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Nonstructural Protein-2 and the Replication of Canine Parvovirus

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The nonstructural protein-2 (NS2) of canine parvovirus (CPV) is produced from the left-hand open reading frame of the viral genome and contains 87 amino-terminal amino acids in common with nonstructural protein 1 (NS1) joined to 78 amino acids from an alternative open reading frame. In the minute virus of mice parvovirus NS2 plays a role in controlling capsid protein assembly and translation in a host-specific manner. The predicted NS2 of CPV is divergent from the proteins of the rodent parvoviruses, and the protein and its functions have not been described. We characterized the large and the small splices of CPV using reverse transcriptase–PCR. NS2 was identified using anti-peptide antibodies against the predicted C-terminal sequence and also by expressing the protein from a plasmid vector. The protein could be detected at low levels in the nucleus and the cytoplasm of a proportion of CPV-infected cells, as well as in cells transfected with the expression plasmid. Virus genomes were prepared with mutations in the splice donor or acceptor sites of the NS2-specific intron or with three different termination codons in the NS2-unique exon. Both splice donor and acceptor mutations resulted in the use of previously cryptic splice sites, and the virus containing the splice donor mutation replicated inefficiently. However, the other four mutant viruses were all viable and replicated efficiently in cat and dog cells, and two mutant viruses that were tested appeared to assemble their capsids in the same manner as did the wildtype. After inoculation of dogs an NS2 mutant virus with a termination codon in the NS2-unique exon replicated to titers similar to those seen for wildtype CPV in several tissues examined. © 1998 Academic Press

INTRODUCTION

Canine parvovirus (CPV) is a member of the feline parvovirus subgroup of the autonomous parvoviruses (Murphy *et al.,* 1995; Parrish, 1990). The autonomous parvoviruses that are similar to minute virus of mice (MVM) all appear to contain genes for two nonstructural (NS) proteins, NS1 and NS2, as well as two viral structural proteins, VP1 and VP2 (Cotmore *et al.,* 1983; Cotmore and Tattersall, 1986, 1987). The structural (VP1 and VP2) and nonstructural proteins are transcribed from separate promoters at about 38 and 4 genome map units (m.u.), respectively (reviewed Cotmore and Tattersall, 1987). The different proteins are derived by alternative splicing of the RNA transcripts and in MVM most transcripts terminate at a single polyadenylation sequence (Clemens and Pintel, 1987).

NS2 is formed from a spliced message (R2) transcribed from the P4 promoter, which in the prototype strain of MVM (MVMp) joins 85 N-terminal residues from reading frame 3 to between 97 and 103 residues from reading frame 2 (Cotmore *et al.,* 1983; Cotmore and Tattersall, 1986; Morgan and Ward, 1986). In MVM 3 different carboxy termini may be present in NS2 depending on the splicing of a small intron linking the first two exons to one or other additional exon sequences (Cotmore and Tattersall, 1990; Morgan and Ward, 1986). Production of the correct proportions of R1 and R2 mRNAs is subject to a variety of controls, which affect the splicing from suboptimal sequences in the R2 splice donor, and splicing also depends on translation of the small exon in the R2 message (Zhao *et al.,* 1995a,b, 1994). Mutants of MVM with splice acceptor or termination mutations in NS2 replicate very poorly in murine cells, although they replicate more efficiently in cells from other hosts (Cater and Pintel, 1992). In murine cells deletion or truncation of NS2 affects efficient capsid protein assembly and causes a decrease in translation of viral mRNA, and the mutant viruses replicate to very low levels in mice (Brownstein *et al.,* 1992; Cotmore *et al.,* 1997; Naeger *et al.,* 1990, 1993). In MVM phosphorylated forms of NS2 are found preferentially in the cytoplasm (Cotmore and Tattersall, 1990), and they also associate with the β and ζ isoforms of the 14-3-3 class of cellular proteins from a variety of hosts, through interaction with phosphoserine interactions (Brockhaus *et al.,* 1996). NS2 functions of the rat parvovirus H1 virus appear similar in many features to those of MVM. The protein is required for efficient replication and virion production in rat cells and to cause disease in rats, but it is not required for the virus to cause disease in hamsters or for replication in human cells. The host-specific effect appears to be on the efficiency of

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translation of the capsid proteins, mediated through sequences in the 3′-nontranslated regions of the viral messages (Li and Rhode, 1991, 1993).

Less is known about the NS2 proteins of the parvoviruses other than MVM and H1. Candidate NS2 proteins have been immunoprecipitated from cells infected with LuIII, bovine parvovirus, and rabbit parvovirus (Cotmore and Tattersall, 1986; Matsunaga and Matsuno, 1983), but in one study no NS2-like protein was immunoprecipitated by an antiserum against the MVM protein sequences from the *in vitro* translation products of mRNA from CPV-infected cells (Cotmore and Tattersall, 1986).

The host ranges of CPV and FPV are complex, and differ both *in vitro* and *in vivo,* and they depend on both the host animal and the differentiated state of the cell (Truyen *et al.,* 1996; Truyen and Parrish, 1992). CPV infection of dog cells or dogs is controlled primarily by the capsid protein gene, with two or three residues which differ between CPV and FPV being particularly important (Chang *et al.,* 1992; Horiuchi *et al.,* 1994; Llamas-Saiz *et al.,* 1996). Those residues are found in three regions of the surface of the capsid and result in a variety of structural differences that can be identified by X-ray crystallography (Agbandje *et al.,* 1993; Llamas-Saiz *et al.,* 1996). The replication of two variants of MVM (MVMi and MVMp) in lymphocytes or fibroblasts is also controlled by small numbers of sequence differences within the capsid protein gene (Ball-Goodrich and Tattersall, 1992; Gardiner and Tattersall, 1988).

