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Molecular Characterization of *Menangle Virus,* a Novel Paramyxovirus which Infects Pigs, Fruit Bats, and Humans

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Menangle virus (MenV), isolated in August 1997 following an outbreak of reproductive disease in a piggery in New South Wales, is the second previously unclassified member of the family *Paramyxoviridae* to be identified in Australia since 1994. Similar to *Hendra virus* (HeV), MenV appears to be a virus of fruit bats (flying foxes) in the genus *Pteropus*. No serological cross-reactivity was detected between MenV and other known paramyxoviruses and to facilitate virus classification a cDNA subtraction method was used to obtain viral-specific cDNA from MenV-infected cells. Cloning and sequencing of the products enabled the entire sequences of the NP, P/V, M, F, and HN genes to be determined. Comparison of the nucleotide and deduced amino acid sequences for each gene with members of the family *Paramyxoviridae,* determination of the P gene mRNA editing strategy, and phylogenetic analyses confirmed that MenV is a new member of the genus *Rubulavirus*. However the deduced protein sequence of MenV HN exhibited only limited sequence homology when compared with attachment proteins of other paramyxoviruses. Key differences within the amino acid residues considered important determinants of neuraminidase activity suggest MenV HN is unlikely to possess the same degree of neuraminidase activity characteristic of other rubulavirus and respirovirus HN proteins.

Key Words: Menangle virus; Rubulavirus; Paramyxoviridae; pigs; fruit bats; *Pteropus;* cDNA subtraction.

INTRODUCTION

Menangle virus (MenV), a recent addition to the family *Paramyxoviridae,* subfamily *Paramyxovirinae,* was isolated in 1997 from stillborn piglets at a commercial piggery in New South Wales, Australia. MenV is the etiological agent of a single outbreak of reproductive disease, which resulted in a reduction in both the farrowing rate and the number of live piglet births per litter, occasional abortions, and an increase in the proportion of mummified and stillborn piglets (Philbey *et al.,* 1998). Affected stillborn piglets frequently had deformities including skeletal abnormalities and severe degeneration of the brain and spinal cord, whereas there was no apparent disease in postnatal pigs of any age (Philbey *et al.,* 1998). Serological evidence indicated that two humans in close contact with infected pigs had contracted an influenza-like illness following infection with MenV (Chant *et al.,* 1998), demonstrating a zoonotic potential that is not yet fully defined. The presence of neutralizing antibodies against MenV in several species of fruit bats (genus *Pteropus*) and their absence in a range of domestic and other wild animals within the vicinity of the piggery suggested that flying foxes are the probable natural host of this virus (Philbey *et al.,* 1998).

MenV is the second member of the *Paramyxoviridae* to be identified in Australia within the last decade. *Hendra virus* (HeV), the cause of an outbreak of fatal respiratory disease resulting in the death of 14 horses and one human, was isolated in September 1994 (Murray *et al.,* 1995; Selvey *et al*., 1995). It has since caused the death of a second human from meningoencephalitis (O'Sullivan *et al.,* 1997), the source of infection having been his exposure to two infected horses that died more than a year earlier (Hooper *et al.,* 1996), and most recently, the death early in 1999 of a mare in Cairns, Queensland, following a short clinical illness (Hooper *et al.,* 2000). Flying foxes were implicated as the natural host of HeV several years ago following the identification of antibodies capable of neutralizing HeV in flying fox sera (Young *et al.,* 1996) and this premise has been further strengthened following additional seroepidemiological studies and the isolation of HeV from several species of flying fox in the genus *Pteropus* (Halpin *et al.,* 1999, 2000; Mackenzie, 1999).

Four additional new members of this virus family have been described since the emergence of MenV. *Nipah virus* (NiV), isolated in March 1999, was the cause of an outbreak of viral encephalitis in Malaysia and Singapore which resulted in 105 human fatalities and the slaughter of over 1,000,000 pigs once it became evident that swine were the source of human infection (Anonymous, 1999a,b; Chua *et al.,* 1999, 2000; Goh *et al.,* 2000; Paton

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et al., 1999). Preliminary serological data demonstrated the presence of antibodies against NiV in Malaysian fruit bats (Field *et al.,* 2001), raising suspicions that, as for HeV, fruit bats are the likely natural host. The recent isolation of NiV from urine samples collected from fruit bats (genus *Pteropus*) in Malaysia adds further compelling evidence to support this notion (Enserink, 2000). NiV is closely related to HeV and it has been proposed that these two viruses represent members of a new genus within the subfamily *Paramyxovirinae* (Chua *et al.,* 2000; Harcourt *et al.,* 2000; Wang *et al.,* 2000).

The remaining three new viruses, in contrast, have an unknown propensity to cause disease. *Tupaia paramyxovirus* (TPMV) was isolated from an apparently healthy Southeast Asian tree shrew (Tidona *et al.,* 1999). *Salem virus* (SalV), although identified while investigating the cause of an unknown equine illness, was not considered to be the etiological agent of the disease in question (Renshaw *et al.,* 2000). Although neither of these viruses has been classified taxonomically, initial phylogenetic comparisons suggested that the closest evolutionary relationships existed between TPMV, SalV, HeV, and members of the genus *Morbillivirus*, with TPMV more closely related to HeV, and SalV more closely related to the morbilliviruses. The most recent virus to be identified, *Tioman virus* (TiV), was isolated in Malaysia from urine samples collected from fruit bats in an attempt to identify the natural host of NiV (Chua *et al*., 2001). A novel member of the genus *Rubulavirus*, TiV, cross-reacts with MenV-specific antisera and appears to be closely related genetically.

Paramyxoviruses are enveloped viruses that contain nonsegmented negative-strand RNA genomes, which are tightly associated with nucleocapsid protein (NP) in the form of helical nucleocapsids. The family *Paramyxoviridae* is divided into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae.* Morphological criteria for inclusion in the subfamily *Paramyxovirinae* include the size and shape of virus particles and the diameter, length, and pitch of nucleocapsids. The biological criteria for inclusion in one of the three genera in this subfamily are antigenic cross-reactivity and the presence (*Respirovirus* and *Rubulavirus* genera) or absence (*Morbillivirus* genus) of neuraminidase activity (Lamb and Kolakofsky, 1996). MenV ultrastructure is consistent with its membership in the subfamily Paramyxovirinae (Philbey *et al.,* 1998) but lack of antigenic cross-reactivity with antiserum to viruses in the *Rubulavirus*, *Morbillivirus*, and *Respirovirus* genera prevented further classification of the virus.

