Construction and nucleotide sequence analysis of an infectious DNA clone of the autonomous parvovirus, mink enteritis virus

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We have cloned the replicative form (RF-) DNA of mink enteritis virus (MEV), constructed an infectious recombinant plasmid containing MEV DNA and determined the nucleotide sequence of the cloned MEV DNA. RF-DNAs were detected and infectious virus was generated when the recombinant plasmid containing the entire MEV genome was introduced into feline kidney cell cultures. The MEV genome was 5094 nucleotides (nt) in length; the 3' end of the virion strand contained a 205 nt palindromic sequence and the 5' end a 62 nt palindromic sequence that could assume Y- and U-shaped configurations, respectively. The 5' end of the virion strand had a direct repeat of 61 nt at the carboxyl terminus of the capsid protein gene. The organization of the MEV genome is similar to those of canine parvovirus (CPV) and feline panleukopenia virus (FPLV); there are two large open reading frames (ORFs), one in the 3' half and the other in the 5' half of the genome, with coding capacities of 668 and 722 amino acid residues, respectively. Both are in the same reading frame and no significant ORFs are apparent in the virion strand (negative-sense strand). Possible functional promoter motifs are located at map unit (m.u.) 4-5 and m.u. 40, and a possible functional poly(A) signal is located at m.u. 96. The nucleotide and amino acid sequence homology with CPV and FPLV is greater than 98%, consistent with the hypothesis that MEV and CPV are host-range variants of FPLV.

Introduction

Mink enteritis virus (MEV) is thought to be a variant of feline parvovirus, which contains a linear negative-sense ssDNA of approximately 5 kb (Hauswirth, 1984). Nucleotide sequences of other variants, feline panleukopenia virus (FPLV) and canine parvovirus (CPV), have been analysed (Carlson et al., 1985; Reed et al., 1988) and comparison of the whole CPV genome with about 80% the 5' end of the FPLV genome has revealed greater than 95% homology (Reed et al., 1988). It has also been reported that CPV shares several structural features with other well characterized autonomous parvoviruses (Astell et al., 1983; Rhode & Paradiso, 1983; Chen et al., 1986; Shade et al., 1986); there are two major open reading frames (ORFs), the ORF in the 3' half of the genome encoding the non-structural (NS) proteins and the ORF in the 5' half encoding the structural proteins (VP). The genes encoding proteins NS and VP are initiated from separate promoters but the mRNAs for the NS and VP proteins have coterminar poly(A) sites (Reed et al., 1988).

Although MEV, FPLV and CPV are closely related viruses, they are distinguishable from each other antigenically when examined with monoclonal antibodies (Parrish et al., 1982; Parrish & Carmichael, 1983; Surleraux et al., 1987), by thermoresistance and haemagglutination reactions (Carmichael et al., 1980; Goto et al., 1984), and by their growth characteristics in cultured cells (Tratschin et al., 1982). It would contribute greatly to the understanding of the evolution of these viruses if the differences in these characteristics were shown to correspond to differences in nucleotide sequences.

With regard to host-range specificity, Parrish et al. (1988) reported a region in which part of the determinant of the CPV host range was related to the capsid protein gene. In the report, the nucleotide and predicted amino acid sequences of the capsid protein genes of CPV, FPLV, MEV and raccoon parvovirus were analysed but the complete nucleotide sequences of MEV and FPLV were not determined.

Several infectious DNA clones of autonomous parvoviruses have been established (Merchlinsky et al., 1983; The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D00765.

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Shull et al., 1988; Diffoot et al., 1989; Bloom et al., 1990). They have been useful in the examination of the function of the viral genome, e.g. the host-range determinant of the genome of minute virus of mice (MVM) was mapped by characterizing the biological properties of hybrid DNA clones (Antonietti et al., 1988).

In this study, we describe the construction of a complete MEV replicative form (RF-) DNA clone which generates progeny MEV in transfected feline cell cultures and the nucleotide sequence of the cloned RF-DNA. The nucleotide sequence is compared with the sequences of other parvoviruses.

**Methods**

*Virus and DNA preparation.* Strain Abashiri of MEV (Higashihara et al., 1981) was propagated in FLF-3 cells, a feline lung fibroblast cell line, as described previously (Goto et al., 1984). MEV RF-DNAs, either free from (f-DNA) or with (p-DNA) the terminal proteins, were prepared from MEV-infected cells as described elsewhere (Shinagawa et al., 1989).