As well as the major host range determinants within the capsid protein gene, less pronounced effects have been mapped to the NS1 and NS2 gene regions of CPV and MVM. In studies of recombinants between CPV and mink enteritis virus (MEV), sequence differences between 22 and 45 m.u. in the genome were shown to affect infection or replication in canine cells, although the precise changes involved were not determined (Horiuchi *et al.,* 1994). The replication of pig parvovirus (PPV) in canine cells is controlled by sequences in both the structural proteins and the NS1 gene region of the genome (Vasudevacharya and Compans, 1992). For MVMp and MVMi recombinants, viruses containing certain combinations of sequences from the NS1 gene region differed in efficiency of cell infection and plaquing (Gardiner and Tattersall, 1988).

Here we have defined the nature of the splicing of the large and small introns of CPV and shown that NS2 is indeed produced by that virus. We examined the functions of NS2 by introducing mutations into an infectious clone which were predicted to alter the NS2 expression or size. The mutations had no consistent effect on *in vitro* infection, replication, or capsid assembly in a variety of cells. One mutant virus with a termination codon in NS2 replicated in dogs as well as its wildtype parent.

RESULTS

Splicing

Splicing of the CPV mRNA transcripts was examined by RT–PCR and sequencing. As shown in Fig. 1, the small splice joined alternative donors at nt 2280 (^{2273}GGATTAAG ↑ GTACG) or nt 2316 (^{2311}GGAGAG ↑ GTAAG) to a single splice acceptor at nt 2389 (^{2384}TATAG ↓ GACTTG) in the sequence of CPV-d (Parrish, 1991). The R2 splice which would form NS2 joins the 5′ exon by the nonconsensus splice donor at nt 532 (^{528}AAAAA ↑ GCAAGT) to a consensus splice acceptor at nt 2005 (^{2001}CCAG ↓ GTTGCA). All R2 transcripts cloned were also spliced at the small intron, as would be expected for the model of parvovirus alternative splicing regulation (Zhao *et al.,* 1995a,b, 1994). In CPV the NS2 open reading frame terminated prior to the small intron, which is in contrast to the situation in the rodent parvoviruses. The 87-residue amino-terminal domain of NS2 shared with NS1 was quite conserved between CPV and the rodent parvoviruses (MVM, H1, MPV) (55% identity between CPV and MVMp), as well as the PPV (Fig. 3). The NS2 carboxy-terminal domain was very conserved among the rodent parvoviruses (100% identity between MVMp and MPV; 76% identity between MVM and H1), but in CPV that sequence was divergent both from the rodent viruses (30% identity with MVM) and PPV (20% identity) (Fig. 3). The NS1 sequence encoded within the alternative reading frame from the NS2 C-terminal domain was more highly conserved (52% identity between CPV and MVM).

NS2 protein expression

The NS2 protein was readily demonstrated in CPV-d-infected A72 cells by immunofluorescence, although it was not detected at the same levels in all cells which expressed capsid proteins (Figs. 2A and 2B). The protein was detected throughout the cell, with increased amounts in the nuclei of some cells. Fluorescent antibody staining of infected synchronized cells indicated that neither the level of NS2 expression nor cell localization was significantly affected by the stage of the cell cycle (results not shown). NS2 protein expressed from a mammalian plasmid expression vector showed a similar distribution within the cells as was seen in the infected cells, but the level of expression was similar in all NS2-positive cells (Fig. 2D).

NS2-related proteins were immune precipitated from [^{35}S]methionine-labeled cells which were infected with CPV-d or transfected with the NS2 gene in a plasmid expression vector (Fig. 4). The protein from the infected cells was present as a single band of 19 kDa, which was the size predicted from the DNA sequence of the gene. After plasmid expression the full-length native NS2 protein was formed, as well as a form about 2000 Da smaller which may represent a product that is less phosphorylated (Clemens *et al.,* 1990) (Fig. 4).

