Characterization of infectious Murray Valley encephalitis virus derived from a stably cloned genome-length cDNA

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An infectious cDNA clone of Murray Valley encephalitis virus prototype strain 1-51 (MVE-1-51) was constructed by stably inserting genome-length cDNA into the low-copy-number plasmid vector pMC18. Designated pMVE-1-51, the clone consisted of genome-length cDNA of MVE-1-51 under the control of a T7 RNA polymerase promoter. The clone was constructed by using existing components of a cDNA library, in addition to cDNA of the 3' terminus derived by RT–PCR of poly(A)-tailed viral RNA. Upon comparison with other flavivirus sequences, the previously undetermined sequence of the 3' UTR was found to contain elements conserved throughout the genus Flavivirus. RNA transcribed from pMVE-1-51 and subsequently transfected into BHK-21 cells generated infectious virus. The plaque morphology, replication kinetics and antigenic profile of clone-derived virus (CDV-1-51) was similar to the parental virus in vitro. Furthermore, the virulence properties of CDV-1-51 and MVE-1-51 (LD50 values and mortality profiles) were found to be identical in vivo in the mouse model. Through site-directed mutagenesis, the infectious clone should serve as a valuable tool for investigating the molecular determinants of virulence in MVE virus.

Introduction

Murray Valley encephalitis (MVE) virus is a member of the genus Flavivirus within the family Flaviviridae (Monath & Heinz, 1996). Flaviviruses are small, lipid-enveloped viruses that contain single-stranded positive-sense RNA genomes approximately 11 kb in length. The genome encodes three structural proteins (the capsid protein C, the membrane protein prM and the envelope protein E) and seven non-structural proteins (NS1, NS2A, NS2B, the helicase/protease NS3, NS4A, NS4B and the viral RNA polymerase NS5). All viral proteins are encoded in a single open reading frame that is flanked by short untranslated regions (UTRs) (see Chambers et al., 1990; Rice, 1996). Viral genomic RNA lacks a poly(A) tail at the 3' terminus, has a 5' methylated cap (type I) and gives rise to infectious virus particles when transfected into susceptible cells (Rice, 1996). Like other flaviviruses such as yellow fever (YF), Japanese encephalitis (JE), Kunjin (KUN), dengue (DEN) and tick-borne encephalitis (TBE) viruses, MVE virus causes clinically significant disease in humans (reviewed in Monath & Heinz, 1996). Occasional cases of encephalitis are seen in the Kimberley region of Western Australia and in north-eastern Queensland, often coinciding with increased mosquito activity during the wet season (December–June; reviewed in Marshall, 1988).

Studies investigating the molecular determinants of virulence in flaviviruses have, in the past, focused on the characterization of attenuated laboratory strains that were derived by cell-culture passage and/or neutralization-escape selection. More recent advances in this field have involved the use of infectious cDNA clones, i.e. bacterial plasmids containing viral cDNA, from which infectious RNA can be transcribed in vitro.

The first such flavivirus clone was produced by Rice et al. (1989) and involved the ligation in vitro of two cDNA fragments of YF virus. Infectious virus was derived from the clone by in vitro transcription of viral RNA and subsequent transfection into BHK-21 cells (Rice et al., 1989). Since this report, infectious clones of flaviviruses have been similarly constructed for JE (Sumiyoshi et al., 1992), various strains of DEN-2 (Kapoor et al., 1995; Kinney et al., 1997; Polo et al., 1997; Gualano et al., 1998), DEN-4 (Lai et al., 1991), KUN (Khromykh & Westaway, 1994) and TBE (Mandl et al., 1997). In some cases, genome-length cDNA was found to be unstable in a bacterial host (YF, JE and DEN-2 strain New Guinea C),
whereas for others (DEN-2 strains 16681, New Guinea C and PUO-218, DEN-4, KUN and TBE), full-length cDNA was stably cloned into *Escherichia coli* or yeast. Success in these latter cases was often dependent on the use of different combinations of plasmid vector and/or bacterial host strain. To date, there has been no report of the construction of an infectious clone for MVE virus.

In this report, we describe the derivation of the 3' terminal sequence of MVE virus prototype strain 1-51 and the construction of a stable genome-length cDNA clone. RNA transcribed from the clone was infectious when introduced into susceptible cells. Furthermore, clone-derived virus (CDV) was found to be indistinguishable from the parental strain with respect to plaque phenotype, antigenicity, replication kinetics and neuroinvasiveness and neurovirulence in mice.