Most viruses in the subfamily *Paramyxovirinae* have genomes which are fairly uniform in size (approximately 15,500 nucleotides (nt) in length) and organization, encoding seven to nine proteins of which two or more are derived from overlapping reading frames in the P gene. Proteins common to the three genera include three nucleocapsid-associated proteins, specifically an RNA-binding protein (NP), a phosphoprotein (P), and a putative polymerase protein (L); and three membrane associated proteins, including an inner membrane or matrix protein (M) and two glycosylated envelope proteins, comprising a fusion protein (F) and an attachment protein (G, or H, or HN) (Lamb *et al.,* 2000). To facilitate classification, we endeavored to obtain nucleotide sequence from the MenV genome using a cDNA subtraction method to selectively amplify MenV-specific mRNAs from virus-infected cells. By comparison with members of the *Paramyxovirinae,* our data revealed that MenV is a new member of the genus *Rubulavirus*. However the HN protein of MenV, although similar to the attachment proteins of viruses in the subfamily Paramyxovirinae, was not characteristic of the attachment proteins of other rubulaviruses, all of which exhibit hemagglutination and neuraminidase activities.

RESULTS

Initial characterization of the MenV genome by cDNA subtraction

Differentially expressed mRNAs in Vero cells infected with MenV were isolated by cDNA subtraction, using a commercial kit as described under Materials and Methods, and amplified by PCR Kit provided synthetic adaptors, each containing two priming sites, were used to provide enrichment. The outer priming sites enabled amplification of the differentially expressed sequences per se, and subsequently, the inner (nested) priming sites were used to further enrich such products. These products were size fractionated by agarose gel electrophoresis, and together with an unfractionated sample containing all of the PCR products, were ligated into pZErO-2 and transformed into TOP10 cells to produce a cDNA library. Forty clones were selected at random (10 from each of the four cloning events) and inserts ranging in length from 100 to 1700 bp were obtained. All 40 inserts were sequenced and homology searches were conducted using the BLAST network service (Altschul *et al.,* 1997) to identify viral specific clones. Six independent putative virus clones were obtained corresponding to regions in the NP, P/V, F, and HN genes of the MenV genome (Fig. 1, clones A–F: 270, 497, 995, 933, 696, and 243 bp, respectively) as determined by alignment with the genome of *Mumps virus* (MuV), the type species of the *Rubulavirus* genus (GenBank Accession No. AB040874). The nucleotide sequence of clones A and B (NP gene) and E and F (HN gene) were thought to be contiguous and this was later confirmed (vide infra). The partial deduced amino acid sequences derived from each of the four genes showed highest similarity to members of the *Rubulavirus* genus (data not shown),

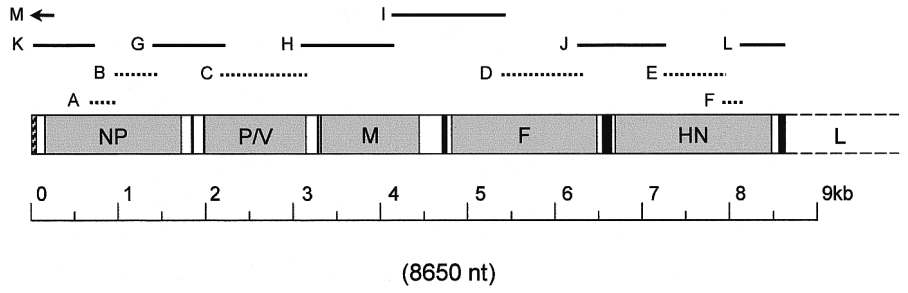

FIG. 1. Schematic representation of the 3′ terminal 8650 nt of the MenV genome and the strategies used to determine its sequence. Clones A–F (dashed lines) were derived by the isolation of differentially expressed mRNAs from MenV-infected cells using a cDNA subtraction strategy. Sequencing of clones A–F facilitated generation of amplimers G and J using MenV-specific primers and amplimers H, I, K, and L by primer walking using viral genomic RNA, instead of mRNA, as template (solid lines). Additional MenV-specific primers permitted generation of overlapping amplimers (not shown) to comfirm the sequence data already obtained and establish the gene order as shown. Amplimer M (left-facing arrow) was generated by 5′ RACE using viral antigenome as template. Shaded boxes represent the predicted ORFs for the NP, P/V, M, F, and HN genes; unshaded boxes represent untranslated regions; black boxes represent intergenic regions of variable length; and the box containing diagonal striping represents the 3′ leader. The open box (L) represents the uncharacterized 5′ portion of the MenV genome predicted to be approximately 7 kb in length, comprising the putative L gene, which encodes the viral polymerase, and the 5′ trailer.

although the HN protein sequence was exceptional in this respect (see Discussion).

Strategy for determining the sequence of genes NP, P/V, M, F, and HN

By assuming that the gene order of the MenV genome would be consistent with that of other rubulaviruses (3′-NP-P/V-M-F-(SH)-HN-L-5′, where SH refers to an additional gene present in the genomes of MuV and *Simian virus* 5 (SV5), but not other members of the genus (Lamb and Kolakofsky, 1996)) virus-specific sequence data had been determined from four of six or seven genes. MenV-specific PCR primers, together with primers designed from highly conserved regions of selected rubulavirus genome sequences, were then used to generate amplimers G–L by RT-PCR using viral genomic RNA, instead of mRNA, as template (Fig. 1). 5′ rapid amplification of cDNA ends (RACE), targeting viral antigenome as template for first-strand synthesis of cDNA, was subsequently used to determine the sequence of the 5′ end of the antigenome (amplimer M), which is complementary to the sequence of the 3′ terminus of the genome. Additional MenV-specific primers were designed to generate overlapping amplimers (not shown in Fig. 1) to confirm the sequence data derived from clones A–F and to establish the gene order in the partially characterized genome as 3′-NP-P/V-M-F-HN-5′. The lack of coding potential for a SH gene between genes F and HN was noted.

Apart from the 13 nt at the 3′ terminus of the MenV genome, the sequence of which was determined by sequencing complementary strands of 12 independent clones derived from the 5′ terminus of the antigenome, every nucleotide depicted in Fig. 1 was sequenced with a minimum, unambiguous fourfold coverage from at least two independent PCR products, in addition to sequence derived from cloned cDNAs.

Untranslated regions in the partially characterized MenV genome

The nucleotide data derived from partial characterization of the MenV genome contained six clearly identified open reading frames (ORFs) within genes coding for putative NP, P/V (two overlapping ORFs), M, F, and HN proteins, respectively, each of which is described in turn in the following sections. The flanking untranslated regions (UTRs), including intergenic regions and the 3′ leader, comprised 16% of the genome characterized thus far, reflecting a coding percentage of approximately 84%.

Putative transcriptional start and stop signals were identified for each gene by comparison with consensus signals for other members of the genus *Rubulavirus* (Table 1). Whereas transcriptional termination signals were readily identifiable, initiation signals were not so immediately obvious, since unlike the start signals for all other viruses in the family *Paramyxoviridae,* which begin with U, those of MenV commenced with C. Furthermore, it was apparent in the NP gene that two putative termination signals immediately adjacent to each other (3′-AAUUUCUUUUGAAUCUUUUUU-5′) might be functional. Confirmation that the putative start and stop signals directed the initiation and termination of transcription, respectively, was achieved using modified 5′ and 3′ RACE protocols as described under Materials and Methods. Results from 3′ RACE clearly demonstrated that the first of the two adjacent termination signals in the NP gene directed the addition of the poly(A) tail to NP gene transcripts. Determining the transcriptional control sequences also revealed the variable length of the intergenic regions, as listed in Table 1.

