Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses

Motohiro Horiuchi,1* Hitoshi Goto,2 Naotaka Ishiguro1 and Morikazu Shinagawa1

1 Department of Veterinary Public Health and 2 Department of Veterinary Microbiology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan

Feline panleukopenia virus (FPLV), mink enteritis virus (MEV) and canine parvovirus (CPV) are classified as host range variants among the feline parvoviruses in the genus Parvovirus (Siegel, 1985). Diseases caused by FPLV and MEV in cats and mink, respectively, have been known for many years, but CPV emerged suddenly in the late 1970s. Retrospective serological studies showed that no anti-CPV antibody was detectable in the sera of domestic dogs or wild canine populations until the mid-1970s, indicating that CPV was a new pathogen for dogs (reviewed by Parrish, 1990). The extensive antigenic and genomic similarities existing between CPV and FPLV/MEV and its sudden appearance strongly suggest that CPV arose by mutation from one of the viruses among the feline parvovirus subgroup.

Parvoviruses contain a linear ssDNA genome of about 5 kb. There are two large open reading frames (ORFs) in the genome; the left-hand ORF encodes at least one non-structural protein (NS) in the feline parvovirus subgroup, whereas the right-hand ORF encodes two capsid proteins, VP1 and VP2, which are translated from alternatively spliced RNA. Partial and almost whole nucleotide sequences of CPV, FPLV and MEV have been determined (Carlson et al., 1985; Parrish et al., 1988a; Reed et al., 1988; Martyn et al., 1990; Kariatsumari et al., 1991). They have more than 98% identity in DNA and predicted amino acid sequences (Kariatsumari et al., 1991); however, CPV and FPLV/MEV are distinguishable in some biological properties such as the pH dependence of haemagglutination (HA; Carmichael et al., 1980) and their host cell specificity in vitro and in vivo. CPV isolates can replicate in canine and feline cell lines; however, FPLV and MEV do not replicate or replicate only poorly in canine cell lines such as A72 canine fibroma, MDCK and C12Th (Tratschin et al., 1982; Mochizuki & Hashimoto, 1986; Parrish et al., 1988a; Horiuchi et al., 1992; Truyen & Parrish, 1992). Viral tropism in cultured canine or feline peripheral blood cells was shown to be similar to that observed in permanent cell lines (Truyen & Parrish, 1992). However, the host range in vivo is not identical to that in vitro; CPV isolates can replicate in dogs but appear not to replicate or

Introduction

Feline panleukopenia virus (FPLV), mink enteritis virus (MEV) and canine parvovirus (CPV) are more than 98% similar in DNA and predicted amino acid sequences, but they show different host-cell specificities; CPV is able to replicate in canine cells in culture, whereas FPLV and MEV cannot or replicate only to a low titre. To map the genomic region responsible for the host range of CPV in vitro, CPV/MEV chimeric viruses were generated by transfecting infectious CPV/MEV chimeric plasmids into a cultured feline kidney cell line, and their host cell ranges were analysed. The 60 to 91 map units (m.u.) region of the CPV genome, which contains a part of the capsid protein (VP) gene encoding from amino acid 91 (in the VP2 sequence) to the carboxy terminus of VP protein, was required to impart the ability to replicate in canine cells to MEV, although the chimeric virus containing the 60 to 91 m.u. region of the CPV genome in the MEV background did not replicate in canine cells as efficiently as did CPV derived from the infectious plasmid of CPV. Not only the VP gene, but also a part of the NS gene of CPV were considered to participate in the full expression of the ability to replicate in canine cells. Within the 60 to 91 m.u. region, five of nine amino acid changes between MEV-Abashiri and CPV-Y1 were thought to be phylogenetically CPV-common; however, a recombinant virus containing all five amino acid changes of CPV in the MEV background replicated minimally in canine cells.

The sequence data presented in this paper have been submitted to the DDBJ sequence database and assigned accession numbers D26079 (CPV-Y1), D26080 (CPV-obl) and D26081 (CPV-CP49).
replicate only to low titres in cats and mink (Barker et al., 1983; Parrish et al., 1987), whereas FPLV has been shown to replicate to a high titre in canine thymus but not in other canine tissues (Truyen & Parrish, 1992). On the other hand, FPLV and MEV are not distinguishable by host range in vivo and in vitro.