*Enzymes.* Restriction endonucleases were purchased from Takara-Shuzo. T4 DNA ligase was purchased from New England Biolabs. Other DNA modification enzymes were purchased from Takara-Shuzo. All enzymes were used according to the suppliers' instructions.

**Construction of MEV RF-DNA clones.** Construction of MEV RF-DNA clones was carried out by two methods (Fig. 1). One was the method of dG . dC-tailing, using p-DNA after proteinase treatment. Proteinase-treated p-DNA was tailed with a short stretch of dC at the 3' terminus and inserted into the PstI site of pUC18, which was tailed with dG (Villa-Komaroff et al., 1978). This clone, pMEV1, was found to have short deletions at both termini of the MEV genome. The other method employed f-DNA. The f-DNA was digested with EcoRI, generating fragments of approximately 4 kb and 1 kb. The 4 kb fragment, containing the 5' terminus of the virion strand, was cloned into the Bluescript M13 + plasmid (SK or KS; Stratagene), using the EcoRI and SmaI sites, to make clone pEl. The 1 kb fragment, containing the 3' terminus of the virion strand, was cloned into pUC18, using the HindIII and EcoRI sites, to make clone pE2. Plasmid pMEV2 was derived from pE2 by inserting an EcoRI-HindIII fragment into the EcoRI and HindIII sites of pMEV2. The region between the EcoRI and BglII sites of pMEV2 was found to have a deletion of 458 bp near map unit (m.u.) 40 (2009 to 2467 bp) so the region between the EcoRI and BglII sites of pMEV2 was replaced with the corresponding region of RF-DNA; this plasmid was designated pMEV3.

**Nucleotide sequence analysis.** Nucleotide sequencing was carried out by the dideoxynucleotide sequencing method (Sanger et al., 1977), using an M13 sequencing kit and a 7-DEAZA sequencing kit (Takara-Shuzo), or by the method of Maxam & Gilbert (1977). Computer analysis of the nucleotide sequence was performed using programs from GENETYX SDC.

**Transfection of cloned MEV DNAs.** CRFK cells, a feline kidney cell line (Crandell et al., 1973), were used in this study. Cells were seeded 24
Construction of an infectious MEV DNA clone

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Fig. 2. May–Gruenwald–Giemsa staining of cells transfected with MEV DNA clones, and MEV-infected cells. Parts (a), (b) and (c) are MEV-infected cells at 24, 48 and 72 h post-infection, respectively. Parts (d), (e) and (f) are pMEV1-, pMEV2- and pMEV3-transfected cells at 72 h after transfection, respectively. (g) Cells incubated with the culture fluid from pMEV3-transfected cells. (h) Mock-infected cells. Arrows, typical intranuclear inclusion bodies; arrowheads, network-like nuclear alterations; triangle, other types of nuclear alterations.

h before transfection at a density of $2 \times 10^4$ cells/cm$^2$ in 60 mm diameter dishes containing coverslips. They were fed 4 h before transfection and each dish was transfected with 5 µg cloned MEV DNA by the calcium phosphate precipitation method (Graham & van der Eb, 1973). At an appropriate time after transfection, coverslip cultures were removed and fixed with either methanol for May–Gruenwald–Giemsa staining or cold acetone for immunofluorescent staining to examine the expression of the transfected viral gene. Anti-CPV dog serum (Chemicon) and fluorescein isothiocyanate-conjugated anti-dog IgG rabbit IgG (Cappel) were used in an indirect immunofluorescence assay (IFA). The transfected cell culture fluids were clarified by centrifugation at 5000 g for 10 min and their infectivity was examined by adding them to freshly prepared CRFK cells.

Detection of virus particles. The transfected culture fluids, harvested 72 h after transfection, were centrifuged on a cushion of 30% CsCl at 100000 g for 2 h. The precipitates were suspended in distilled water, negatively stained with silicotungstic acid and examined by electron microscopy for the presence of virus particles.

Detection of RF-DNA in transfected cells. Low $M_r$ DNA was extracted from cells 72 h after transfection or infection using the procedure described (Shinagawa et al., 1989). The DNA was electrophoresed in 1.0% agarose gels, transferred onto a nitrocellulose filter by the method of Southern (1975) and hybridized with $^{32}$P-labelled MEV DNA.