The 3′ leader of the MenV genome immediately preceded the NP gene and was determined to be 55 nt in length. By comparison with 3′ leader sequences of other members of the *Paramyxovirinae* (data not shown), the 3′

TABLE 1

Transcriptional Control Signals and Intergenic Regions (IGR) of MenV[a]

Genes	Gene stop	IGR	Gene start
/NP			CUCGGGUCUUC
NP/P	AAUUUCUUUU	18	CUCGGG-CUUG
P/M	AAAUUCUUUUUU	5	CUCGGG-CUUG
M/F	AAAUUAUUUUUU	38	CUCGUG-CUUG
F/HN	AAGUUCUUUUUU	85	CUCGGG-CUUA
HN/L	AAAUUCUUUUUU	68[b]	CCCGGU-CUUA[b]
Consensus[c]			
MenV	$AAaUUCU_{4-6}$	5–85	CUCGGGNCUUN
MuV	$aAauUCU_{6-7}$	1–6	UUCGgNNCUUN
MeV	$auUaU_{4-6}$	GAA	UCCuNg-gUuC
SeV	$NAUUCU_{5}$	GAA	UCCCAc-UUUC

[a] Genomic sequences (3′ to 5′) are shown.

[b] The putative start sequence for the L gene was not confirmed by 5′ RACE. The size of the HN-L IGR is therefore deduced.

[c] Consensus sequences and variability in IGR length (nt) are compared with the type species of the rubulavirus (MuV), morbillivirus (MeV), and respirovirus (SeV) genera, respectively. Consensus sequences are derived from Bellini *et al.* (1998): capital letters indicate conservation with no more than one exception; lowercase letters indicate conservation with no more than two exceptions; and N indicates differences at that position in more than two sequences.

terminal 13 nt of the MenV genome were found to match exactly the consensus sequence 3′-UGGUUCCCCUYUU-5′, derived from the 3′ termini of the rubulaviruses MuV, SV5, *Human Parainfluenza virus type 2* (hPIV2), and *Simian* virus 41 (SV41).

The nucleoprotein (NP) gene

The mRNA for the NP gene was predicted to encode a protein of 519 amino acid residues (aa) in length with a calculated molecular mass (M_r) of 58.9 kDa (Table 2). This assumed the first available AUG codon from the 5′ end of the mRNA, despite being in a suboptimal context (Kozak, 1991), is used for the initiation of translation. Considering the alignment of the deduced protein sequence with that of other known rubulavirus NP proteins, all of which have the conserved N-terminal motif MSSV (Fig. 2), and that the next available AUG codon is 148 nt further downstream, this appears likely.

Pairwise alignments of the deduced protein with NP proteins of the *Paramyxovirinae* revealed the closest relationships with members of the *Rubulavirus* genus. Percentage identities ranged between 43 and 50% for *Human Parainfluenza virus type 4a* (hPIV4a), hPIV4b, *Mapuera virus* (MPRV), MuV, SV5, SV41, and hPIV2, decreased to 34% for *Newcastle Disease virus* (NDV), but was as high as 78% for the recently described TiV (Chua *et al.,* 2001). Percentage identities with NP proteins of the respirovirus and morbillivirus genera were significantly lower (19–20 and 23–25%, respectively), but were slightly higher for the unclassified SaIV, TPMV, HeV, and NiV (24–28%).

The phosphoprotein (P/V) gene

The mRNA for the P/V gene was determined to be 1423 nt long and contained two overlapping reading frames of lengths 684 nt (ORF1) and 747 nt (ORF2). ORF1, beginning at the first AUG codon from the 5′ end of the mRNA, was predicted to encode a protein of 227 aa in length (Table 2). Identification of a Cys-rich C-terminal domain and the results of homology searches confirmed ORF1, as for all members of the genus *Rubulavirus* with the exception of NDV, encoded the V protein. Pairwise alignments with *Paramyxovirinae* V proteins demonstrated amino acid identities ranging between 19 and 29% for the rubulaviruses, except for TiV, which was more closely related at 57%. Percentage identities with V proteins of other *Paramyxovirinae* members ranged from 18% for *Sendai virus* (SeV), *Rinderpest virus* (RPV), SaIV, and TPMV to 20% for NiV. By restricting the comparison to the highly conserved Cys-rich domain of each protein (Fig. 3A), the much closer relationship with TiV was again

TABLE 2

Products of Transcription and Translation for Each Gene in the Partially Characterized MenV Genome

	mRNA				Deduced protein	
Gene	Length (nt)	5′ UTR (nt)	ORF (nt)	3′ UTR (nt)	Length (aa)	Calculated M_r (kDa)
NP	1787	108	1560	119	519	58.9
P/V (V)[a]	1423	125	684	614	227	25.1
P/V (W)[a]	1424	125	465	834	154	16.9
P/V (P)[a]	1425	125	1167	133	388	41.8
M	1426	35	1122	269	373	42.0
F	1804	65	1665	74	554	60.4
HN	1924	49	1788	87	595	65.9

[a] The P/V gene of MenV has the potential to code for three proteins, all of which share identical N-termini 151 aa in length, by RNA editing (see text for details).

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MenV    MSSVFRAFELFTLEQEQNEHGNDIELPPETLRTNIKVCTLNNQEPQARHDMMCFCLRLIASNSARAAHKTGAILTLLSLPTAMMQNHLRI    90
TiV     .................Q.L.....I......S.....I..S.D..T.............................N.........I..    90
MuV     ....LK...R..I...LQDR.EEGSI.....KSAV..FVI.TPN.TT.YH.LN....I.C.QN...S.RV..LI..F...S.G....I.L    90
SV5     ....LK.Y.R...T..LQDQSEEGTI..T..KPV.R.FI.TSNN.EL.SRLLL....IVL..G..DS.RF..L..MF...S.T.L..VKL    90
MPRV    ....LSM..R..M...LQDR.AEGT.....IKST...FI..SDD.RL.WK..N......M.DA.KISR.V..MI..F...ASG....V.L    90
hPIV4a  ....LA.Y.Q.LQTT.DRGF.DQQFVQSD..KAE.P.FV..TND..Q.FTL.N....QAV.S..KS.I.Q..L.S....QATS.....M.    90
hPIV4b  ....LA.Y.Q.LQTT.DRSF.DQQFVQSD..KAE.P.FV..TND..Q.FTL.N.....AV.S..KS.I.Q..L.S....QATS.....M.    90
SV41    ....LKT..R..IQ..LQD.EE.TPV.L..I.PL.R.FVV.SND.AL.AQLLL.N..I.M..T..ES.....L.SMF...A.A.G...KL    90
hPIV2   ....LKT..R..IQ..LQ.QSD.TPV.L..IKPT.R.FVI..ND.VV.SRLLF.N..I.M..T..EG.RA..L.S.....S.A.S..IKL    90
NDV     .....DEY.QLLAA.TRPNGAHGGGEKGS..KVDVP.F...SDD.ED.WSFVV....IAV.ED.NKPLRQ..LIS..CSHSQV.R..VA.    90