### Methods

#### Cells and virus.

Vero (ATCC CCL81 P130–P145), BHK-21 (ATCC CCL10 P56–P59) and *Aedes albopictus* C6/36 (ATCC CRL1660 P5–P20) cells were grown in M199 medium supplemented with 2 mM-l-glutamine and 10% foetal calf serum (FCS) and were incubated either at 37 °C in an atmosphere containing 5% CO₂ (Vero, BHK-21) or at 28 °C under standard atmospheric conditions (C6/36). MVE-1-51, the prototype strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working strain of MVE virus (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952).

To enable the use of the *Xhol*1101 site for linearization at the 3' terminal of the viral genome, a DNA fragment encompassing the 3' terminal sequence of the MVE-1-51 genome was derived by reverse transcription of poly(A)-tailed MVE RNA and subsequent amplification of MVE cDNA by PCR. Briefly, MVE RNA was isolated from purified virus as described previously (Rice et al., 1989) and polyadenylated at the 3' terminal by using *E. coli* poly(A) polymerase (Bresates) according to the manufacturer's instructions. First-strand cDNA was synthesized by oligo(dT)-primed reverse transcription of polyadenylated RNA with AMV reverse transcriptase (Gibco-BRL). After cDNA synthesis, the MVE virus 3' UTR was amplified by PCR with Vent DNA polymerase (New England Biolabs). Oligonucleotides used for PCR amplification were oligo(dT)₁₂,₁₈ (New England Biolabs) and a primer corresponding to MVE virus nt 9940–9955 (Lee et al., 1990). The resultant PCR products were isolated from low-melting-point agarose, methylated with BamHI methylase and ligated to BamHI linkers (New England Biolabs). Ligation products were isolated on low-melting-point agarose, digested with BamHI and subcloned into BamHI-digested M13mp18. The sequence of the 3'-terminal 85 nucleotides (nt 10930–11014) of MVE virus RNA was obtained by sequence analysis of single-stranded M13 DNA isolated from a total of ten separate recombinants, generated from two independent PCRs.

The predicted stem–loop structure at the 3' terminal of MVE virus RNA (see Fig. 1a) was derived by using MFOLD and SQUIGGLES software, available on-line at WebANGIS GCG (http://www.angis.org.au/WebANGIS/WAG).

#### Construction of genome-length cDNA.

Clones from an MVE-1-51 cDNA library (Dalgrano et al., 1986) were used in the construction of a full-length cDNA clone. Superscript numbers represent the nucleotide positions in the full-length MVE virus sequence of 11014 nucleotides. Initially, cDNA of the 5' terminal (spanning nt 1–1943) was generated by PCR by using clone p1/1/12 (Dalgrano et al., 1986) as a template. The 5'-end primer (containing a Sall restriction enzyme site for cloning, a T7 RNA polymerase promoter and the first 22 bases of the 5' MVE virus sequence) was designed so that the first nucleotide of the MVE cDNA sequence would be adjacent to the T7 promoter with one extra base (G) added (see Fig. 1b; primer sequence 5' GAATTCGCAGTAAATACGACTCTATAGAGACGGTCGACGTTGAGGACGTTGAGGACGTG3'). The PCR product isolated (Sall → Xhol1143) was ligated to an Xhol1143 → Clal1150 fragment excised from clone p1/1/12 and subsequently cloned between the Sall and Clal sites of pBR322 (designated pBR/5'MVE; see Fig. 1a).

A cDNA copy of the 3' terminal (spanning nt 9951–11014) was generated by RT–PCR of viral RNA using a high-fidelity *Pfu* polymerase (Stratagene). The reverse primer contained a Sall site for cloning as well as the last 43 bases of the MVE virus sequence. In addition, an Xhol1101 site was incorporated adjacent to the 3' terminal to enable linearization of the clone and subsequent run-off transcription of full-length RNA (see Fig. 1a, b; primer sequence 5' GCCCGCGCTCGA-CTTGAAGATCTGTTGTCTTCTCCCCATACGTACGTCGTGGCCGC3'). The isolated RT–PCR product (Sphi1993 → Sall) was ligated to a Clal1947 → Sphi1993 fragment excised from clone p2/2/6 (Dalgrano et al., 1986) and subsequently cloned between the Clal and Sall sites of pBR322 (designated pBR/3'MVE; see Fig. 1a).

Having constructed two sub-genomic clones spanning nt 1–5407 (pBR/5'MVE) and 5407–11014 (pBR/3'MVE) of the viral genome, a full-length clone was generated by ligating an excised Sall → Clal1947 fragment from pBR/5'MVE to an excised Clal1947 → Xhol1101 fragment from pBR/3'MVE. This full-length cDNA was then cloned between the Sall and Xhol sites of pMC18 (for plasmid reference see Gualano et al., 1998) to give the full-length clone pMVE-1-51.