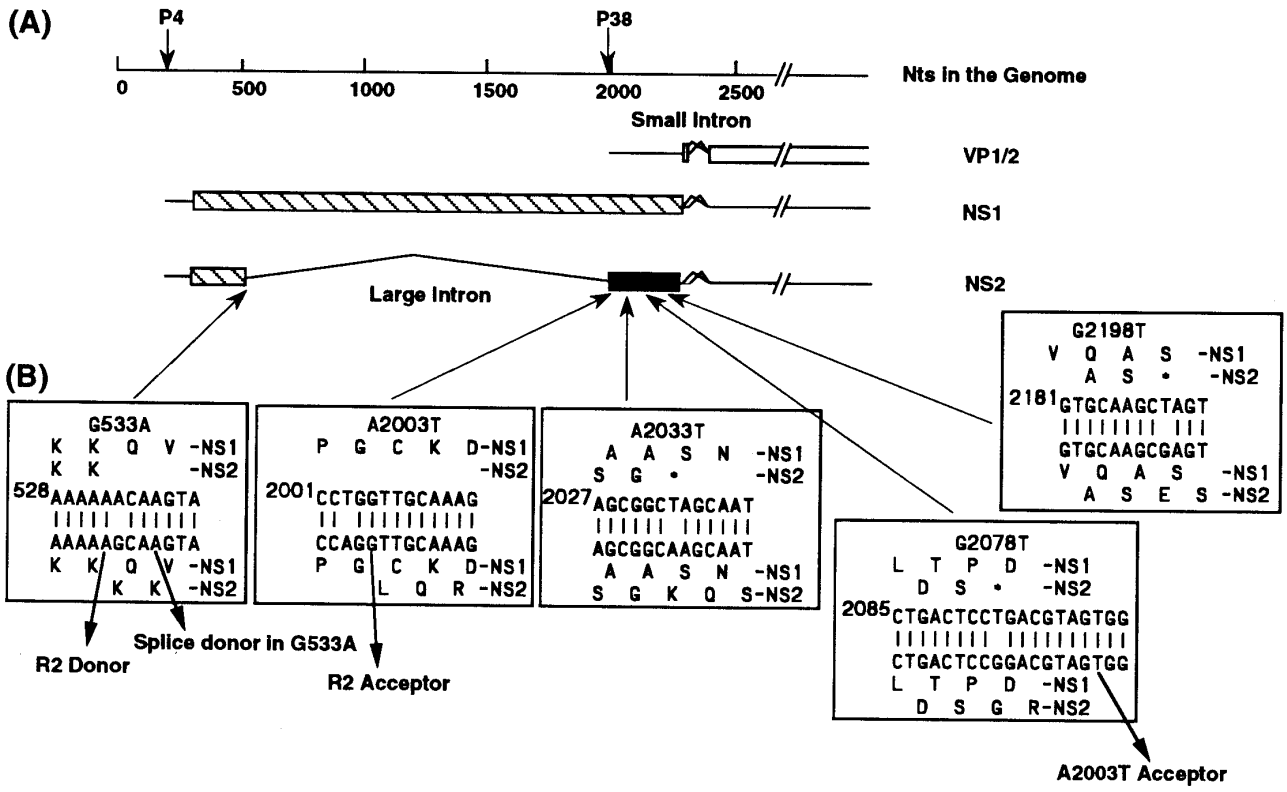

FIG. 1. (A) Genetic structure of the left-hand 60% of the CPV genome, showing the P4 and P38 promoters, the locations of the splice sites in the R2 and R3 mRNAs, and open reading frames of NS1 and NS2. (B) Mutations prepared in these studies. The mutations are identified by the wildtype nucleotide, the nucleotide number, and the new nucleotide in the mutant. The wildtype and mutated virus sequences and the NS1 and NS2 translations are also shown. The normal R2 splice donor and acceptor sequences identified in these studies are indicated, as is the alternative donor used in the mutant virus G533A.

NS2 mutant viruses

Mutations were prepared which introduced termination codons at various positions in the NS2-specific portion of the open reading frame or which were designed to inactivate the R2 splice donor or acceptor (Fig. 1). All five mutants would likely produce some amount of the NS2 amino-terminal domain, as RT–PCR of mRNA from cells inoculated with mutant G533A (R2 splice donor) or mutant A2003T (R2 splice acceptor) showed that some mRNA was spliced using previously cryptic splice donor and acceptor sites, respectively (Fig. 1). After transfection of NLFK or A72 cells the G533A infectious plasmid replicated very inefficiently, but it did form low levels of viral DNA and virus.

To verify the presence of the NS2 mutants, the accumulations of NS2 and capsid proteins were examined by immunoprecipitation of infected A72 cells labeled for 3 h with [^{35}S]methionine (Fig. 5). With the exception of G533A, the NS2 mutants all produced amounts of VP1 and VP2 approximately equal to those produced by CPV-d. The NS2 protein could be precipitated from the CPV-d-infected but not from the mutant infected cells, confirming that intact NS2 protein was not expressed by the mutant viruses.

Forty-eight hours after transfection of wildtype or mutant infectious plasmids, four of the mutants produced similar amounts of capsids or virions in HA, ELISA, and TCID50 assays, and similar amounts of RF-DNA were recovered from cells transfected with those plasmids (Fig. 6A). Although the G533A plasmid was viable and infectious virus was recovered after transfection, the production of capsid protein was less than 1/10 that of the other mutants examined (Fig. 5B). We do not have an explanation for that difference, but it may be related to the alternative splicing of that sequence once the R2 splice donor was mutated. There were no additional mutations in G533A that would explain the differences in infectivity or replication, as the region containing the mutation was recloned into a new infectious plasmid background, the region replaced was sequenced, and the virus derived had the same properties as G533A.

When viruses were prepared from transfection of A72 cells and used to inoculate A72 or NLFK cells, the four mutants besides G533A all produced both double- and single- stranded DNA at levels similar to those of CPV-d in A72 cells. In NLFK cells the DNA levels of the mutant viruses were consistently slightly lower than those in CPV-d, but again they produced both the double- and single-stranded DNA forms in similar proportions (Fig. 6B).