Results

Construction of MEV RF-DNA clones and the properties of MEV DNA clones in cultured cells

pMEV1 was constructed using p-DNA and had deletions of 161 and 74 bp at the left and right ends of the genome, respectively. pMEV2 and pMEV3 were constructed using f-DNA; pMEV2 had a deletion of 458 bp around m.u. 40 and pMEV3 was considered to contain the entire MEV genome.

Morphological changes of cells transfected with the cloned DNAs were compared with those of MEV-infected cells. CRFK cells were stained with May–Gruenwald–Giemsa 72 h after transfection with either pMEV1, pMEV2 or pMEV3, and 24, 48 and 72 h after infection with MEV. The nuclei of pMEV1-transfected cells showed rough network-like patterns (Fig. 2d); such patterns were also observed in cells 24 h after MEV infection (Fig. 2a). The pMEV2-transfected cells showed some nuclear alterations that were never observed in mock-infected cells (Fig. 2e) and some pMEV3-transfected cells showed clear intranuclear inclusion bodies, observed in MEV-infected cells 48 and 72 h post-infection (Fig. 2f, b and c, respectively). Formation of
clear inclusion bodies was not observed when pMEV1- or pMEV2-transfected cells were incubated for longer periods (data not shown).

Transfected cells were examined for the synthesis of viral proteins by IFA 72 h after transfection. Cells transfected with each DNA clone synthesized viral proteins; however, some differences were observed in the nuclear fluorescence patterns of these cells. The nuclear fluorescent signal of cells transfected with pMEV1 or pMEV2 was somewhat weaker than that of MEV-infected cells and the fluorescence pattern was granular (Fig. 3a, b). The fluorescent signal of cells transfected with pMEV3 was similar to that of MEV-infected cells: strong nuclear fluorescence and some round cells with strong fluorescence, suggesting a c.p.e. of MEV (Fig. 3c, d).

When newly prepared CRFK cells were incubated with the culture fluid from pMEV3-transfected cells, obtained 72 h after transfection, clear intranuclear inclusion bodies were observed (Fig. 2f). Furthermore, virus-specific antigens, similar to those from MEV-infected cells, were observed in the cells (Fig. 3e). This infectivity was blocked by incubation with anti-CPV dog serum (for 30 min at room temperature) before adding culture fluid to the CRFK cells (data not shown). No virus-specific antigens were observed in cells incubated with the culture fluids from pMEV1- or pMEV2-transfected cells (data not shown). These results suggested that infectious virions were generated only from pMEV3. Mock transfection was performed with vector DNA (Bluescript M13+ plasmid) and neither nuclear alteration nor virus-specific antigen were observed in mock-transfected cells (data not shown).

Detection of RF-DNA in transfected cells

To examine whether viral DNA replication occurred in cells transfected with MEV DNA clones, low Mr DNA was harvested 72 h after transfection and analysed by Southern blotting. As shown in Fig. 4, three MEV DNA species, monomeric and dimeric RF-DNA, and ssDNA, were detected in MEV-infected or pMEV3-transfected cells. No evidence of DNA replication was seen in cells transfected with pMEV1 or pMEV2 72 h (Fig. 4) or 144 h (data not shown) after transfection.

Detection of virus-like particles from pMEV3-transfected cells

Since the transfection studies suggested that the cells transfected with pMEV3 produced infectious virus particles, the culture fluids of pMEV3-transfected cells were examined electron microscopically and virus-like particles 22 nm in diameter were observed (Fig. 5). In addition to the virus-like particles, small particles of a
uniform size (4 to 5 nm in diameter) were observed. At present, we have no data to enable the origin of the small particles to be identified.

**Nucleotide sequence of pMEV3**

It was found that pMEV3 was an infectious DNA clone. This indicated that pMEV3 contains all the sequences required for the replication of viral DNA and generation of progeny MEV, i.e. pMEV3 seems to contain an almost or completely full-length MEV genome; the nucleotide sequence of MEV DNA inserted in pMEV3 was determined. Fig. 6 shows the sequencing strategy for MEV DNA and the restriction maps used for subcloning. The entire sequence of 5094 bases is shown in Fig. 7. The sequence shown is that of the plus-sense strand which is complementary to the minus-sense strand of MEV.
Identification of potential promoter motifs and polyadenylation sites

A computer search for possible promoter regions was done by using the information on eukaryotic promoters reported by Bensimhon et al. (1983). In the MEV genome, eight potential TATA boxes were found (Table 1). Among these, TATA boxes at m.u. 4-5 and m.u. 40 were the most likely candidates for the functional promoters because their positions are similar to two functional promoters described for other autonomous parvoviruses (Pintel et al., 1983; Carlson et al., 1985) and they contained all the appropriate promoter components (enabler region, activator region and TATA box). Polyadenylation signals (AATAAA) were identified at m.u. 32 and m.u. 96.