Competent *E. coli* DH5α cells, prepared by the calcium chloride method of Sambrook et al. (1989), were used for the cloning and amplification of sub-genomic and full-length plasmids.

#### PCR mutagenesis of pBR/5'MVE.

To enable the use of the *Xhol*1101 site for linearization at the 3' terminal in a full-length clone, the *Xhol*1101 site of pBR/5'MVE was abolished by replacing a short region of pBR/5'MVE (Sall → PhiI1056) with a new fragment derived by PCR. The reverse primer used to generate this fragment was designed with a single mismatch at nucleotide 1943, resulting in a T → A transition mutation that did not alter the encoded amino acid sequence (GTT and GTA both encode Val).
Infectious cDNA clone of MVE virus

Fig. 1. (a) Construction of the genome-length MVE virus cDNA clone pMVE-1-51. The cDNA library was prepared by Dalgarno et al. (1986). Both the 5' and 3' termini were synthesized by RT–PCR from MVE-1-51 RNA. A T7 RNA polymerase promoter (grey box) was incorporated upstream of the 5' terminus to facilitate transcription. In addition, an XbaI site was incorporated at the 3' terminus to allow linearization of the clone. Other restriction enzyme sites used in construction of the clone are shown. Note that the XbaI** site of clone pBR/5'MVE was abolished by PCR. (b) The 5' and 3' termini of cDNA contained in the clone pMVE-1-51. An additional G residue was added immediately downstream of the promoter (highlighted by larger font) to improve the efficiency of transcription. Four non-viral nucleotides are predicted to be added to the 3' terminus of the viral RNA following in vitro transcription. (c) The predicted stem–loop structure at the 3' terminus of MVE virus RNA. The secondary structures formed by the last 100 nucleotides of the genome, including the putative pseudoknot (indicated by dashed lines) are conserved throughout the genus Flavivirus. The predicted structure for KUN is shown for comparison (Proutski et al., 1997).

In vitro transcription. Approximately 1 µg pMVE-1-51 containing full-length cDNA was linearized by digestion with XbaI and prepared for in vitro transcription by overnight incubation with protease K (200 µg/ml) and SDS (0.5%). DNA was subsequently purified by phenol–chloroform extraction and ethanol precipitation and resuspended in 8 µl nuclease-free water. Transcription was carried out by using a MEGAscript T7 kit (Ambion) according to the manufacturer’s instructions. The reaction contained 7.5 mM each of dATP, dCTP and dUTP, 2.5 mM dGTP and the cap analogue m7G(5')ppp(5')GTP at 5 mM in a final volume of 20 µl. The reaction was incubated at 37 °C for 3 h and
RNA products were precipitated by the addition of 2.5 M LiCl at –20 °C for 1 h. RNA was then pelleted by centrifugation in a bench-top microfuge for 10 min, washed with 70% ethanol and resuspended in 25 µl nuclease-free water. The efficiency of the reaction was determined by running 1 µl of the resuspended RNA on a denaturing formaldehyde–agarose gel and staining with ethidium bromide.

**RNA transfection.** RNA transfections were carried out by using a protocol slightly modified from that outlined by Mandl et al. (1997). Briefly, sub-confluent BHK-21 cells were collected by trypsinization, washed with ice-cold PBS and resuspended at 6·25 × 10⁶ cells/ml in ice-cold PBS. Aliquots of 800 µl were then transferred to pre-chilled, 0·4 cm gene pulser cuvettes containing 100 µl ice-cold PBS and 1·5 µg resuspended RNA from *in vitro* transcription. After incubation on ice for 5 min, cells were electroporated by two successive pulses (settings 1·5 kV, 25 µF, 0° D), resulting in a time constant of approximately 0·8 ms. A 1 ml aliquot of M199 supplemented with 5 % FCS was added immediately and the cells were gently resuspended before being transferred to an 80 cm² tissue culture flask containing 20 ml of the same medium. Cells were incubated at 37 °C in 5 % CO₂ and observed daily for signs of cytopathic effect (CPE).

Virus derived from the above transfection was passaged three times in C6/36 cells (72 h per passage) and designated CDV-1-51. This third-passage stock was subsequently used for phenotypic characterization of the virus.