The existence of the host cell specificity of these viruses has been noticed because CPV is considered to have gained the ability to replicate in dogs by mutation from FPLV or some other closely related virus. A knowledge of their cell tropism would contribute to the investigation of the relationship between CPV and FPLV/MEV, and the sudden emergence of CPV. Virus binding to the cell surface and late stages in the virus life cycle do not appear to be involved in the restrictive infection of FPLV and MEV in canine cells (Horiuchi et al., 1992). Studies of chimeric constructs of CPV and FPLV indicated that the 59 to 73 map units (m.u.) region of CPV, within the common region of VP1 and VP2, could impart the ability to replicate in dogs as well as in canine cells to CPV/FPLV chimeric viruses (Parrish et al., 1988a; Parrish, 1991). More recently, the canine host range has been shown to be determined by at least two amino acid residues, which are encoded between 59 and 73 m.u. of the CPV genome (Chang et al., 1992). The fibrotrropic and lymphotropic determinants of the two strains of minute virus of mice (MVM), MVM(p) and (MVM(i), have also been mapped to the 68 to 73 m.u. region within the capsid protein gene (Gardiner & Tattersall, 1988b; Antonietti et al., 1988). These results indicate that the determinants of host range are located within small regions of the capsid protein gene.

Here we show different results using chimeric viruses between CPV and MEV. The CPV strain used here belongs to CPV-2a, a categorization proposed by Parrish et al. (1988b), on the basis of the reactivity of monoclonal antibodies, whereas previous studies by Parrish and his colleagues were conducted with CPV-2, the original CPV type. In the CPV/MEV chimeric virus system, a region larger than 59 to 73 m.u. of the CPV genome seemed to be responsible for the in vitro canine host range. In addition, we discuss the implication of the NS gene in the full expression of the growth properties of CPV in canine cells.

Methods

Cells and viruses. Crandell feline kidney cells (CRFK; Crandell et al., 1973) were grown in Dulbecco’s modified Eagle’s MEM (DMEM) containing 5% fetal calf serum (FCS). A72 canine fibroma cells (Bunn et al., 1980) were grown in Leibovitz L-15 medium with 10% FCS. The Abashiri strain of MEV (Higashihara et al., 1981) and the Y1 strain of CPV (Senda et al., 1986) were used in this study.

Construction of recombinant genomic clones. Recombinants were constructed from pMEV, an infectious plasmid clone of the MEV-Abashiri strain (Kariatsumari et al., 1991), and pCPVY1, an infectious plasmid clone of the CPV-Y1 strain (Horiuchi & Shinagawa, 1993), according to standard procedures (Sambrook et al., 1989). The 3’ and the 5’ ends of the CPV genome are 44 bp shorter and 63 bp longer than those of the MEV genome, respectively, and nucleotides (nt) 4593 to 4633 of the MEV sequence are not found in CPV (depicted in Fig. 1); hence nt numbers are not identical in the two viruses. In this report, we have followed the nt numbering system of MEV in cases where there is no annotation. Maps of the recombinant plasmids are shown in Fig. 1. The identities of the individual clones were checked by confirming the nucleotide sequence(s) in the inserted fragment which differed from that in the replaced fragment. Map unit numbers have been rounded down to the nearest whole number in this text.

Site-directed mutagenesis. The positions of primers are depicted in Fig. 2. Four changes (nt 3132, 3791, 4515 and 4526) were introduced using the method described by Kunkel (1985). Psrl (60 m.u.) and EcoRV (79 m.u.) and EcoRV to BamHI (100 m.u., in the multiple cloning site of Bluescript SK+) fragments of pMEV were cloned into a phagemid vector. Single-stranded DNA template was synthesized in Escherichia coli CJ236, and a mutant DNA strand synthesized by oligonucleotide priming was selected in E. coli BHM71-18ms. One change (nt 3103) close to the Psrl site was introduced by polymerase chain reaction (PCR). One picomole of primer 2 was annealed to 1 μg of heat-denatured pMEV at 60°C for 30 min, and then a mutant DNA strand

---

Fig. 1. Genetic map of recombinant plasmids. The two ORFs are shown at the top of the figure, and the two lines below the ORFs indicate the genomes of MEV-Abashiri and CPV-Y1 in each infectious plasmid. Restriction sites used for construction of chimeric plasmids are indicated in the MEV genome. Regions enclosed by broken lines correspond to sequences that are not in the CPV-Y1 genome, and the solid bar at the 5’ end corresponds to a sequence that is not found in MEV-Abashiri. To represent the genetic map of the recombinant plasmid, MEV sequences are denoted with open blocks, and CPV sequences with shaded blocks. Closed circles indicate the amino acid residues of CPV-Y1 that are different from those of MEV-Abashiri. Closed circles with a vertical line denote a site-directed change artificially introduced into the MEV sequence.
was synthesized with the Klenow fragment of DNA polymerase I. One-hundredth of the product was used as a template for PCR. PCR was performed using primers 2 and 3, and the resulting fragment was cloned into the EcoRV site of Bluescript SK+. After confirmation of the change by DNA sequencing, the fragments were used to reconstruct infectious plasmids by substitution for corresponding fragments using appropriate restriction sites. The maps of recombinant virus genomes are shown in Fig. 1. The primer sequences used are as follows: primer 2, 5’ TAAAACGAGTTAACCGGAAACATG 3’ (nt 3088 to 3112, for nt 3103); primer 3, 5’ TTGAATAATAATCTCCATG 3’ (complementary to nt 3444 to 3463); primer 4, 5’ TCTACCAAGTATA-TTGGAG 3’ (nt 4507 to 4525, for nt 4515); primer 5, 5’ ATGGAGGTATGAAAATTG 3’ (nt 4519 to 4537, for nt 4526); primer 6, 5’ CTCATGCACAAATTGTAACAC 3’ (nt 3126 to 3146, for nt 3132); primer 7, 5’ AATACAAACTATATTACTGAAG 3’ (nt 3785 to 3806, for nt 3791). The underlined bases indicate nucleotides targeted for site-directed mutagenesis.