MenV    ADRSPDADIERLEIDGFEPGTFRLRANARTPMTNGEVTALNLMAQDLPDTYSNDTPFLNPNTETEQCDEMEQFLNAIYSVLVQVWVTVCK   180
TiV     ..........I.V.......Y...P....SL....I...S...N......T.....T.HRA.G.N...T.............L......   180
MuV     .....E.Q...C.........Y..IP...ANL.AN.IA.YA.L.D...P.IN.G..YVHADV.GQP...I....DRC....I.A..M...   180
SV5     ..Q..E.....V......E.S...IP...SG.SR..IN.YAAL.E.....LNHA...VDSEV.GTAW..I.T..DMC....M.A.IVT..   180
MPRV    .......Q...I..E..A.DS.K.IL.E.SS..QE.INS.DF..RE..KGFQGK.VY..VDA.GLV...V....DRA.T..L...ILA..   180
hPIV4a  .A.A...ALRII.V.AID.PDYT.TI.P.SGWDDIKIR.YRALSR...ISLADR.V.VSRDA.HAV..D.DTY..R.F...I...IM...   180
hPIV4b  .A.A...ALRII.V.AID.QDYT.TI.P.SGWDDIKIR.YRALSR...ISLADR.V.VSRDA.HAV..D.DTY..R.F...I.I.IM...   180
SV41    .T...E.S.D.V..T...GRS..VVPD..ST.SRA..L.YEAI.E.I...LNHK...V.ADV.QGDY..T.G..ELC....M.A.IVT..   180
hPIV2   .MH..E.S.D.V..T...NNS..VIPD..ST.SR...L.FEAL.E.I...LNHQ...V.NDV.DDIF..T.K..DVC....M.A.IVT..   180
NDV     .GKQNE.TLAV......AN..--PQF.N.SGVSEERAQRFAMI.GS..RAC..G...VTAGA.DDAPEDITDT.ER.L.IQA......A.   178

MenV    CMTAHDQPTGSDERRLAKYQQQGRLDQRYALQPELRRQIQTCIRSSLTIRQFLTHELQTARKQGAITGKYYAMVGDIGKYIDNAGMSAFF   270
TiV     ........................K.............A...G.........Y.............R.....................   270
MuV     ....Y...A..AD..F.........EA..M....AQ.L...A..K..VV..Y..F...L..R..LLSNR.............E.S.LT...   270
SV5     ....P...AA.I.K..Q..R....INP..L....A..I..NV..KGMVV.H...F...L..A.SLVSNR.......V....E.C..GG..   270
MPRV    ....Y...AA.IKK.TD..K....FLAT.T..AQAQATL.NV..Q..IV...MAY...IS.H..T..NR...L........E........   270
hPIV4a  ....Y......E.......K....MLE..Q..TDA.KI..LV..E.MV.....VQ.ML..D.V..Y.NR........A...A.V......   270
hPIV4b  ....Y......E.......K....MLEK.Q..TDA.KI..LV..E.MV.....VQ.ML..D.V..Y.NR........A...A.V......   270
SV41    ....P...PI.I.K.M.........INP.FI....A..I..NA..KAMVV.H...Y...M.QSKTLLANR.......V....EHS..GG..   270
hPIV2   ....P...PV.V-AKM.........INA..V....AQ.L..NA..K.MVV.H.M.Y....LSQSRSLLANR............EHS..GG..   269
NDV     A...YETADE.ET..IN..M....VQKK.I.Y.VC.ST..LT..Q..AV.I..VS..KRG.NTAGG.ST..NL...VDS..R.T.LT...   268

MenV    MTMRFALGTKWPPLALAAFSGELLKLKSLMQLYRSLGDRARYMALLEMSEMMEFAPANYPLCYSYAMGIGSVQDPMMRNYTFARPFLNPA   360
TiV     .........R......S................N..EK.........P.....................................   360
MuV     L.LKY......S..S....T...T..R...M...D..EQ...L....APQI.D...GG...IF.....V.T.L.VQ.....Y......GY   360
SV5     L.LKY....R..T.........T......A..QT..EQ...L....SPHL.D..A.....L........Y.L.VN....A.S.SYM.KT   360
MPRV    L.IKY....R.Q..........T.I....M...D..EN...L....APQ..........L.........T.L.AQ....KYG.D....V   360
hPIV4a  L.LK.G..NR.K..........V......S...R....S..L....SP.L..........LF.....V......LI...Q.G.N...TS   360
hPIV4b  L.LK.G..NR.K..........V....F.S...R....S..L....SP.L..........LF.....V......LI...Q.G.N...TS   360
SV41    C.LKYG...R..T.........Q...A..LH.Q...PM.K......SPKL.D....E...M........T.I.TN....AYG.SY...Q   360
hPIV2   L.LKYG...R..T.........Q...A..LH.Q...PM.K......SPKL.D.V.SE...D........T.L.TN....AYG.SY..QQ   359
NDV     L.LKYGIN..TSA...SSL..DIQ.M.Q..R...MK..N.P..T..GD.DQ.S....E.AQL..F...MA..L.KGTGK.Q...D.MSTS   358

MenV    YFQLGVETANRQQGSVDKAMAAELGLTEDEKRDMSAAVTRLTTGRGGNQAQELINVMGARQGRDQGRRGNRDYDVVEENEETESDSDNDE   450
TiV     .................S..E......E.R.....T.........AG...DM..I.....AAGGRGAQG.ALRI...D.T.DDE.VD.Q   450
MuV     ...I.....R....T..NRV.DD....PEQRTEVTQLID.                                                   400
SV5     .....M...RK...A..MR..ED....QA.RTE.ANTLAK                                                   400
MPRV    F..F.....RK..SA..VK......V.TTD.EE.AQTL..                                                   400
hPIV4a  ...Y.....MKH..T..PKF.S...I.DEDRV.IMQS.EK                                                   400
hPIV4b  ...Y.....MKH..T..PKL.L...I.DEDRV.IMQS.EK                                                   400
SV41    .........RK...A..HRT.ED..MSQAD.VELA.TLAK                                                   400
hPIV2   .........RK...A..NRT.ED..M.AAD.A.LT.TISK                                                   399
NDV     FWR....Y.QA.GS.INED.....K..PAAMKGLA..AQ.                                                   398

MenV    EQEIQNRPLPPIPQMPQNIDWEVRLAEIERRNQQMAARDRPQAVVTADVHQEPADARVDEQDMLLDLDM    519
TiV     ADD..G.....V.APIRE....A.I....EQG.R.RDQGVGRL.DNQAPNVQNTRQNTQDNQA.....I    519
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FIG. 2. Comparison of the NP proteins of MenV and other members of the *Rubulavirus* genus. The complete sequences of MenV and TiV are aligned, together with the more conserved N-terminal region (amino acid residues 1–400) of members of the *Rubulavirus* genus. Amino acid residue numbers for each protein are shown to the right of each sequence. Dots indicate identical residues and dashes represent gaps introduced during alignment to maximize sequence similarities.

apparent with 74% identity, compared with only 34–52% for the remaining rubulavirus sequences.