**Immunofluorescence assay.** Sub-confluent C6/36 cells were seeded onto glass coverslips in 24-well tissue culture trays (containing M199 supplemented with 10% FCS) and left overnight at 28 °C prior to infection with MVE-1-51 or CDV-1-51. At 60 h p.i., cells were washed twice with cold PBS and fixed in 80% acetone at −20 °C for 10 min. After air-drying, cells were rehydrated with cold PBS and incubated in a 1:100 dilution of primary antibody (anti-MVE virus hyperimmune ascites fluid; a gift from M. Kroeger, Department of Microbiology, University of Western Australia) at room temperature for 1 h. Cells were subsequently washed three times in cold PBS and further incubated for 1 h in a 1:100 dilution of secondary antibody (FITC-conjugated goat anti-mouse IgG). Cells were then washed again (three times) in cold PBS and the coverslips were mounted onto clean microscope slides with PBS–glycerol. Immunofluorescence was visualised under UV light by using a Leitz Orthoplan microscope.

**Sequence analysis.** Complete sequence analysis of pMVE-1-51 was performed by using an ABI-Perkin Elmer automated sequencing system that incorporates fluorescently labelled dideoxynucleotides. Multiple analyses were performed to confirm any differences observed between the published sequence of MVE-1-51 (Dalgarno et al., 1986; Lee et al., 1990) and the sequence of the full-length clone. All primers were supplied by Gibco-BRL as 20-mers and were used at a final concentration of 0·8 nM. Details of primer sequences are available from R.J.H. on request.

**Labelling of polypeptides in infected cells.** Sub-confluent monolayers of Vero cells in 60-mm tissue culture dishes were infected with either MVE-1-51 or CDV-1-51 at an m.o.i. of approximately 10. At 24 h p.i., cells were washed twice in amino acid-deficient medium (diluted 1:10) and incubated in the same medium for 24 h at 37 °C. Amino acid-deficient medium was then removed and replaced with fresh medium + Labelling of polypeptides in infected cells.

**Immunoprecipitation of radiolabelled virus.** Immunoprecipitation of radiolabelled virus from cytoplasmic lysates was achieved by incubating the lysate at 4 °C for 1 h in the presence of pre-immune mouse ascitic fluid (1:100 dilution). Immune complexes were then removed by adding protein A-bearing *Staphylococcus aureus* cells (strain Cowan I, Gibco-BRL) and incubating on ice for 30 min prior to centrifugation at 4 °C for 5 min in a bench-top microfuge. The supernatant was then incubated in the presence of a 1:40 dilution of anti-MVE virus hyperimmune ascites fluid for 2 h at 4 °C and immune complexes were again removed by the addition of *S. aureus* cells as described above, except that the suspension was incubated on ice for 1 h. The suspension was then layered onto 1 ml of 10% sucrose solution in NTE buffer and centrifuged at 4000 r.p.m. for 10 min in a bench-top microfuge. Pellets were washed twice in NTE buffer containing 0·1% Triton X-100 and resuspended in 30 µl electrophoresis sample buffer (60 mM Tris–HCl, pH 6·8; 2% SDS; 0·1% bromophenol blue; 10% glycerol). Samples were heated to 60 °C for 20 min and then centrifuged for 2 min in an Eppendorf tube to pellet *S. aureus* cells and the supernatant was transferred to a fresh Eppendorf tube for storage at −20 °C.

**SDS–PAGE of viral polypeptides.** Prior to electrophoresis, immunoprecipitated polypeptides were reduced by the addition of 100 mM DTT and 6 % (v/v) 2-mercaptoethanol and incubated at 95 °C for 5 min. Samples were then loaded onto Tris–HCl 10–20% polyacrylamide gradient gels (Bio-Rad) and electrophoresed at 130 V for 1 h. Gels were fixed and stained in Coomassie blue, destained and treated for fluorography with Amplify reagent (Amersham) prior to drying under reduced pressure and exposing to X-ray film.

**Virulence in mice.** Litters of 21-day-old ARC/Swiss mice (five per litter; obtained from the Animal Resources Centre, Murdoch, Western Australia) were injected intracranially (i.c.) or intraperitoneally (i.p.) with 10 µl or 50 µl, respectively, of a tenfold dilution of MVE-1-51 or CDV-1-51. Mice were counted daily and deaths were recorded. Humane endpoints were employed to minimize distress to experimental animals, a method that does not significantly alter LD₅₀ values in models of viral encephalitis (Wright &Philippotts, 1998). The 50 % ‘humane end-point’ dose (HD₅₀) was calculated for each group by the LD₅₀ method of Reed &Muench (1938).