NS2 mutants of MVM appear to assemble poorly in cells (Cotmore *et al.,* 1997). The assembly of mutants

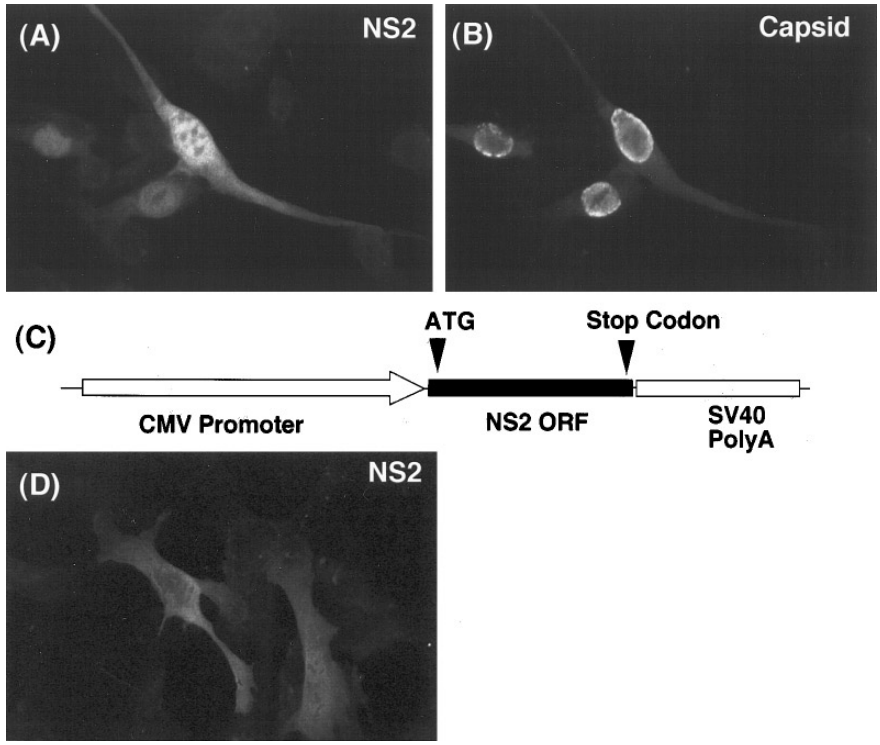

FIG. 2. Identification of the NS2 protein in infected cells and expression from a plasmid vector. (A) CPV-d-infected A72 cells stained with mouse anti-NS2 peptide serum and detected with FITC-labeled anti-mouse IgG. (B) The same cells as in (A) stained with RITC-conjugated rabbit anti-capsid antibodies. (C) Expression vector for transient expression of NS2. The plasmid was prepared in the pCEP4 vector and contains a complete cDNA copy of the NS2 message, as well as the CMV immediate early promoter and the SV40 polyadenylation signal. (D) A72 cells 48 h after transfection with the NS2 plasmid, stained with mouse anti-NS2 peptide serum, and detected with FITC-labeled anti-mouse IgG.

A2003T and A2033T in A72 cells was therefore compared to that in CPV-d by [^{35}S]methionine labeling for 30 min and then incubation of the cells with complete medium for 2 h. Capsid formation was analyzed by sucrose gradient centrifugation, and similar levels of empty and full capsids were seen for the three viruses (Fig. 7).

Inoculation of feline CRFK and 3201 cells, or canine A72 and MDCK cells, showed only small differences among CPV-d, A2003T, and A2033T in the levels of virus recovered (Fig. 8). None of the viruses infected COS-1, NB324K, or L cells.

Mutant A2033T replicated efficiently in dogs, with titers that were similar to or higher than those seen for CPV-d in several tissues examined (Fig. 9).

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CPV-d    1 MS.GNQYTEEVMEGVNWLKKHAENEAFSFVFKCDNVQLNGKDVRWNNYTKPIQNEELTSL
H1       1 MA.GNAYSDEVLGVTNWLKDKSSQEVFSFVFKNENVQLNGKDIGWNSYRKELQDDELKSL
MPV      1 MA.GNAYSDEVLGTTNWLKEKSNQEVFSFVFKTEDVQLNGKDIGWNNYKKELQEDELKSL
MVMp     1 MA.GNAYSDEVLGATNWLKEKSNQEVFSFVFKNENVQLNGKDIGWNSYKKELQEDELKSL
PPV      1 MAAGNTYSEEVLKATNWLQDNAQKEAFSYVFKTQKVNLNGKEIAWNNYNKDTTDAEMINL

                                  Exon1 ──┼── Exon2

CPV-d   60 IRGAQTAMDQTEEEEMDWESEVDSLAKKLQRLRDT.SGKQSSESRPSSNSSDSGRSGPCT
H1      60 QRGAETTWDQSEDME..WESAVDDMTKKFNALTISDSEKYASQPKLRSYSTCIGPCGPSS
MPV     60 QRGAETTWDQSEDME..WESTVDEMTKKFGTLTIHDTEKYASQPELCTNSTCIGSRGPGF
MVMp    60 QRGAETTWDQSEDME..WETTVDEMTKKFGTLTIHDTEKYASQPELCTNSTCIGSRGPGF
PPV     61 QRGAETSWDQATDME..WESEIDSLTKRLSDFHIS..ENFASGQQLRSNSNTGGP.GFSF

CPV-d  119 GTVEYSRYAYQRNC.KSTIKPTWRYSQRRAS.ESDMVRNRGRPESHLYF...........
H1     118 RALEHTKYSCCGHCSKPKHWGGWFHSLPRCSTEPNLVRDRGGFESLLQSRTVGERLQRGA
MPV    118 RALEHTKYSCCGHCRNPEHWGSWFQSLPRWSTEPNLVRDRGGFESVLRCGTVEERLQRAA
MVMp   118 RALEHTKYSCCGHCRNPEHWGSWFQSLPRWSTEPNLVRDRGGFESVLRCGTVEERLQRAA
PPV    116 S.LGAVERANNTNFHQPALN.SNTARFSNTDTKSNLVGNRNRHKSLLW...........