Organization of the MEV genome

All parvovirus genomes examined so far have two major ORFs, one located on the left half and the other on the right half of the genome; they occur in different frames in most parvoviruses except CPV-N and FPLV (Reed et al., 1988; Carlson et al., 1985). Fig. 9 shows the occurrence of termination codons in the three reading frames of the plus-sense strand. In frame 2 there are two major ORFs, one located on the left half (left ORF) and the other on the right half (right ORF) of the genome, with coding capacities of 668 and 722 amino acid residues, respectively. No ORFs of significant size were found in the negative-sense strand (data not shown).

Nucleotide and amino acid sequence homology between MEV and other parvoviruses

The homology between the right and left ORFs of MEV and those of other parvoviruses are shown in Table 2. Both the nucleotide and the amino acid sequences of MEV obtained here showed greater than 98% homo-
Table 2. Nucleotide and amino acid sequence homology between MEV and other parvoviruses

<table>
<thead>
<tr>
<th>Virus (m.u.)</th>
<th>Left ORF</th>
<th>Right ORF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleotide (Amino acid)</td>
<td>Nucleotide (Amino acid)</td>
<td></td>
</tr>
<tr>
<td>FPLV (20 to 96)</td>
<td>100 (100)</td>
<td>99.8 (99.7)</td>
<td>Carlson et al. (1985)</td>
</tr>
<tr>
<td>CPV-N (0 to 100)</td>
<td>99.4 (99-1)</td>
<td>99-6 (99-3)</td>
<td>Reed et al. (1988)</td>
</tr>
<tr>
<td>MEV-d (45 to 100)</td>
<td>NT*</td>
<td>NT</td>
<td>Parrish et al. (1988)</td>
</tr>
<tr>
<td>H-1 (0 to 100)</td>
<td>54-2 (73-2)</td>
<td>36-3 (53-8)</td>
<td>Rhode &amp; Paradiso (1983)</td>
</tr>
<tr>
<td>B19 (0 to 100)</td>
<td>33-8 (23-2)</td>
<td>30-5 (34-9)</td>
<td>Shade et al. (1986)</td>
</tr>
</tbody>
</table>

* NT, Not tested.

![Fig. 9. Genomic organization of the complementary strand. Each line represents a stop codon position.](image)

Discussion

It has been reported that protein(s) associated covalently with the 5' end of the RF-DNAs of MVM, H-1 and Kilham rat virus (Chow et al., 1986; Cotmore & Tattersall, 1988; Revie et al., 1979; Wobbe & Mitra, 1985). Recently, the protein attached to the 5' termini of RF-DNAs of MVM was identified as the major NS protein, NS-1. We have reported that most of the RF-DNAs of MEV in productively infected cells bind to protein but that some are free from protein (Shinagawa et al., 1989). It is thought to be difficult to clone p-DNA because a few amino acid residues or an oligopeptide may still be attached at the 5' end even after proteinase treatment (Shinagawa et al., 1989). Thus, we cloned both protein-bound and protein-free RF-DNA. We could not obtain an infectious clone using proteinase-treated p-DNA (representative: pMEV1). Sequence analysis revealed that the MEV DNA of pMEV1 has deletions at both ends of the genome. On the other hand, using protein-free RF-DNA allowed us to construct an infectious clone (representative: pMEV3); a clone (pMEV2) deleted in the region in which the possible functional promoter at m.u. 40 was contained was isolated while constructing pMEV3. The biological properties of these clones were characterized in cultured cells.