Alternatively, mean time to death was determined by injecting mice (10 per group) i.c. or i.p. with 1000 p.f.u. MVE-1-51 or CDV-1-51 and recording survival of mice over a period of 21 days. Statistical significance was determined by using Student’s paired t-test.

Mouse experiments were undertaken by using protocols approved by the UWA Animal Experimentation Ethics Committee. Mice were kept on a clean litter of sawdust and given food and water ad libitum.

**Results**

**Derivation of the 3’-terminal sequence of MVE-1-51**

A cDNA copy of the 3’ terminus of MVE-1-51 was derived by polyadenylation of viral RNA with *E. coli* poly(A) polymerase and subsequent RT–PCR with an oligo(dT) primer. The resultant PCR products were successfully cloned into M13mp18 by using BamHI linkers and the nucleotide sequence of the terminal 85 nucleotides was obtained by sequence analysis of single-stranded M13 DNA. The accuracy of this sequence was confirmed by sequencing ten separate clones generated from two independent PCRs. The sequence obtained (GenBank accession no. AF161266) was further subjected to
Table 1. Differences between the published sequence of MVE-1-51 and that of the infectious cDNA clone pMVE-1-51

The published nucleotide and amino acid sequences were obtained from Dalgarno et al. (1986) and Lee et al. (1990). Nucleotide and amino acid differences are indicated in the form MVE-1-51 → clone.

<table>
<thead>
<tr>
<th>Genome region</th>
<th>Nucleotide</th>
<th>Difference</th>
<th>Corresponding residue in related flaviviruses</th>
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<td>E</td>
<td>1196</td>
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</tr>
<tr>
<td></td>
<td>1943*</td>
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</tr>
<tr>
<td></td>
<td>4964</td>
<td>C → T</td>
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</tr>
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</tr>
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</tr>
<tr>
<td></td>
<td>10665†</td>
<td>G → G</td>
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* Abolished XbaI site in pMVE-1-51.
† Dinucleotide inversion.
‡ Single nucleotide insertion in pMVE-1-51.

secondary RNA structure analyses by using the genetic algorithms implemented in the MFOLD and SQUIGGLES software programs (for reference see Zuker, 1989). The optimal secondary structure for the 3′-terminal 100 nucleotides of MVE virus is depicted in Fig. 1(c). As expected, the double stem–loop structure predicted for MVE virus was similar to that predicted for flaviviruses in general (Brinton et al., 1986; Proutski et al., 1997). Furthermore, the derived sequence had conserved elements that have been shown to be important in the formation of putative pseudoknot structures in a range of different flaviviruses such as JE, West Nile virus (WNV) and YF (Shi et al., 1996).

Construction and sequencing of the full-length MVE virus cDNA clone

The full-length MVE-1-51 clone was assembled by using cDNA derived from an existing cDNA library (Dalgarno et al., 1986). The genome was cloned in two halves into the low-copy-number vector pBR322; the first half containing nt 1–5407 and the second half containing nt 5408–11014 (see Fig. 1a). The two halves were subsequently joined at a unique Clal site (nt 5407) to form full-length cDNA, which was then inserted into the plasmid pMC18. This plasmid was designated pMVE-1-51.

The plasmid vector pMC18 was chosen because of its low copy number (approximately six per cell; Birnboim, 1983) and because full-length cDNA could not be cloned into the vector pBR322 (copy number greater than 25 per cell; Holmes & Quigley, 1981), a problem encountered during the construction of clones of other flaviviruses such as YF (Rice et al., 1989), JE (Sumiyoshi et al., 1992) and DEN-2 (Kapoor et al., 1995).

The sequences at both termini are depicted in Fig. 1(b). A T7 RNA polymerase promoter was positioned immediately upstream of the 5′ terminus and an additional G residue was included to improve transcription efficiency (Milligan et al., 1987). An XhoI site was incorporated to form part of the 3′ terminus and was utilized to generate RNA transcripts with authentic 3′ termini (plus four non-viral nucleotides; see Fig. 1b).

The nucleotide sequence of pMVE-1-51 was analysed by complete sequence analysis and compared to both the published sequence of MVE-1-51 (GenBank accession no. M24220; Dalgarno et al., 1986; Lee et al., 1990) and the sequence of the 3′ terminus derived in this laboratory. The differences between the two sequences are shown in Table 1.
Nineteen differences were found and confirmed by second-round sequencing. Seven of these differences led to amino acid changes and three were located in the 3’ UTR. None of the seven amino acid changes altered the charge or polarity of the encoded amino acid significantly. Furthermore, the three changes in the 3’ UTR were located well upstream of known flavivirus consensus sequences and predicted stem–loop structures (Hahn et al., 1987; Lee et al., 1990; Shi et al., 1996).