**Generation of recombinant virus stocks and determination of virus titres.** The recombinant infectious plasmids were transfected into CRFK cells by the calcium phosphate precipitation method (Graham & van der Eb, 1973). Resulting viruses in the culture fluid were propagated in newly prepared CRFK cells once or twice to obtain virus stocks whose titres were greater than 1.0 x 10⁵ p.f.u./ml. The prefix ‘p’ indicates a chimeric infectious plasmid, whereas ‘v’ indicates the corresponding viral stock. Viral titres were determined using the MEV rabbit serum, and then plaques were visualized by the avidin-biotin-peroxidase complex method using a VECTASTAIN ABC kit (Vector Laboratories).

**Analysis of replicative form (RF) DNA.** Low M₀ DNA was prepared from virus-infected cells as previously described (Horiuchi et al., 1993). In brief, 100 µl of diluted virus solution was adsorbed to CRFK cells prepared in 35 mm dishes for 1 h at 37 °C, and the cells were overlaid with DMEM containing 1% methyl cellulose and 3% FCS. Five days after infection, the cells were fixed with cold methanol for 20 min. The fixed cells were incubated with a 1:2000 dilution of anti-MEV rabbit serum, and then plaques were visualized by the avidin-biotin-peroxidase complex method using a VECTASTAIN ABC kit (Vector Laboratories).

**Direct sequencing.** The 59 to 90 m.u. region was amplified by PCR in three sections, using three sets of primers (primers 1 and 3, 5 and 9, and 8 and 10, Fig. 2). Generation of ssDNA from PCR products was performed as described by Higuchi & Ochman (1989). In brief, DNA was amplified using a set of primers, one of which was phosphorylated and the other non-phosphorylated. The amplified fragment was treated with λ-exonuclease (BRL), a 5’ to 3’ exonuclease that specifically attacks a 5’-terminal phosphate, to generate an ssDNA template for DNA sequencing. Sequencing was performed with Sequenase (USB) according to the instructions of the manufacturer. The primer sequences used were as follows: primer 1, 5’ GTACATTTAAATATCTCCATG 3’ (nt 3088 to 3463); primer 2, 5’ TAAAACGAGTTAACCGGGAAACATG 3’ (nt 3088 to 3112, for nt 3103); primer 3, 5’ TTGAATAATAATCTCCATG 3’ (complementary to nt 3444 to 3463); primer 4, 5’ TCTACCAAGTATA-TTGGAG 3’ (nt 4507 to 4525, for nt 4515); primer 5, 5’ ATGGAGGTATGAAAATTG 3’ (nt 4519 to 4537, for nt 4526); primer 6, 5’ CTCATGCACAAATTGTAACAC 3’ (nt 3126 to 3146, for nt 3132); primer 7, 5’ AATACAAACTATATTACTGAAG 3’ (nt 3785 to 3806, for nt 3791). The underlined bases indicate nucleotides targeted for site-directed mutagenesis.

**Host-range analysis.** Each virus was inoculated at an m.o.i. of 0·1 onto A72 and CRFK cells prepared 24 h before in 60 mm dishes. After DNA was electrophoresed in a 0·9% agarose gel, and transferred onto nylon membranes by Southern transfer (Southern, 1975). Filters were hybridized with 3²P-labelled MEV DNA.

**Analysis of replicative form (RF) DNA.** Low M₀ DNA was prepared from virus-infected cells as previously described (Horiuchi et al., 1993). In brief, 100 µl of diluted virus solution was adsorbed to CRFK cells prepared in 35 mm dishes for 1 h at 37 °C, and the cells were overlaid with DMEM containing 1% methyl cellulose and 3% FCS. Five days after infection, the cells were fixed with cold methanol for 20 min. The fixed cells were incubated with a 1:2000 dilution of anti-MEV rabbit serum, and then plaques were visualized by the avidin-biotin-peroxidase complex method using a VECTASTAIN ABC kit (Vector Laboratories).
adsorption of the virus, the cells were washed with DMEM twice and cultured with 4 ml of medium. Seventy-two hours after infection, the viral titres in culture fluids were determined in CRFK cells by plaque assay. Simultaneously, low Mr DNA was extracted from the cells for the detection of RF DNA. One-fourth and one-tenth equivalents of DNA samples from A72 and CRFK cells cultured in 60 mm dishes, respectively, were used for electrophoresis.