Only pMEV3 generated infectious virions which were released into the culture fluids of the transfected cells. No infectivity was detected in the culture fluids of cells transfected with either pMEV1 or pMEV2 although virus-specific antigens were detected by IFA in the transfected cells. The results of IFA suggest that virus-specific antigens are translated from the virus-specific mRNAs transcribed from pMEV1 or pMEV2, in spite of their inability to replicate in the transfected cells. Furthermore, the products encoded by pMEV1 or pMEV2 may cause nuclear alterations in transfected cells (Fig. 2d, e) similar to the alterations observed soon after MEV infection (Fig. 2a).

It has been reported for infectious recombinant plasmids of adeno-associated virus (AAV) that mutants with deletions of part of the rep gene (rep corresponds to NS genes of autonomous parvovirus) failed to replicate AAV DNA, whereas mutants with deletions of part of the capsid gene could accumulate RF-DNA in transfected cells (Tratschin et al., 1984). Moreover, recombinant AAV plasmids with deletions of both terminal repeats failed to replicate in transfected cells (Samulski et al., 1983). Our finding that pMEV1 (both ends deleted) and pMEV2 (C terminus of the NS gene deleted) could not replicate in transfected cells, as judged by the production of RF-DNA, are consistent with the observations on AAV. Some infectious DNA clones of parvoviruses have
been reported (Samulski et al., 1982; Merchlinsky et al., 1983; Shull et al., 1988; Diffoot et al., 1989; Bloom et al., 1990) and it is noteworthy that such clones always have palindromic sequences at both ends of the viral DNA; mutants with deletions of the palindromic sequences cannot replicate (Samulski et al., 1983; PMEV1 in this paper). These results suggest that the palindromic sequences are essential for replication and excision of the viral DNA from the vector DNA.

Terminal structures have been thought to play a pivotal role in parvovirus DNA replication (Hauswirth, 1984) and palindromic sequences of one or both termini have been found in other paroviruses (Astell et al., 1983; Reed et al., 1988; Bloom et al., 1988; Shade et al., 1986; Rhode & Klaassen, 1982; Ranz et al., 1989; Diffoot et al., 1989). The nucleotide sequence of the 3' end of the virion strand of MEV DNA revealed that the intact 3' end palindromic sequence had been cloned and that its Y-shaped configuration resembled those of other paroviruses. The 3' end palindromic sequence, consisting of 205 nt, is the largest described so far, although the precise 3' end of the virion DNA is not known. The 5' end palindromic sequence could assume a U-shaped secondary structure; this is the first report of a 5' end palindromic sequence for host-range variants of FPLV.

MEV displays greater than 98% homology with CPV-N, FPLV and MEV-d in both nucleotide and amino acid sequences (Table 2); homology with rodent and human paroviruses was less than that with CPV-N and FPLV. However, the regions conserved among all paroviruses so far examined were retained in MEV. In the GKRN region (Fig. 7), a small region (amino acids 393 to 422 in MEV) that is partially identical to the large T antigens of polyomaviruses and the E1 proteins of papillomaviruses is found in MEV as it is in other paroviruses (Anton & Lane, 1986). The amino acid sequence of this region contains a purine nucleotide binding motif, such as that proposed in the study of ATP synthetase and other ATP-requiring enzymes by Walker et al. (1982). If the NS protein has ATPase activity, it will be of interest to determine whether it has any helicase-like activity, as suggested by Cotmore & Tattersall (1989). Recently, Kim & Muzyczka (1990) demonstrated that the rep68 protein of AAV contains an ATP-dependent site-specific endonuclease activity and a DNA helicase activity.

It has been reported that the genomic region that associates with part of the determinant of the canine host range maps to between m.u. 59 and m.u. 64 of the CPV genome (Parrish et al., 1988). Comparing our sequence data with CPV-d (Parrish et al., 1988), we found that three predicted amino acid residues were different in that region (data not shown); we have no evidence as to whether this region affects the host-range specificity. The region around m.u. 60 is thought to encode an antigenic site located on the surface of the virus capsid because it encodes one of the neutralizing antigenic sites (Parrish et al., 1988). Thus, this region might be related to the ability of each virus to infect host cells.

The homology of MEV sequences with those of CPV-N and FPLV strongly supports the hypothesis that they may be a group of host-range mutants with a common unidentified ancestor. The infectious MEV plasmid facilitates the production of mutant clones by in vitro mutagenesis. Moreover, this plasmid allows us to produce chimeric viruses constructed using MEV and CPV, or FPLV. Thus, it will be possible to examine the genomic regions which affect the host specificity or pathogenicity of the viruses more clearly.

References