The P/V gene of MenV, like most members of the *Paramyxovirinae*, contained a conserved UC-rich editing site (3′-UUCUCCCCCUUUCCU-5′) that allows for the addition of nontemplated G residues to mRNA products during transcription (Thomas *et al.,* 1988). The putative editing site was located in the region of overlap between ORF1 and ORF2 and required the addition of a minimum of two nontemplated G residues to permit access to ORF2 downstream of the editing site during translation. To confirm this sequence-directed RNA editing and to characterize the number of G insertions in P/V gene transcripts, a pair of primers flanking the putative editing site was designed to generate a 520-bp RT-PCR product, using mRNA purified from MenV-infected cells as template. The product was cloned and the number of G insertions was determined by sequence analysis of 35

A

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MenV     ---------------------------KGFETWPKRHRTGGHRREIAIDWIGGRPRVTEWCNPICHPISQSTFRGSCRCGNCPGICSLCE   214
TiV      ---------------------------STST...R.............S.AT.T.................F.Y..T....C..DV.....   212
MuV      ---------------------------A.SGCSRPDNPR......WSLS.VQ.EV..F.......S..TAAARFH..K.....AK.DQ..   218
SV5      ------------------------------------.DTG.F....YS.G.V.DEVK.......S.S..TAAAR.FE.T.HQ..VT..E..   219
PoRV     ----------------------------ADRKGANPGEP.....YS.G.VC.TV..L.....A.S...MEPRYYQ.T..T..AK.PQ.A   229
hPIV4a   -----------------EYRYQERGLGYTESEIKNTTP.PR....YS.S.VN..TTIS.....C.A.VKSTASVEK.T..R..K..E..I   226
hPIV4b   -----------------EYSHQERGLGYTESEIKNTIFIPR....HS.S.VN..TTIS.....C.A.VKSIASVEK.T..R..K..E..I   226
SV41     ------------------------------GEIDGRHK.R....WS.A.V.DEVK.Y.....T.A.VTATDRKF..T..T..DR.GE..   222
hPIV2    ------------------------------GANRERAR.N....WS.A.V.DQVK.F.....R.A.VTA.ARKFT.T..S..S..GE..   222
NDV      MVKPPGRASSTSDSTAGESTKPRKQSRETA.PGQG.PWKP.....HS.S.TM.GVTTIS....S.S..RAEPRQY..T..S..AT.R..A   225

MenV     RDYTLLTDSGDSD-------   227
TiV      ...A..A.IR.P.CES----   228
MuV      ...GPP---------------   224
SV5      ..T-----------------   222
PoRV     G.NGIVESNRGPHDGAGNES   249
hPIV4a   ..P-----------------   229
hPIV4b   ..P-----------------   229
SV41     G.N-----------------   225
hPIV2    G.H-----------------   225
NDV      S.DVYDGGDITEGK------   239
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B

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MenV     -------------------------GKDLRHGPSDIGPGAIGGRSQLTGLAGGRESQSGATQYVTQSPSQPSEVAADVETAPASAPY--VK   215
TiV      -------------------------.VPPP..RGAT.Q.DT..K.PSA.QPEP.G..N........Y.NS.TGEP.GAGAVQM...C--.R   213
MuV      -------------------------.PGAAAQGQT.QEEG.D.NGASA.SKERSG.L....P.AHL.LP.QDSTP.N.GI..Q..IS--AN   219
SV5      ---------------------------------AG.PA.S.E.ST.SD.WEMKSR.L...IHP.L...L.QGDLN.L.TSVQSL.LN--.N   220
PoRV     ----------------------------PTGKELIPANQ.TVENIASG.SV.PSG.SN....H.P.Y.WNQDTTN.P.AP..Q..LN--.P   229
hPIV4a   --------------GNIDTRKEAWVTQNQRSRTQPPLQD.EESTRFH..TEEPQYP...IHAAH..NQL.LSKN.H..DV.KF.N.--AS   227
hPIV4b   --------------GNIATKKEAWVTQNQRSKIQSSFQD.EESTRFH.SMEEPQY....IHVAH..NQL.PSKNVH..DV.KF.N.--AL   227
SV41     ----------------------------GEKLT.ATKAD...SGASP.SETKLRFM...I.H.P.LLPLTASSPVL..P..IG.EN--..   223
hPIV2    ----------------------------GELIEKEQEAT..ENGV.H.SEIRSK.S..VIPG.P..RP.LASSP.HADP.....EN--..   223
NDV      WSSPQEGNHQRPTQQQGSQPSRGNSQERPQNQVKAA..NQ.TDVNTAYHGQWE...LS.GATPHALR.RQ.QDNTL.SADHVQP.VDF.Q   225

MenV     EIIHYLQTLETRINNLDWKVDKILSQQSVITQIKHEQHAIKAGIATLEGLITTIKIMDPGVGDGATAAKSKRLFKEAPVVVSGPVIGDNP   305
TiV      ..M.........A........L....TT.....ND..T...SL..I..............P....SQA..I.......I...IL....   303
MuV      ..MDL.RGMDA.LQH.EQ....V.A.G.MV....N.LSTV.TTL..I..MMA.V......NPT.VPVDELR.S.SDHVTI....GDVSFS   309
SV5      ..LNTVRN.DS.M.Q.ET...R...S..L.QT..NDIVGL...M.....M...V.......PSNV.VEDVRKTLSNHA...PESFNDSFL   310
PoRV     ..MEL.KAI.G.MMA.EM...RV.A.G..L....N.VTTL..TT..I......VR......PSNM..QIARNQIA.V.LI.T..GPVPQY   319
hPIV4a   ..LDAIKA..V.LDRIEG.....MLT.NT.Q.T.NDTQQ..GSL..I......M.......PSKVSLRSLNKESEQV.II.T.NGDVSKF   317
hPIV4b   ..LDAIKA..V.LDRIEG.....MLT.NT.Q.T.NDTQQ..GSL..I......M.......PSKVSLRSLNKGPEQV.II.T.TGDVSKF   317
SV41     ...EI.RG.DL.MQS.EG......ATSAT..AL.N.VTSL..NV..V..MM..M.....STPTNVPVE.IRKNL.DT..II...LSESHI   313
hPIV2    ...EL.KG.DL.LQTVEG......ATSAT.INL.N.MTSL..SV..M..M.........STPTNVPVEEIRKSLHNV...IA..TS.GFT   313
NDV      AMMSMMEAISQ.VSKV.YQL.LV.K.T.S.PMMRS.IQQL.TSV.VM.ANLGMM..L...CANISSLSDLRAVARSH..L....GDPSPY   315