Results

Mapping the genomic region responsible for the canine host range

To map the minimum region of the CPV genome responsible for the ability to replicate in canine cells, chimeric infectious plasmids were constructed from pMEV and pCPVY1, and CPV/MEV chimeric viruses were generated by transfecting the recombinant plasmids into CRFK cells. Fig. 3 and Table 1 show the replication of the chimeric viruses in A72 and CRFK cells. Some variability may exist in the assay although the experiments were conducted carefully. Therefore, we judged viral replication in A72 cells not by virus yield, but on the basis of the ratio of A72/CRFK viral titres and the detection of RF DNA in order to eliminate the effects of variability. vMEV retaining biological properties of the parental virus replicated little in A72 cells; the ratio of A72/CRFK viral titres was in the order of 10⁻⁵, whereas vCPVY1 replicated in A72 cells as did the parental virus, as the ratio was in the order of 10⁻¹. In preliminary experiments, viral titres obtained from vMEV-infected A72 cells ranged from 10¹ to 2.9 x 10² p.f.u./ml, whereas those from vCPVY1-infected CRFK cells were from 5 x 10⁴ to 3.6 x 10⁵ p.f.u./ml (data not shown). It was speculated that the recombinant viruses that showed the CPV-Y1-type host range would replicate in CRFK cells with an efficiency similar to vCPVY1. Therefore, A72/CRFK ratios below 1·0 x 10⁻³ [i.e. approx. (2·9 x 10¹)/(3·6 x 10⁵)] were considered not to be significant in this assay. Two of six chimeric viruses in Table 1, vX5 and vP5, appeared to replicate in A72 cells, although the ratios were lower than that of vCPVY1. The results of Southern blot analysis also demonstrated that vX5 and vP5 could replicate in A72 cells, which is in agreement with the results of plaque assay (Fig. 4). CPV/FPLV chimeric virus carrying the 59 to 73 m.u. region of the CPV genome in the FPLV background has been reported to replicate in canine cells (Parrish et al., 1988a). However, the chimeric virus vPA, which contains the 60 to 85 m.u. region of the CPV genome, appeared not to replicate efficiently in A72 cells (m.u. 60 in this study is identical to m.u. 59 in their study; the difference was caused by the different nt numbering systems), as determined by the undetectable level of RF DNA (Fig. 3) and the less than 1·0 x 10⁻⁵ order of the A72/CRFK titre ratio. To eliminate the possibility that the 5' non-coding region was implicated in the host cell tropism, a chimeric virus carrying the 60 to 91 m.u. region of the CPV genome was generated using the PstI (60 m.u.) and PacI (91 m.u.) sites (vPP). No significant difference was observed between vP5 and vPP in replication in A72 cells (Fig. 4 and Table 2). The vPP still possessed a 112 base 5' non-coding region of CPV-Y1 sequence from which a

### Table 1. Viral yields in A72 and CRFK cells infected with CPV/MEV chimeric viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>A72 cells</th>
<th>CRFK cells</th>
<th>Yield ratio (A72/CRFK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMEV</td>
<td>5 x 10¹</td>
<td>1 x 10⁶</td>
<td>5 x 10⁻⁵</td>
</tr>
<tr>
<td>vCPVY1</td>
<td>2·5 x 10⁴</td>
<td>1·5 x 10⁸</td>
<td>1·7 x 10⁻³</td>
</tr>
<tr>
<td>v3P</td>
<td>2 x 10⁴</td>
<td>1·9 x 10⁶</td>
<td>1·1 x 10⁻³</td>
</tr>
<tr>
<td>vX5</td>
<td>5·8 x 10⁴</td>
<td>2·9 x 10⁸</td>
<td>2·0 x 10⁻²</td>
</tr>
<tr>
<td>vP5</td>
<td>1·4 x 10⁴</td>
<td>5·0 x 10⁸</td>
<td>2·8 x 10⁻²</td>
</tr>
<tr>
<td>vPB</td>
<td>1·3 x 10⁴</td>
<td>8·6 x 10⁶</td>
<td>1·5 x 10⁻³</td>
</tr>
<tr>
<td>vV5</td>
<td>2·5 x 10⁴</td>
<td>6·1 x 10⁸</td>
<td>4·1 x 10⁻⁴</td>
</tr>
<tr>
<td>vPBV5</td>
<td>2·2 x 10⁴</td>
<td>1·2 x 10⁸</td>
<td>1·8 x 10⁻³</td>
</tr>
<tr>
<td>vPA</td>
<td>8·5 x 10⁴</td>
<td>8·8 x 10⁵</td>
<td>9·7 x 10⁻⁴</td>
</tr>
</tbody>
</table>

**Fig. 4. Replication of vPP in A72 cells.** The size markers and abbreviations are the same as in the legend to Fig. 3. The corresponding viral yields are shown in Table 2.