The stability of pMVE-1-51 after propagation in E. coli was confirmed by comparing restriction enzyme profiles of the plasmid over three separate passages in E. coli strain DH5α. In each case, the restriction profile remained the same. Furthermore, RNA transcribed from pMVE-1-51 of different passages and transfected into BHK-21 cells generated CPE with equal efficiency.

In vitro synthesis of infectious RNA and recovery of recombinant virus

Capped RNA transcripts were transcribed from linearized pMVE-1-51 (XbaI digested) by using T7 RNA polymerase and an optimized 2:1 ratio of methylated cap analogue [m7G(5′)ppp(5′)GTP] to dGTP. Increasing the proportion of cap analogue in the reaction above this level resulted in a substantial decrease in the yield of RNA. Transcribed RNA was then precipitated in the presence of 2.5 M LiCl and washed with 70% ethanol prior to resuspension in nuclease-free water. An aliquot was electrophoresed on a 1% denaturing formaldehyde–agarose gel to check for efficient transcription of full-length product and to estimate RNA concentration (data not shown). Approximately 1-5 μg full-length RNA was subsequently transfected into BHK-21 cells by electroporation. CPE indistinguishable from that of the parental virus MVE-1-51 was visible at 3–4 days p.i. The specific infectivity of the RNA was estimated to be between 10⁶ and 10⁸ p.f.u./μg based on the assumption that, at most, only two-thirds of the transfected RNA was capped due to the proportion of cap analogue in the reaction (as described above). A high-titre stock of this virus, designated CDV-1-51, was generated by passage on C6/36 cells and used for subsequent virulence studies.

Confirming the presence of clone-derived virus (CDV-1-51)

During virulence studies (outlined below), the presence of CDV-1-51 was confirmed by extracting viral RNA from the brains of encephalitic mice and performing a RT–PCR/restriction enzyme analysis. Approximately 1-6 kb of the viral genome (spanning the abolished XbaI restriction enzyme site at nt 1943) was amplified and digested with XbaI. The results are shown in Fig. 2. When digested with this enzyme, MVE-1-51-derived cDNA (lane 3) is cleaved into two fragments, approximately 1-0 kb and 0-6 kb in length. CDV-1-51-derived cDNA (lane 2), however, is not cleaved due to the abolition of the XbaI site during construction of the full-length clone, as described in Methods.

Biological characterization of CDV-1-51

In order to confirm the antigenicity of CDV-1-51, monolayers of C6/36 cells were infected at an m.o.i. of 10 and compared to cells infected with MVE-1-51. All cells were found to show immunofluorescence at 60 h p.i. by using anti-MVE virus hyperimmune ascites fluid (see Fig. 3). The antigenic profile of CDV-1-51 was examined further by infecting Vero cells with the virus and labelling viral proteins 24 h later with tritiated amino acids. Cytoplasmic lysates and tissue culture supernatants were collected at 48 h p.i. and immunoprecipitated in the presence of anti-MVE virus hyperimmune ascites fluid. Immunoprecipitates were analysed by SDS–PAGE and compared to those obtained for positive and negative controls. As shown in Fig. 4, a range of MVE virus-specific proteins was visible in both the cell lysates and cell culture supernatants of MVE-1-51- and CDV-1-51-infected cells. At least five viral proteins, including the E protein (~57 kDa), NS1 (~49 kDa) and prM (~26 kDa), were identified in cell lysates on the basis of their size (Lee et al., 1990), and the electrophoretic mobility of these proteins was identical for the two viruses. Two other virus-specific proteins (~42 and 15 kDa) were also identified; however, their identities are unknown. Mature E (and possibly NS1) proteins were also detected in the cell culture supernatants of both viruses. The antigenic profiles of MVE-1-51 and CDV-1-51 (intra- and extracellular virus) were therefore said to be identical.

In addition to the above observations, the plaque size and morphology of CDV-1-51 were compared to those of MVE-1-51 by standard plaque assay on Vero cells. The viruses
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Fig. 3. Results of immunofluorescence assays. C6/36 cells were infected at an m.o.i. of 10 and assayed for immunofluorescence at 60 h p.i. (a) Positive control (MVE-1-51); (b) negative control; (c) CDV-1-51.