MenV     IVDADTIQLDELARPSLPKTKSQKSSAASPA--ALSGYKMTLLALIKECIPNQAKRQKFEMQVGGIRNEQDFKNLRREIIRSAAQ   388
TiV      .IG.EA..........PA.PRQV.Q.GP.SS--.IV...S..QS.V....S.PSM....DLAISN.KS.....QV..D.....T-   385
MuV      SGEEP.LY.......VPKPRPAKQPKPQPVK--D.A.R.VMITKM.TD.VA.PQMK.V..QRLAKAST.DALNDIK.D.....I-   391
SV5      TQSE.V.S.......TATSV.KIVRKVPPQK--D.T.L.I..EQ.A.D..SKPKM.EEYLLKINQASS.AQLID.KKA.....I-   392
PoRV     KR.T.L.V........IAPLPAAQTQKPA..KQ.TD.ARLMVSRM.SS.VT.DSA.KR..ARL.SCTTMDQIQA.KNDV..Y.S-   403
hPIV4a   VDQDN..T..S....I.SG..QKTDERRAGV--RIDAL.I.VSEM.RDLFGDCD.SK.LLESINMATT...INLIKTNAL..IT-   399
hPIV4b   VDQDN..T..P....I.SG..QITDERRAGV--RIDAL.I.VSEM.RDLFGDCD.SR.LLESINMATT...INSIKTNAL..IT-   399
SV41     TEGS.M.V.........SS..KIVRRPEPKK--D.T.M.LM.IQ.AND.MGKPDQKAEIVAKIHAATR.AQLLDIK.S..K..I-   395
hPIV2    AEQVIL.SM......T.SS..RITRKPE.KK--D.T.I.L..MQ.AND..SRPDTKTE.VTKIQAATT.SQLNEIK.S.....I-   395
NDV      VTQGGEMA.NK.SQ.VPHPSELI.PAT.CGP--DIGVE.D.VR...MSRPMHPSSSA.LLSKLDAAGSIEEIRKIK.LALNG---   395
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FIG. 3. Comparison of the V protein specific (A) and P protein-specific (B) C-terminal domains of MenV and other rubulavirus V and P proteins. Amino acid residue numbers are shown to the right of each sequence. Identical residues are shown as dots, and dashes represent gaps introduced during alignment to maximize sequence similarities. Conserved cysteine residues in the V protein C-terminus (A) are shaded.

clones selected at random. Twenty-seven clones lacked the presence of any additional G residues at the editing site, whereas seven clones contained two additional G residues and one clone a single G insertion. Resulting from a frameshift during translation, two further proteins could therefore be produced from this gene in addition to V (Table 2). The insertion of two G residues at the editing site would result in a putative P protein 388 aa in length, the first 151 aa of which are amino coterminal with V. In contrast, the addition of a single G would result in a protein of only 154 aa in length, the N-terminal 151 of which are common to the V and P N-termini.

Pairwise alignments of the deduced P protein with other *Paramyxovirinae* P proteins revealed, similarly to V, a greater diversity than that found within related NP proteins. The most related was the P protein of TiV with

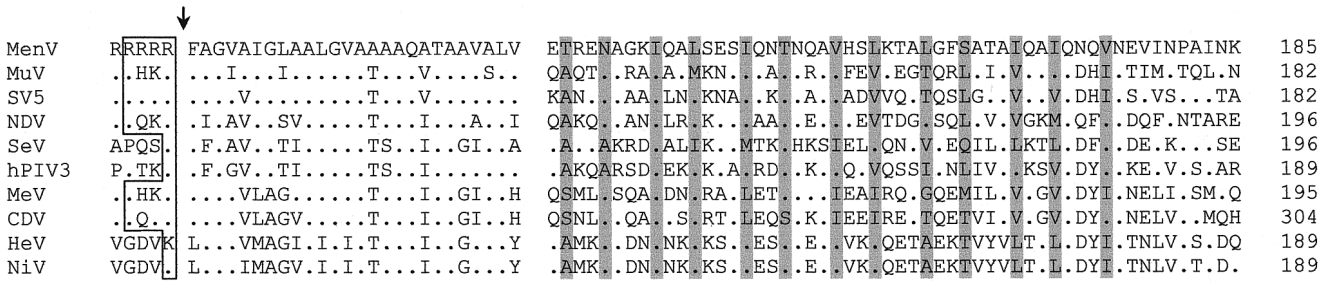

FIG. 4. Alignment of MenV and selected *Paramyxovirinae* F protein cleavage, fusion peptide, and adjacent heptad repeat A (HRA) sequences. The arrow is positioned at the cleavage site, to the right of the cleavage recognition motifs which are boxed to distinguish single from multibasic cleavage sequences. The hydrophobic fusion peptide which is at the new N-terminus of F_1 generated by cleavage of F_0 (see text for details) is to the right of the arrow. A gap has been introduced into the alignment between the fusion peptide and the adjacent HRA, in which amino acid residues with bulky and hydrophobic or neutral side chains with a 4–3 periodicity are shaded. Dots indicate identical residues and amino acid residue numbers are shown to the right of each sequence.

a 56% identity by pairwise alignment, followed by 16–31% for the remaining rubulavirus P proteins. If the P-specific domain alone was compared (Fig. 3B), these identity values increased marginally to 61% and 19–37%, respectively.

The matrix (M) gene

The mRNA for the M gene was predicted to encode a protein of 373 aa in length (Table 2). The first AUG codon from the 5′ end of the mRNA was predicted to initiate translation based on the following: this codon is in a favorable context for initiation of translation (Kozak, 1991); the resulting protein is consistent in size and homology with other *Paramyxovirinae* M proteins (data not shown); and the next available AUG codon is 227 nt further downstream and is in a less favorable context for initiation of translation. Comparison with other members of the *Paramyxovirinae* revealed the deduced protein was most related to M proteins of the *Rubulavirus* genus. hPIV4a and hPIV4b shared 45% identity, MuV and *Porcine Rubulavirus* (PoRV) 44% identity, while SV41, SV5, hPIV2, and NDV demonstrated 39, 37, 35, and 25% identity, respectively. Percentage identities with morbilliviruses (17–20%), respiroviruses (16–18%), and the unclassified HeV, NiV, and TPMV (17–18%) were significantly lower, consistent with the placement of MenV in the genus *Rubulavirus*.

The fusion (F) gene

The mRNA for the F gene was determined to be 1804 nt in length and contained a single ORF coding for the F protein (Table 2). Three in-frame AUG codons were present in the region coding for the first eight amino acid residues (codons 1, 2, and 8, respectively), none of which were in an optimal context for the initiation of translation (Kozak, 1991). Assuming the first codon is used to initiate translation, the predicted protein would be 554 aa in length. Homology studies showed the closest relationships existed with fusion proteins from members of the *Rubulavirus* genus, with percentage identities ranging from 30% for NDV to 37% for MuV. The remaining members of the *Paramyxovirinae* demonstrated identities ranging between 21% for *Human Parainfluenza virus type 1* (hPIV1) and *Dolphin Morbillivirus* (DMV) to 26% for TPMV.