### Table 2. Comparison of viral yields of vPP with vP5

<table>
<thead>
<tr>
<th>Virus</th>
<th>A72 cells</th>
<th>CRFK cells</th>
<th>Yield ratio (A72/CRFK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMEV</td>
<td>1·0 x 10⁴</td>
<td>5·3 x 10⁵</td>
<td>1·9 x 10⁻⁵</td>
</tr>
<tr>
<td>vCPVY1</td>
<td>3·4 x 10⁴</td>
<td>3·3 x 10⁸</td>
<td>1·0 x 10⁻¹</td>
</tr>
<tr>
<td>vP5</td>
<td>5·5 x 10⁴</td>
<td>4·6 x 10⁸</td>
<td>1·2 x 10⁻²</td>
</tr>
<tr>
<td>vPP</td>
<td>6·9 x 10⁴</td>
<td>4·1 x 10⁸</td>
<td>1·7 x 10⁻²</td>
</tr>
</tbody>
</table>
41 base sequence (nt 4593 to 4633) of MEV was deleted (Fig. 1). However, we could not rule out the possibility that the sequence difference in the non-coding region adjacent to the carboxy terminus of the VP gene affected the cell tropism. Thus, in the CPV/MEV chimeric virus system, we concluded that the 60 to 91 m.u. region of the CPV genome was basically responsible for the host range in canine cells. Although vP5 and vPP replicated in A72 cells, the ratio of viral titres was lower than that of vCPVY1 (Tables 1 and 2). This result indicates that an additional region(s) may be required to confer the complete canine host range in vitro.

Comparison of nucleotide and predicted amino acid sequences between MEV and CPV

Nucleotide and amino acid differences between MEV-Abashiri and CPV-Y1 are delineated in Fig. 5. Eight of 18 nucleotide differences in the NS ORF, and 12 of 21 of those in the VP ORF, were involved in coding changes. Nine of 12 coding changes in VP were located in the 60 to 91 m.u. region that confers canine cell tropism. To attempt to determine CPV tropism-specific amino acid residues, nt sequences of the 60 to 89 m.u. region of other CPV strains determined by direct sequencing of PCR products derived from virion DNA, and the previously reported MEV and FPLV sequences were compared (Fig. 5). Two of nine coding changes [amino acids (aa) 300 and 305] in CPV-Y1, CPV-Kushiro and CPV-ob1 were identical to two of three CPV-2a-specific changes (Parrish et al., 1988a); however, the other CPV-2a-specific change (aa 555; CPV-2/CPV-2a: V/I) was not found in this study. Two aa residues (aa 411 and 562) of the CPV strains that differ from MEV-Abashiri were found in the MEV-A, -D and FPLV-193 strains, indicating that these residues are not specific to CPV. Thus, within the 60 to 91 m.u. region, five of nine coding changes (aa 93, 103, 323, 564 and 568) seemed to be phylogenetically CPV-common.

Replication of recombinant viruses containing site-directed changes

To determine the aa residues necessary for acquisition of the host range of CPV, various recombinant viruses containing site-directed change(s) were generated and their host ranges in vitro were analysed (Fig. 6 and Table 3). v28 and vPB8, containing the two aa of the CPV sequence, Asn-93 and Asn-323, that have been shown to be required to impart the canine host range to FPLV (Chang et al., 1992), replicated little in A72 cells as the ratios of viral yields were not greatly different from that of vMEV. Nor did vPB846 replicate efficiently in A72 cells although it contains all of the five aa changes that are considered to be phylogenetically CPV-specific. We have confirmed that the recombinant viruses v28, vPB8 and vPB846 possessed the changed nucleotides that were introduced by in vitro mutagenesis, by direct sequencing of PCR products derived from their virion DNA. Both vB52 and vP5 replicated with similar efficiency, indicating that Ala-103 was not important for the in vitro canine host range. Compared with v28, v78, vPB8 and vPB846, recombinant viruses vB57, vPBV58, vPV46 and vPA46 appeared to replicate to some extent as the ratios were in the order of 10⁻³ (Table 3) and RF DNAs were detected (Fig. 6), although the efficiencies of replication were obviously lower than that of vP5. Consequently, in the CPV/MEV recombinant virus system, we could not specify the amino acids determining the host range for canine cells; the host range in vitro appeared not to be determined by only two or three amino acids, rather most of the nine amino acid coding changes in the region from 60 m.u. to the carboxy terminus coding area of the VP ORF appeared to be required to impart canine cell tropism to MEV.