Fig. 4. Antigenic profiles of MVE-1-51 and CDV-1-51. Virus-infected Vero cells (and uninfected controls) were labelled with tritiated amino acids and immunoprecipitated in the presence of either hyperimmune (HIAF) or pre-immune (NIAF) ascites fluid. Lane 1: CDV-1-51-infected cell lysate immunoprecipitated with HIAF; 2, CDV-1-51-infected cell lysate immunoprecipitated with NIAF; 3, CDV-1-51-infected cell culture supernatant immunoprecipitated with HIAF; 4, CDV-1-51-infected cell culture supernatant immunoprecipitated with NIAF. Lanes 5–8 are identical to lanes 1–4 except that MVE-1-51 was used in place of CDV-1-51. Lanes 9–12 are negative controls; uninfected cell lysates (lanes 9–10) and cell culture supernatants (lanes 11–12) immunoprecipitated with HIAF (lanes 9 and 11) or NIAF (lanes 10 and 12). Proteins E, NS1, and prM are indicated by arrows and the relative positions of molecular mass markers are given in kDa.

generated indistinguishable plaque phenotypes at 3–4 days p.i., with mean plaque diameters of approximately 2–2.2 mm (Fig. 5). Furthermore, single-step growth curves of MVE-1-51 and CDV-1-51 in Vero cells (Fig. 6) showed that the replication kinetics of the two viruses were very similar.

Pathogenicity of CDV-1-51

The virulence of MVE-1-51 and CDV-1-51 were compared in the mouse model by injecting litters of ten 21-day-old mice i.p. or i.c. with tenfold dilutions of virus. Mice were observed daily for signs of encephalitis. The LD₉₀ values by the i.p. route for MVE-1-51 and CDV-1-51 were 4 and 8 p.f.u., respectively, whilst by the i.c. route they were 1 and 9 p.f.u., respectively. The LD₉₀ values were thus very similar for the two viruses by either route of administration.

In order to characterize the virulence of CDV-1-51 further, a mortality profile was determined by injecting groups of ten mice i.p. or i.c. with 1000 p.f.u. MVE-1-51 or CDV-1-51. Mean time to death (humane end-point) was calculated for each group. The differences in the profiles for each group (Fig. 7a) were not statistically significant by Student’s t-test, indicating that the neuroinvasiveness and neurovirulence profiles of CDV-1-51 were similar to those of MVE-1-51.

Finally, to determine whether the central nervous system growth kinetics of MVE-1-51 and CDV-1-51 were the same,
groups of twenty 21-day-old mice were injected i.c. with 1000 p.f.u. of each virus. Three brains from each group were collected each day and assayed separately by plaque assay on Vero cells. The titres of the two viruses were found to be comparable throughout the course of the infection (days 2–5) and were therefore said to be identical (Fig. 7b). 100% mortality was evident in both groups by day 6 p.i.

**Discussion**

A stable full-length clone of MVE virus prototype strain 1-51 was constructed. Viral RNA transcribed from the clone gave rise to infectious virus and, in all respects, this virus (CDV-1-51) had an almost identical phenotype to wild-type virus. The specific infectivity of clone-derived RNA, estimated to be between $10^5$ and $10^6$ p.f.u./µg, was similar to that described for other flavivirus clones such as YF and TBE (Rice et al., 1989; Mandl et al., 1997). The presence of an introduced mutation in the clone was confirmed by RT–PCR and restriction enzyme analysis of viral cDNA derived from the brains of encephalitic mice, showing that the virus was indeed clone derived.

Construction of the clone was made possible by determining the 3′-terminal sequence of the virus. In combination
with the work of others, the full genomic sequence of MVE virus is now known (Dalgaro et al., 1986; Lee et al., 1990). It is our belief that the cDNA sequence of CDV-1-51 (reported in this paper) is more accurate than that reported by Lee et al. (1990), due only to the improved accuracy of automated sequencing over the older Maxam and Gilbert method used in their study (Maxam & Gilbert, 1980). This is supported by the fact that five of the seven predicted amino acid changes in CDV-1-51 appear to resemble more closely the sequences of related flaviviruses than do those reported in the original published sequence. For example, the serine residue encoded by nt 9531–9533 of the original published sequence is an asparagine residue in JE virus, KUN, YF virus, DEN-2 and WNV (Lee et al., 1990) and is also an asparagine in CDV-1-51. The presence of cloning artifacts, introduced during the construction of the full-length clone, cannot be excluded, however, even though their presence appears to have little effect if any on the phenotype of the virus.