The MenV F protein, as for other members of the *Paramyxovirinae,* was predicted to be a type I integral membrane protein, produced as a biologically inactive precursor (F_0) requiring cleavage by host-cell enzymes into two disulfide-linked peptides F_1 and F_2 before functioning in viral infectivity (Lamb and Kolakofsky, 1996). Three hydrophobic domains were identified: a putative C-terminal transmembrane domain located at amino acid residues 489–508 preceding a cytoplasmic tail 46 aa in length; a putative signal sequence within the hydrophobic N-terminal 21 aa of the protein; and the putative fusion peptide, located at amino acid residues 106–131, immediately following the predicted cleavage site for the MenV F_0, which is multibasic. Two heptad repeat (HR) sequences, HRA adjacent to the fusion peptide and HRB proximal to the putative transmembrane domain, characteristic of other *Paramyxovirinae* F proteins, were also identified (Buckland *et al.,* 1992; Chambers *et al.,* 1990). The MenV cleavage site, fusion peptide domain, and adjacent HRA are presented in Fig. 4 together with corresponding regions from selected *Paramyxovirinae* fusion proteins.

The attachment (HN) gene

The mRNA for the HN gene, which codes for the putative attachment protein, contained a single ORF encoding a protein of 595 aa in length (Table 2). Like other attachment proteins of the *Paramyxovirinae,* the MenV HN was predicted to be a type II integral membrane protein with a putative N-terminal transmembrane domain located at amino acid residues 46–65. Pairwise alignments of attachment proteins within the subfamily *Paramyxovirinae* demonstrated that the HN of MenV was

TABLE 3

Percentage Sequence Identity between Attachment Proteins of Selected *Paramyxovirinae,* Determined by Pairwise Alignment[a]

	Rubulavirus								*Respirovirus*			*Morbillivirus*							
Virus	MenV	MuV	SV5	SV41	hPIV2	hPIV4a	PoRV	NDV	SeV	hPIV1	hPIV3	CDV	PDV	DMV	MeV	RPV	HeV	NiV	TPMV
MenV	—																		
MuV	19	—																	
SV5	19	**44**	—																
SV41	20	**41**	**51**	—															
hPIV2	19	**39**	**45**	**61**	—														
hPIV4a	19	**35**	**37**	**36**	**37**	—													
PoRV	18	**40**	**42**	**41**	**38**	**34**	—												
NDV	16	29	**31**	**33**	**30**	29	**30**	—											
SeV	12	19	20	21	19	19	19	22	—										
hPIV1	13	19	19	19	19	21	19	21	**73**	—									
hPIV3	13	20	20	21	20	20	19	21	**45**	**46**	—								
CDV	11	11	11	11	10	13	12	11	13	12	12	—							
PDV	10	12	12	11	12	12	12	11	12	12	12	**74**	—						
DMV	11	12	11	10	9	11	10	13	12	12	13	**42**	**40**	—					
MeV	10	11	11	11	12	12	11	12	13	12	14	**35**	**36**	**44**	—				
RPV	10	12	10	10	12	12	12	13	13	13	14	**35**	**35**	**46**	**60**	—			
HeV	12	13	13	14	14	13	14	13	17	20	17	12	11	13	12	14	—		
NiV	13	12	13	14	12	13	15	14	19	20	18	12	11	13	13	13	**79**	—	
TPMV	12	13	12	14	13	12	12	13	21	21	19	13	12	12	11	11	15	15	—

[a] Sequence identities of 30% and greater are shown in bold.

not significantly related to attachment proteins of any of the existing three genera (Table 3). The MenV HN exhibited only 16–20% amino acid sequence identity with other rubulavirus proteins (no sequence data are available for TiV HN), which is not significantly different to the sequence identities seen with other *Paramyxovirinae* (10–13%), particularly when considering identities between 34–61, 45–73, and 35–74% are observed for attachment proteins within the rubulavirus (with the exception of NDV), respirovirus, and morbillivirus genera, respectively. The putative MenV HN was also larger than that of any other rubulavirus, the closest in size being the HN of MuV, which contains 13 fewer aa.

Recently, Langedijk *et al.* (1997) conducted a comprehensive structural analysis of the globular head domains of *Paramyxovirinae* HN proteins and by comparison with the sequence and structure of influenza virus neuraminidase (N) and a range of bacterial and eukaryotic neuraminidases, identified seven active site residues common to most of the neuraminidases examined. Six of these seven residues, which are considered important determinants of neuraminidase activity, are conserved in respirovirus and rubulavirus attachment proteins. The hexapeptide NRKSCS, also conserved in HN proteins of both genera, has been proposed to form part of the neuraminidase active site (Jorgensen *et al.,* 1987; Mirza *et al.,* 1994). An unexpected finding was that only two or three of the seven active site residues and only the last two amino acids in the sequence motif NRKSCS were conserved in MenV HN (Fig. 5).

DISCUSSION

To facilitate classification of MenV, we derived the nucleotide sequence for the 3′ half of the viral genome, from the 3′ genome terminus to the beginning of the 3′ UTR of the putative L gene. Initial sequence data were obtained from MenV-specific mRNAs, isolated from virus-infected cells using a cDNA subtraction strategy, and subsequently from viral genomic RNA. By this approach five genes, from a genome predicted to contain six in total, were characterized. As discussed herein, analysis of the UTRs and the predicted ORFs provided clear evidence for the classification of MenV within the genus *Rubulavirus*. However, although the genome organization was more or less as anticipated, several novel findings were apparent, the most significant of which is the limited sequence homology exhibited by the MenV HN to attachment proteins of other paramyxoviruses.

The first 12–13 nt of *Paramyxovirinae* genome ends are genus specific and are thought to play a critical role as the promoter sequence for directing transcription as well as genome replication, both of which are functions mediated by the viral RNA-dependent RNA polymerase (Bellini *et al.,* 1998; Lamb and Kolakofsky, 1996). In this study, the sequence of the MenV 3′ genome end was determined by sequencing the 5′ terminus of the antigenome and was found to match the consensus sequence derived from the 3′ genome ends of representative rubulaviruses. An intermediate in RNA replication, the antigenome is a full-length positive-sense copy of the