Involvement of NS gene

Although vP5 replicated in A72 cells, the viral growth was not as efficient as that of vCPVY1 (Tables 1 and 2). This suggested that another region was required for full expression of the in vitro canine host range. To assess the NS gene for its host range determining capacity, vE5 containing the 22 to 100 m.u. region of the CPV genome was generated and viral yields were compared with those of chimeric viruses containing all or part of the VP ORF. The results are shown in Table 4. The recombinant virus vX5, with the 45 to 100 m.u. region of the CPV genome in a MEV background, contains an extreme carboxy-terminal coding region of the CPV NS ORF that encodes eight aa, although the aa sequence is identical to that of MEV. Thus, vX5 was regarded as carrying the complete CPV VP ORF and MEV NS ORF. As shown in Table 4, vE5 appeared to replicate more efficiently in A72 cells compared with vX5 and vP5. When the ratios of A72/CRFK viral titres were compared, the difference between vE5 and vCPVY1 was not statistically significant (P > 0.05), whereas the differences between vE5 and vX5 (P < 0.05) and vE5 and vP5 (P < 0.05) were significant. These results indicated that the VP gene alone was not sufficient but that the additional 22 to 45 m.u. region of the CPV genome assisted in the nearly full expression of the ability to replicate in canine cells. The 22 to 45 m.u. region encodes part of the NS protein from aa 277 to the carboxy terminus. Hence the NS protein is suggested to contribute to the efficacy of virus replication in canine cells. In this region, four of 11
Fig. 5. Comparison of the nucleotide and predicted amino acid sequences of MEV-Abashiri and CPV-Y1 with other CPV strains and the previously reported MEV and FPLV strains. (a) Genetic map of MEV-Abashiri. NS, VP1 and VP2 initiation codons are denoted by arrows. The region between the PstI (60 m.u.) and PacI (91 m.u.) sites that seemed to confer the CPV host cell range is also marked. (b) Differences between MEV-Abashiri and CPV-Y1. The upper line indicates the MEV-Abashiri genome, and the lower the CPV-Y1 genome. Nucleotide differences within the two large ORFs are shown; short vertical lines indicate silent nt differences, long lines with nt numbers indicate coding changes. The aa are numbered from the initiation codon of VP2. (c) Differences in the aa sequence between 60 m.u. and the carboxy terminus of the VP gene. Among the nine coding changes shown in (b), aa residues identical to those of CPV-Y1 are denoted by closed circles. Amino acid residues different from MEV-Abashiri except for these nine are indicated by arrowheads. DNA sequences of CPV-Kushiro (Goto et al., 1984), CPV-CP49 (Azetaka et al., 1980) and CPV-obl were determined by direct sequencing of PCR products; those of MEV-A and -D, FPLV-Carl and FPLV-193 were reported by Parrish et al. (1988a), Carlson et al. (1985) and Martyn et al. (1990), respectively.
nucleotide differences between MEV-Abashiri and CPV-Y1 were involved in coding changes (Fig. 5).

This region also contains a p40 promoter which controls transcription of VP mRNAs. One possibility that therefore arose was that a difference within the p40 promoter region between MEV and CPV affected expression of the VP genes in canine cells. However, efficient transcription of the VP gene has been observed in A72 cells transfected with pMEV (Horiuchi et al., 1992). Therefore, the involvement of the p40 promoter region in the full expression of the in vitro canine host range seemed to be negligible.

Discussion

In this study we investigated the genomic region which controls the canine host range of CPV using CPV/MEV chimeric viruses. The 60 to 91 m.u. region of the CPV genome was shown to be required to confer the ability of MEV to replicate in A72 canine fibroblasts, although vPP replicated in A72 cells at reduced efficiency compared with vCPVY1 (Table 2). This region contained a part of the VP ORF encoding aa 91 to the carboxy terminus of VP2 and the 112 bp 5′ non-coding region adjacent to the VP ORF. We could not exclude the participation of this non-coding region in determining the host range. In addition, part of the NS gene appeared to participate in the acquisition of the nearly complete ability to replicate in canine cells.