CPV-d      ...........
H1     178 DLGLRPEITWF
MPV    178 ELGLRPEITWF
MVMp   178 ELGLRPEITWF
PPV        ...........
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FIG. 3. Alignment of the translated CPV-d NS2 sequence with those of H1, MVMp, mouse parvovirus (MPV), and PPV. Shading indicates that the sequence is identical between CPV and the other virus. The MVM, H1, and MPV sequences all terminate after the small intron in the sequence and hence have at least three alternative NS2 carboxy-terminal sequences, two of which are introduced by alternative splicing. For H1, MPV, and MVM the most common "P" isoform is shown (Cotmore and Tattersall, 1990).

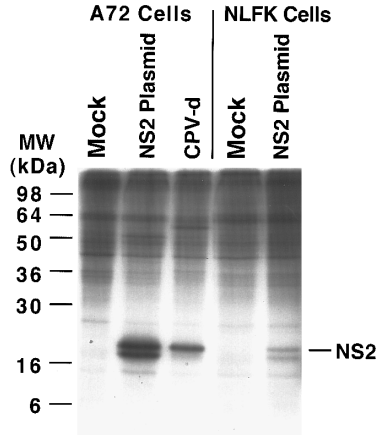

FIG. 4. Immune precipitation of proteins from [^{35}S]methionine-labeled cells using a mouse anti-NS2 peptide serum. A72 or NLFK cells were mock infected, infected with CPV-d, or transfected with the plasmid expressing NS2.

DISCUSSION

Here we show that the predicted NS2 protein gene of CPV is functional and that a single NS2 protein is produced in virus-infected cells. The size of the protein was close to the 19,076 predicted from the translated DNA sequence. Five mutants of CPV were prepared, and all were viable. Analysis of the effects of altering the splice donor or acceptor sequences was complicated by the fact that in each case an alternative sequence used to splice the message was detected by RT–PCR. Analysis of four of the five mutants which would affect NS2 transcription or translation showed that intact NS2 was not required for efficient virus DNA replication or single-stranded DNA production. When two mutants (A2003T and A2033T) were examined for capsid assembly in the A72 cells they assembled in a fashion similar to that of wildtype virus. One other mutation (G533A) which altered the R2 splice donor sequence resulted in inefficient DNA replication after plasmid transfection, and the virus grew to only low titers. Although we cannot explain why that virus replicates poorly by comparison with the other mutants, recloning of the mutation showed that the effect seen was due to the mutation introduced, and it is possible that this was related to the use of an alternative splice acceptor that alters the regulation of RNA transcription or splicing.

The role of the NS2 in *in vivo* infection was examined by inoculation of dogs with one of the mutants (A2033T) which contained a termination mutation in the second NS2 exon, close to the splice acceptor. That virus grew efficiently in several tissues of the dogs (Fig. 9), indicating that the intact NS2 was also not required for efficient replication *in vivo* in the host from which the virus was isolated. There are a number of possible explanations for this apparent lack of effect of the four NS2 mutations. Read through of the termination codon does not occur at levels where the protein can be detected in the RIP assay (Fig. 5A). A truncated form of NS2, containing only the amino-terminal domain, may provide NS2 functions, although that is unlikely based on similar studies of MVM NS2 (Brownstein *et al.,* 1992; Cater and Pintel, 1992; Cotmore *et al.,* 1997; Naeger *et al.,* 1990). A further possibility is that the dogs or the cat, mink, or dog cells tested here may not represent the original host of the CPV. Perhaps there is another (as yet unidentified) host from which CPV originally derived, in which NS2 is required for efficient replication. This would be similar to the situation reported for H1, where NS2 is required for the virus to replicate and cause disease *in vivo* in rats but not in hamsters (Li and Rhode, 1991).

The function(s) of NS2 in the replication of autonomous parvoviruses are still poorly understood. Most studies have been conducted on MVM and H1 parvoviruses, where NS2 mutations result in the viruses replicating poorly in their normal host cells, although they replicate with greater but variable efficiency in cells from other hosts (Brownstein *et al.,* 1992; Cater and Pintel, 1992; Li and Rhode 1991; Naeger *et al.,* 1990). The NS2 proteins of the rodent parvoviruses are highly conserved in sequence (Fig. 3), with 97% identity in NS2 sequence between MVM and MPV, indicating that there is a strong sequence dependence for their function(s) in rodents. NS2 mutants in MVM rapidly revert to wildtype sequence or acquire compensating mutations (Cotmore *et al.,* 1997), further indicating that the NS2 protein plays an important role in efficient replication of that virus. The cell restriction for MVM or H1 NS2 mutants appears to be due to an inefficient assembly of VP1 and VP2 into capsids in mouse or rat cells, with apparently a subsequent decreased translation of the capsid proteins (Cotmore *et al.,* 1997; Li and Rhode, 1991).

The finding that the NS2 of MVM and H1 was required

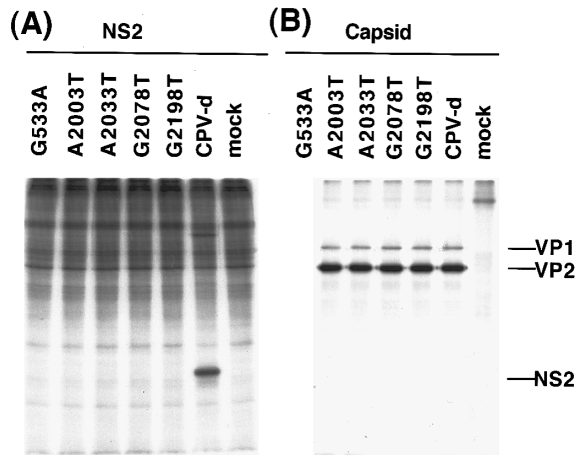

FIG. 5. Immune precipitation of [^{35}S]methionine-labeled proteins from mock-infected A72 cells or from A72 cells inoculated with 10^5 $TCID_{50}$ of CPV-d or each of the five different mutant viruses. (A) Cell lysates precipitated with the mouse anti-NS2 peptide serum. (B) Proteins from the same cells immune precipitated with a rabbit anti-capsid serum.

