tandem repeat present in NADL-2. This suggests that a different cell tropism among PPV strains is at most partially responsible for the different pathogenicities. Moreover, this tropism determinant does not explain the difference between classical virulent and more virulent dermatitis strains.

The origin and role of the vaccine-like PPV strains associated with mummification are obscure. NADL-2 is widely used as an inactivated vaccine. Even when inactivation would be incomplete, NADL-2 is expected to be safe (24). However,

experimental conditions may differ from field conditions, and coinfections may condition the susceptibility of the host. Second, inconsistent mutations in the vaccine-like genomes (e.g., IAF-3) suggest that they may have been present in the population for some time already. These mutations may have arisen from non-inactivated vaccine in the host or during repeated passages by the vaccine producers. Whether the loss of the repeat in these vaccine strains may convert them to virulent strains remains to be established.

While the permissivity of many viruses is solely determined by the presence or absence of virus attachment molecules, the dependence of parvoviruses on host-cell functions is greater than that of most viruses because of their small coding capacity, which imposes further prerequisites for permissivity. Oraveerakul et al. (21) showed that cell lines differing in their ability to support PPV replication internalize radiolabeled PPV (in both nonpermissive [MDCK] and permissive [ST] cells). Their studies confirm flow cytometry results (26) that demonstrated that PPV is internalized by both permissive and nonpermissive cells. The capsid protein could have a role in uncoating or could interact with a specific host cell factor involved in DNA transcription (particularly NS genes). Earlier, Gardiner and Tattersall (12) presented evidence that MVM gene expression is modulated by the viral particle and that different mechanisms regulate viral gene expression in transfected and infected cells. Since cellular factors have no effect during transfection, a modified capsid may extend (possibly by modulation of the ability to interact with such cellular factors) the tropism to other cells (4, 34). The impact of cellular factors on the restriction of PPV replication and their role in viremia and the modulation of the ability of certain strains to pass the placental barrier remain to be established.

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