The results in this study differ somewhat from the results obtained using CPV/FPLV chimeric viruses. The 59 to 73 m.u. region of the CPV genome has been shown to determine the canine host range using CPV/FPLV chimeric viruses generated by transfecting recombinant RF DNAs between the two viruses into CRFK cells (Parrish et al., 1988a). In the recent report by Chang et al. (1992), at least two aa within the 59 to 73 m.u. region (aa 93 and 323 in the VP2 sequence) have been shown to be responsible for the canine host range, and the chimeric viruses containing the 59 to 91 m.u. region of CPV in the FPLV genetic background replicated in A72 cells with an efficiency similar to that of parental CPV. There are several possible reasons for these differences. First, the different combinations of viruses might cause different results. We used the MEV-Abashiri and CPV-Y1 strains in this study. Although FPLV and MEV are indistinguishable in their host range in vitro, it is possible that a latent difference exists between the two viruses which might become apparent as a result of constructing CPV/FPLV or CPV/MEV recombinant viruses. The CPV strains were also different: CPV-Y1 belongs to CPV-2a, a category proposed by Parrish et al. (1988b) on the basis of the reactivity of monoclonal antibodies, whereas their work was done with CPV-2, the original CPV type. It has been reported that three CPV-2a-specific coding changes exist in the 59 to 91 m.u. region compared with CPV-2 (Parrish et al., 1988a), and CPV-Y1 actually possessed two of them (aa 300 and 305, Fig. 5). Hence the aa changes between CPV isolates, which are not CPV-common phylogenetically, could affect some biological properties such as stability of the capsid structure or the maintenance of certain functional epitopes. For instance, one CPV-2a-specific coding change in the VP2 sequence at aa 87, which is encoded outside the region of 59 to 91 m.u., has been suggested to form an epitope with two CPV-2a-specific coding changes at aa 300 and 305 (Parrish et al., 1991). Second, for the assessment of viral replication, it should be noted that the evaluation systems for viral replication in canine cells are different. We could not perform a direct plaque assay because no clear plaques could be generated in A72 cells; therefore viral titres of the culture fluid of virus-infected A72 cells were determined in CRFK cells. In such cases, the different efficiencies of virus production should be emphasized compared with direct plaque assay if viral titres are determined only by plaque numbers. Third is the relatively low titre of some virus stocks. Because some recombinant viruses did not grow well in CRFK cells, we barely obtained virus stocks whose titres were $1 \times 10^6$ p.f.u./ml. Therefore, the viruses could be inoculated at an m.o.i. of only 0.1 for the analysis of the host cell range. However, under these conditions, virus production in A72 cells was not as efficient and the relatively low titres made it difficult to assess the replication of viruses in A72 cells.

The results presented in Table 4 show that the VP gene is basically essential but that the NS gene might cooperate to confer full expression of the growth properties of CPV in canine cells. The fibrotropic and lymphotropic determinants of MVM(p) and MVM(i) have also been mapped to a small region within the VP gene (Antonietti et al., 1988; Gardiner & Tattersall, 1988b). However, a recombinant virus containing only that region of MVM(p) in an MVM(i) genetic background has been reported to generate pinpoint-sized plaques, whereas viruses containing a large region of the MVM(p) genome, including the NS gene of MVM(p) in addition to the minimum region responsible for the fibrotropism, generated wild-type plaques in mouse fibroblasts (Gardiner & Tattersall, 1988b). A similar observation has been reported on analysis of the determinants of fibrotropism of hr 101, an extended host range variant of MVM(i) (Ball-Goodrich et al., 1991). These reports seem to support our finding in the CPV/MEV system that the NS gene is involved in the full expression of the growth properties of CPV in canine cells. Furthermore, vX5, which contains a heterologous combination of NS and VP genes, was about one-tenth
Table 3. Viral yields in A72 and CRFK cells infected with CPV/MEV recombinant viruses containing site-directed changes

<table>
<thead>
<tr>
<th>Virus</th>
<th>A72 cells (p.f.u./ml)</th>
<th>CRFK cells (p.f.u./ml)</th>
<th>Yield ratio (A72/CRFK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMEV</td>
<td>$1.9 \times 10^5$</td>
<td>$5.7 \times 10^4$</td>
<td>$3.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>vCPVY1</td>
<td>$4.8 \times 10^4$</td>
<td>$2.4 \times 10^5$</td>
<td>$2.0 \times 10^{-1}$</td>
</tr>
<tr>
<td>vP5</td>
<td>$6.8 \times 10^5$</td>
<td>$3.3 \times 10^5$</td>
<td>$2.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>v28</td>
<td>$1.0 \times 10^5$</td>
<td>$3.2 \times 10^5$</td>
<td>$3.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>v78</td>
<td>$1.4 \times 10^5$</td>
<td>$4.4 \times 10^5$</td>
<td>$2.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>vPB8</td>
<td>$2.6 \times 10^6$</td>
<td>$4.8 \times 10^6$</td>
<td>$5.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>vB52</td>
<td>$3.6 \times 10^4$</td>
<td>$1.3 \times 10^5$</td>
<td>$2.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>vB57</td>
<td>$9.9 \times 10^2$</td>
<td>$1.7 \times 10^5$</td>
<td>$5.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>vPBV58</td>
<td>$9.0 \times 10^2$</td>
<td>$2.1 \times 10^4$</td>
<td>$4.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>vPA46</td>
<td>$6.0 \times 10^2$</td>
<td>$9.0 \times 10^4$</td>
<td>$6.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>vPB846</td>
<td>$9.2 \times 10^2$</td>
<td>$2.7 \times 10^5$</td>
<td>$3.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>vPB846</td>
<td>$2.9 \times 10^3$</td>
<td>$1.5 \times 10^5$</td>
<td>$1.9 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

As efficient at replicating in A72 cells as vCPVY1 (Table 4). This suggests that a direct or indirect interaction of NS and VP proteins, although no evidence for the interaction of the two viral proteins has yet been demonstrated, occurs during the virus life cycle. Further, the possible interaction between heterologous combinations may be less effective than that of homologous combinations for the production of a mature virion. Single-stranded DNA synthesis is concomitant with packaging in the autonomous parvoviruses (Richards et al., 1977), and NS-1 has been reported to be covalently attached to the 5' termini of RF DNA and newly synthesized ssDNA (Cotmore & Tattersall, 1988). It is tempting to speculate that the NS and VP proteins interact at the packaging stage. Otherwise, because NS protein is known to be essential for viral DNA replication (Rhode, 1989), it is also conceivable that an interaction between NS protein and a host cell factor(s) concerned with viral DNA replication may be involved in the efficient replication of viral DNA. The NS protein of CPV may be more suitable for canine cells than that of MEV.