Molecular modelling of the 3′-terminal sequence of MVE virus suggests that it forms a stem–loop structure similar to that found in other flaviviruses. Furthermore, it appears that the conserved elements for the putative pseudoknot structure, involving a tertiary interaction between the loop of the smaller 3′ stem–loop and the stem of the larger 5′ stem–loop, are also conserved (Shi et al., 1996). This supports the hypothesis that a conserved secondary structure at the extreme 3′ terminus of viral RNA is important for the replication, transcription and/or translation of the flavivirus genome (Blackwell & Brinton, 1997; Zeng et al., 1998).

This is the first report of an infectious clone of MVE virus. It is likely that we were able to clone full-length cDNA into a low-copy-number plasmid vector as a result of our increased understanding of the difficulties involved in such cloning. In the past, the construction of flavivirus clones has been hampered by the instability of full-length cDNA in a bacterial host. This was probably due to the choice or combination of vector and host strain, a hypothesis used to explain difficulties encountered during the construction of clones of other flaviviruses such as YF and JE (Rice et al., 1989; Sumiyoshi et al., 1992). As a result, other groups opted for an in vitro ligation approach, where full-length cDNA was excised from two half-clones and ligated in vitro as a template for transcription. It is our belief that the choice of the low-copy-number vector pMC18, which has a copy number of approximately six per cell, was essential to our success in obtaining a full-length clone of MVE virus. In addition, the use of the E. coli DH5α host strain, which has the deoR genotype engineered to allow the uptake of large plasmids, may also have contributed to our success.

Attempts to clone full-length cDNA into the plasmid vector pBR322, which has a copy number of more than 25 per cell, were unsuccessful. In the hands of others, however, this vector has been used successfully in the construction of full-length clones (Khromykh & Westaway, 1994; Kapoor et al., 1995; Mandl et al., 1997). It is possible that the use of pBR322 represents the extreme limit of bacterial tolerance of full-length flaviviral cDNA. The use of higher-copy-number vectors such as pGEM may pose serious toxicity problems for the host, resulting in cell death or genomic instability. This may or may not be the result of the expression of flaviviral proteins in the bacterial host, a deleterious effect that would be minimized by the use of a lower-copy-number vector such as pMC18. In any event, the early difficulties faced during the construction of full-length flavivirus clones appear now to have been largely circumvented, making the generation of clone-derived RNA a much simpler process.

It is envisaged that the infectious cDNA clone of MVE virus will serve as a useful tool for elucidating the molecular determinants of virulence in flaviviruses. The mouse model of MVE virus infection is well established and is arguably one of the best working models for the encephalitic flaviviruses. The availability of such a model will allow the use of the infectious clone in the identification of molecular determinants of both neuroinvasiveness and neurovirulence. In particular, the clone is currently being used to confirm that a Ser → Ile change at position 277 in the envelope protein is responsible for the low neuroinvasiveness of the BHV-1 strain. This change also inhibits the ability of the virus to haemagglutinate across the pH range 5.5–7.5 (McMinn et al., 1995a). In separate experiments, the clone will also be used to examine more rigorously a mutation in the putative receptor-binding site (amino acid position 390 of the envelope protein) of a different strain of MVE virus. This mutation alters an RGD motif thought to be important in the interaction of the virus with host-cell integrins (Lobigs et al., 1990; Chen et al., 1997).

To date, no neurovirulence determinants have been identified for MVE virus. However, McMinn et al. (1995b) identified a strain of the virus that appears to have one or more changes in the non-structural genes and/or 3′ UTR of the genome. These changes appear to affect neuron-specific replication. The virus (C7P9) is only seven passages removed from the prototype strain 1-51 and is therefore unlikely to contain more than a few mutations. The mutation(s) is of interest because it seriously inhibits the ability of the virus to replicate in vivo in the brains of 21-day-old mice and in vitro in mouse neuroblastoma cells (McMinn et al., 1995b). By constructing intratypic chimeras with the infectious cDNA clone, it may be possible to identify the precise location of this mutation(s) and confirm its role in the attenuation of neurovirulence. Such findings may have implications for future flavivirus vaccine design and could be used in combination with other molecular determinants of virulence to re-engineer current live-attenuated vaccines, reducing the risk of their reversion to wild-type. Recent work, involving the construction of a chimeric clone containing the prM and E genes of JE virus strain SA14-14-2 within the backbone of a YF virus 17D infectious clone (Chambers et al., 1999), has shown that mice inoculated subcutaneously with 10^3 p.f.u. of chimeric
virus (ChimeriVax-JE) are solidly protected against i.p. challenge with a virulent strain of JE virus (Guirakhoo et al., 1999), as are rhesus monkeys immunized with a similar dose (Monath et al., 1999). Such studies provide good evidence that infectious clone technology can be applied successfully to the design and construction of safe and efficacious vaccines.

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