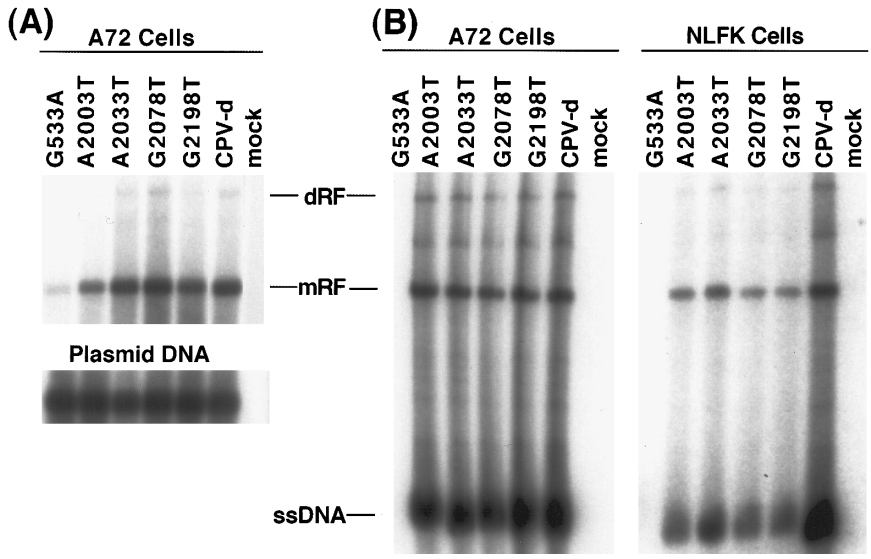

FIG. 6. DNA replication of CPV-d or the NS2 mutants after inoculation or transfection of A72 or NLFK cells. DNA recovered using a modified Hirt-extraction method was electrophoresed in a 1% agarose gel with 1 μg/ml of ethidium bromide, Southern blotted, and probed with a ^{32}P-labeled full-length viral DNA fragment. The dimer replicative form (dRF), monomer RF (mRF), and single-stranded (ss) DNAs are indicated. (A) DNAs recovered after transfection of A72 cells with infectious plasmids. The DNA recovered was treated with *Dpn*I prior to electrophoresis. The input DNA of each plasmid added is represented by a *Dpn*I fragment which is present in all the plasmids. (B) Viral DNAs recovered 48 h after inoculation of A72 or NLFK cells with the various viruses.

for replication in the cells and tissues of the normal host animal also indicates that NS2 plays a role in controlling the viral host range. Major determinants of host range of CPV, MEV, MVM, or PPV have all been mapped to specific sequences within the capsid protein genes (Ball-Goodrich and Tattersall, 1992; Bergeron *et al.,* 1996; Chang *et al.,* 1992; Horiuchi *et al.,* 1994; Vasudevacharya and Compans, 1992). However, determinants which enhance growth in certain host cells have also been mapped to the NS1 or NS2 genes of those viruses (Gardiner and Tattersall, 1988; Horiuchi *et al.,* 1994; Vasudevacharya and Compans, 1992). The finding that NS2 of MVM assists in capsid assembly in the homologous host cell suggests that there could be an interaction between the host range determining mutations of the capsid protein gene and the NS2 sequence, at least in the rodent parvoviruses.

Although the NS2 gene sequence is present as an intact open reading frame in all CPV, FPV, and MEV sequences that have been reported, and we show here that it is expressed from CPV, it differs significantly in amino acid sequence from the NS2 of the rodent viruses and PPV. The NS2 open reading frame of CPV also terminated prior to the small intron in the genome, so

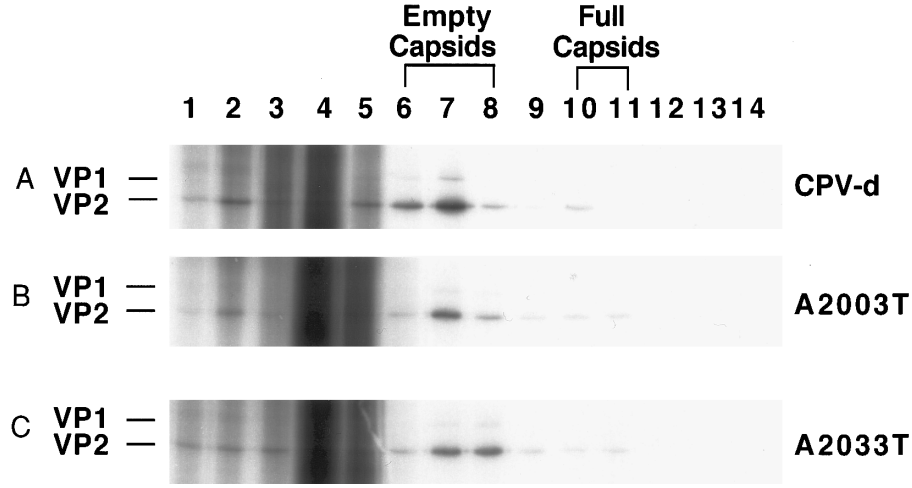

FIG. 7. Assembly of the capsid proteins of (A) CPV-d, (B) A2003T, or (C) A2033T in virus-infected cells. A72 cells were incubated with viruses for 24 h, labeled for 30 min with [^{35}S]methionine, and then incubated for 2 h with complete medium. Proteins were harvested and banded on 10–40% sucrose gradients, and then virus was immunoprecipitated from gradient fractions using a rabbit anti-capsid antiserum and electrophoresed in SDS–PAGE. The positions in the gradients of empty and full capsids are indicated.