Table 4. Virus production in A72 and CRFK cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean virus yield (p.f.u./ml)*</th>
<th>Yield ratio† (A72/CRFK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMEV</td>
<td>$1.9 \times 10^5 \pm 1.0 \times 10^5$</td>
<td>$7.0 \times 10^5 \pm 5.0 \times 10^6$</td>
</tr>
<tr>
<td>vCPVY1</td>
<td>$2.7 \times 10^4 \pm 1.0 \times 10^4$</td>
<td>$1.1 \times 10^5 \pm 0.7 \times 10^5$</td>
</tr>
<tr>
<td>vE5</td>
<td>$1.1 \times 10^5 \pm 0.2 \times 10^5$</td>
<td>$8.3 \times 10^4 \pm 3.3 \times 10^4$</td>
</tr>
<tr>
<td>vX5</td>
<td>$2.7 \times 10^5 \pm 2.2 \times 10^5$</td>
<td>$1.3 \times 10^5 \pm 0.7 \times 10^5$</td>
</tr>
<tr>
<td>vP5</td>
<td>$2.9 \times 10^5 \pm 2.7 \times 10^5$</td>
<td>$2.6 \times 10^5 \pm 1.8 \times 10^5$</td>
</tr>
<tr>
<td>vV5</td>
<td>$3.9 \times 10^5 \pm 2.8 \times 10^5$</td>
<td>$3.5 \times 10^5 \pm 2.4 \times 10^5$</td>
</tr>
</tbody>
</table>

* Titres are expressed as mean p.f.u./ml (±S.E.M.) from three independent experiments.
† The A72/CRFK virus yield ratios are expressed as mean values (±S.E.M.) from the three experiments.
directed mutagenesis. Both results revealed that only two amino acid changes are responsible for tropism and that these changes appeared to be on the surface of the virion (Tsao et al., 1991). In this study, even though vPB846 contains five amino acid changes that seemed to be CPV-common in the MEV background, it did not show an apparent CPV phenotype in its host range in vitro (Fig. 6). After its first appearance, CPV seems to have evolved continuously to escape immune pressure (Parrish et al., 1991) and probably to adapt further to dogs. Such evolution would apply to MEV and FPLV. Therefore, a possible explanation of the different results in site-directed mutagenesis is that the phylogenetic relationship between CPV-Y1 and MEV-Abashiri may be more distant than that between CPV-d and FPLV-b which were used by Chang et al. (1992), and thus they might contain some minor changes which affect certain biological properties.

At which stage and by what mechanism is VP protein involved in the restrictive infection? It has been suggested that the restrictive infections of some paroviruses are not mediated at the cell surface level (Spalholz & Tattersall, 1983; Ridpath & Mengeling, 1988; Horiiuchi et al., 1992; Oraveerakul et al., 1992). Late stages in the virus replication cycle (after viral transcription) are not involved in restrictive infection either (Gardiner & Tattersall, 1988a; Horiiuchi et al., 1992). Synthesis of the complementary strand of incoming virion DNA to provide a duplex DNA template precedes viral transcription (Cotmore & Tattersall, 1987). This occurs in the reciprocal restrictive infection of MVM(p) and MVM(i) but transcription occurs only in a permissive host, suggesting that neither penetration nor uncoating of the virion is affected, but transcriptional initiation is blocked in the restrictive host (Spalholz & Tattersall, 1983; Gardiner & Tattersall, 1988a). It is not known whether this can be extended to explain the host cell specificity of the feline parovirus subgroup. It is also likely that uncoating may be one stage at which virus replication is regulated in restrictive cells, although the mechanism of paroviral uncoating remains to be elucidated. A cellular factor(s) is expected to be involved in viral uncoating; thus, more speculatively, a mechanism similar to protease-dependent tropism, which has been proposed for paramyxoviruses (Nagai et al., 1976), could regulate the host range of the feline parovirus subgroup. A hypothetical endopeptidase in canine cells might not efficiently cleave MEV or FPLV capsid protein at the uncoating step.

This work was supported by a Grant-in Aid for Encouragement of Young Scientists (05760217) from the Ministry of Education, Science and Culture of Japan.

References


(Received 7 October 1993; Accepted 20 December 1993)