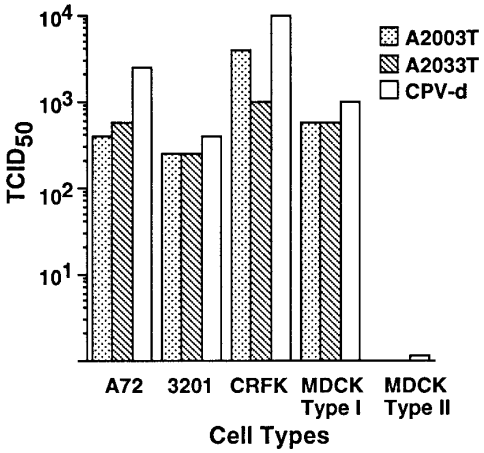

FIG. 8. Virus $TCID_{50}$ titers in NLFK cells for viruses recovered from cells inoculated with CPV-d, A2003T, and A2033T. Cells tested were canine A72 and MDCK type I and type II cells and feline 3201 or CRFK cells.

that the different isoforms of NS2 seen in MVM-infected cells would not be formed (Fig. 1). Four mutations which removed varying amounts of the carboxy-terminal domain of the protein did not affect the DNA replication of CPV or its protein translation or capsid assembly and also did not greatly affect the infection or replication of the virus in tissue cultured cells or in dogs. This indicates that the NS2 of CPV has properties different than those of the rodent parvoviruses and that its functions for the virus life style or replication must be relatively subtle.

MATERIALS AND METHODS

Viruses and cells

The CPV-d strain of CPV type-2 was derived from an infectious plasmid clone and propagated in Norden laboratory "feline" kidney (NLFK) or A72 canine cells (Binn *et al.,* 1980; Parrish, 1991). The NLFK cells in our laboratory are the most susceptible cells for infection and growth of all the carnivore parvoviruses we have tested to date, but recent genetic analyses indicate that these NLFK cells are not feline cells, but appear to be closely related to mink lung cells (C. R. Parrish, unpublished results).

Other cells tested in these studies included CRFK feline cells (Crandell *et al.,* 1973), a 3201 feline thymoma-derived cell line (Rojko *et al.,* 1989), type I and type II strains of the MDCK canine kidney cell line (Nichols *et al.,* 1986), human NB324K cells, African green monkey COS cells, and mouse L cells. Cells were grown either in a 50% mixture of McCoy's 5A and Leibovitz L15 with 5% fetal bovine serum (FBS) or in Dulbecco's minimal essential medium with 10% FBS.

Mutations of the CPV-d sequence were prepared to alter the NS2-specific R2 splice donor or the R2 splice acceptor or to introduce termination codons into the second NS2 exon (Fig. 1). The NS1 polypeptide sequence was left unchanged, and the altered NS1 codons were ones that were commonly used in the CPV NS1 and VP gene sequences. Mutations were introduced using specific oligonucleotide primers and uracil-containing M13 DNA, as described by Kunkel (1985). The regions containing the mutations were sequenced and introduced back into the CPV-d infectious clone. Plasmids were transfected into NLFK or A72 cells using Lipofectamine (Gibco/BRL, Gaithersburg, MD), and viruses recovered were passaged twice before being frozen as stocks. Stock viruses were confirmed as being mutant by PCR amplification and sequencing of the region containing the mutation. Mutant A2033T contained a unique new *Nhe*I site (GC*T*AGC), and the presence of that mutation was therefore also monitored by *Nhe*I digestion of RF-DNA or PCR products.

Transcription analysis

RNA recovered from virus-infected cells was pretreated with RNase-free DNase (Promega, Madison, WI), reverse transcribed, and then the cDNA amplified with primers flanking the predicted large or small splices. The small splice products digested with *Taq*I and *Hin*dIII were cloned into the mp18 vector digested with *Acc*I and *Hind*III. RT–PCR products spanning the large splice were recovered from agarose gels, incubated with DNA polymerase I (Klenow fragment) and dNTPs, and then ligated into *Sma*I-digested pGEM3Z plasmid. Clones were sequenced using the M13, T7, or SP6 primers and the Sequenase method (Amersham, Arlington Height, IL). The NS2 sequences predicted for CPV-d were compared to those of MVMp, H1, LuIII, mouse parvovirus (MPV), and PPV. Sequences obtained from GenBank were aligned using the Clustal W program of DNAStar (DNAStar Inc., Madison, WI).

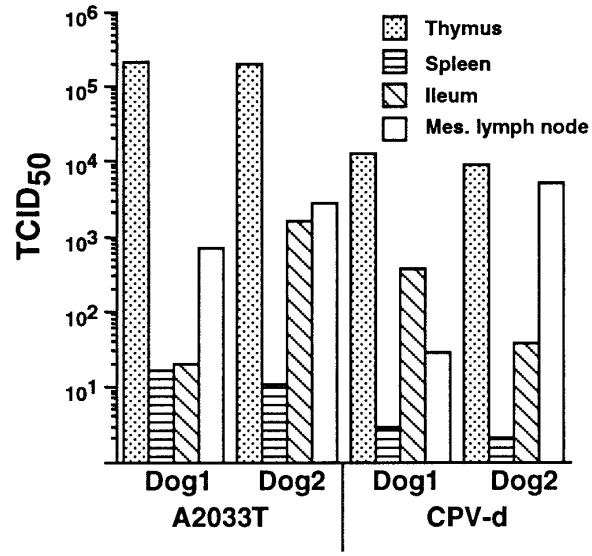

FIG. 9. Virus titers in tissues of 12-week-old dogs 5 days after inoculation with 10^5 $TCID_{50}$ of either A2033T or CPV-d. Tissues sampled were the thymus, spleen, ileum, and mesenteric lymph nodes, and titers were determined in NLFK cells.

Protein expression, antibodies, and immune staining

The spliced form of the R2 message was isolated from a cloned RT–PCR product, and the intact NS2 open reading frame was cloned into the eucaryotic expression vector pCEP4 (Invitrogen, San Diego, CA), under the control of the CMV promoter (Fig. 2C). That plasmid was transfected into A72 cells using Lipofectamine. Antibodies were prepared against a peptide containing the carboxy-terminal sequence of NS2 (NS2 residues 153 to 165). The peptide was conjugated to keyhole limpet hemocyanin and inoculated once into mice in Freund's complete adjuvant and then repeatedly in Freund's incomplete adjuvant. Virus-infected or plasmid-transfected cells were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS, and incubated first with the mouse anti-peptide sera and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. In some cases the cells were costained with rhodamine isothiocyanate (RITC)-conjugated rabbit IgG prepared against purified CPV capsids.

Cells infected with CPV-d or with NS2 mutants were labeled between 24 and 27 h postinfection with [^{35}S]methionine (Amersham) at 0.2 mCi/ml. The cells were lysed into radioimmune precipitation (RIP) buffer (50 mM Tris · HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin). The lysate was incubated with the mouse anti-NS2 peptide serum and then with Immunoprecipitin (Gibco/BRL). The precipitates were washed three times with RIP buffer, the Immunoprecipitin was pelleted and boiled in SDS-loading buffer, and then the proteins were electrophoresed in 12% SDS–polyacrylamide gels, which were then dried and exposed to X-ray film.

Virus replication

Viral DNA replication was monitored after virus infection or after transfection with infectious plasmid DNA. CRFK or A72 cells seeded at $1 \times 10^4/cm^2$ in 9-cm^2 wells were transfected with 5 μg of plasmid DNA using 8 μl of Lipofectamine. DNA was recovered from the cells 72 h after infection or transfection by proteinase K digestion and then phenol and chloroform extraction. DNA samples from the plasmid transfected cells were incubated for 3 h with 10 U of *Dpn*I in 50 mM Tris · HCl (pH 8.0), 10 mM $MgCl_2$, 0.21 M NaCl, and 1 mM dithiothreitol. DNA was electrophoresed in 1% agarose gels containing 1 μg/ml of ethidium bromide, Southern blotted to nylon membranes, and probed with ^{32}P-labeled DNA containing the complete CPV genome.

Capsid production after plasmid transfection or virus infection was measured by hemagglutination (HA). $TCID_{50}$ titers were determined in NLFK cells seeded in 96-well trays, where wells containing infected cells were identified by immunohistochemical staining, and titers calculated by the method of Reed and Muench (1938). To determine the efficiency of capsid assembly, NLFK cells inoculated with CPV-d, or mutants A2003T or A2033T, were labeled after 24 h with [^{35}S]methionine for 30 min. After incubation with complete medium for a further 2 h the cells were lysed into RIPA buffer, layered over 10–40% sucrose gradients in 50 mM Tris · HCl (pH 8.0), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA buffer, and centrifuged at 100,000 *g* for 6.5 h at 4°C. Proteins were immune precipitated from gradient fractions with polyclonal rabbit anti-capsid antibodies which recognize both assembled and nonassembled capsid proteins and electrophoresed in 10% SDS–PAGE, and then the gels were dried and exposed to X-ray film.

Host range

CPV-d or the various viable NS2 mutants were titrated in NLFK cells, then inoculated at a virus/cell multiplicity of infection of 1 into A72, CRFK, 3201, MDCK, NB324K, COS, or L cells. $TCID_{50}$ titers recovered from the cultures after freezing and thawing were determined in NLFK cells. To confirm that the mutations were still present after replication, virus in the supernatant of infected cells was used directly as a template in PCR reactions, amplifying the region containing the mutations. The PCR products were then sequenced or, in the case of A2033T, the mutation was confirmed by digestion with *Nhe*I.

Two pairs of 10-week-old specific-pathogen-free beagle dogs were inoculated intramuscularly with 10^5 $TCID_{50}$ of either CPV-d or the NS2 mutant A2033T. Five days later the animals were euthanized and the virus titers in the thymus, mesenteric lymph node, spleen, and ileum determined by $TCID_{50}$ assay in NLFK cells. The mutant nature of the viruses recovered from the thymus of the dogs was confirmed by PCR amplification of DNA of virus recovered from the tissues and *Nhe*I digestion of the product.

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