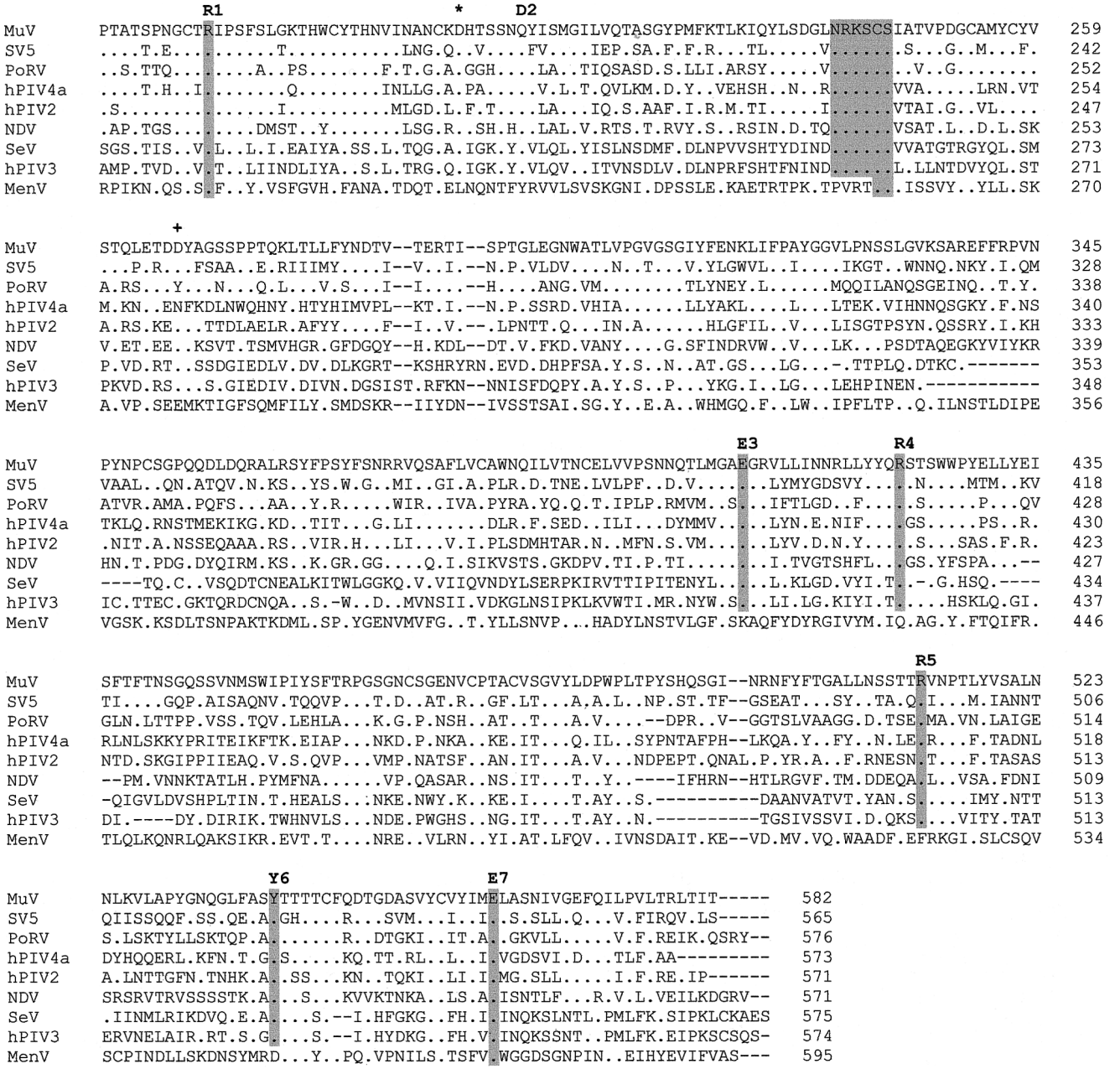

FIG. 5. Sequence alignment of the globular head region of representative rubulavirus and respirovirus attachment proteins. The seven active site residues considered important determinants of neuraminidase activity are numbered and shown in bold above the corresponding amino acid residues, which are shaded if conserved, for each protein in the alignment. The two alternative candidates for the second active site residue are indicated by an asterisk and plus sign, respectively. The hexapeptide NRKSCS, conserved in all rubulavirus (with the exception of MenV) and respirovirus HN proteins, is highlighted with shading. Amino acid residue numbers are shown to the right of each sequence. Identical amino acid residues are shown as dots, and dashes represent gaps introduced during alignment to maximize sequence similarities.

viral genome and has been shown to constitute up to 40% of ribonucleoprotein in virus-infected cells (Kolakofsky and Bruschi, 1975). This approach is thus a simple and efficient method of determining the 3′ genome end sequence of any paramyxovirus.

A comparison between the transcriptional control signals of MenV and selected *Paramyxovirinae* demonstrated the greatest similarity between those of MenV and members of the *Rubulavirus* genus (consensus sequences for the type species in each of the three genera are shown in Table 1). A significant difference was the finding that the sequences controlling the start of transcription for MenV all commenced with C, which is in contrast to those of all other members of the *Paramyxovirinae,* except for TiV (Chua *et al.,* 2001), which commence with U. Determination of the transcriptional control signals also permitted the intergenic regions of the MenV genome to be clearly defined. These regions are

precisely three nucleotides long for the respiroviruses and morbilliviruses, but are variable in length for the rubulaviruses, ranging in size from 1 to 47 nt (Lamb and Kolakofsky, 1996). Consistent with the genome organization of other rubulaviruses, the intergenic regions of MenV varied in length from 5 nt between genes P and M to 85 nt between genes F and HN (Table 1).

Analysis of the deduced gene products from the NP, P/V, M, and F genes revealed further compelling evidence for the placement of MenV within the genus *Rubulavirus*. The N-terminal sequence motif MSSV, which is unique to all rubulavirus NP proteins, was conserved in MenV NP (Fig. 2). As for other members of the *Paramyxovirinae,* MenV NP could be divided into a relatively well-conserved N-terminal domain of approximately 400 aa and a much more variable C-terminal domain, which is negatively charged. The variability in the NP C-terminal domains is in part due to the difference in size between the proteins, which range from 489 aa for NDV to 551 aa for hPIV4a and hPIV4b (not shown in Fig. 2). The sequence identity levels between the MenV NP protein and those of other rubulaviruses ranged between 14 and 19% in this region. In the case of TiV, this was significantly higher at 41%. However, the much closer relationship between the NP proteins of MenV and TiV, both of which are 519 aa in length, was more readily appreciated when comparisons were made within the N-terminal 421 aa. The percentage identity was then 89% and multiple regions of identity were present, the longest stretching for 59 aa between residues 320 and 378.

The mRNA editing site in the P/V gene of MenV was identical to that found in other rubulavirus P/V genes, and as for all members of the *Rubulavirus* genus, with the exception of NDV, faithful transcripts of the MenV P/V gene encoded the V protein, whereas the addition of two Gs resulted in a +1 frameshift allowing access to the P ORF downstream of the editing site. Of interest was the finding of one clone derived from an edited transcript that had resulted in the insertion of only a single G. This generates a frameshift allowing access to the putative W ORF, which in MenV, terminates after the addition of only three further amino acids, resulting in a protein of 154 aa in length. The potential to code for a W protein has been noted for SeV (Delenda *et al.,* 1998; Vidal *et al.,* 1990), *Measles virus* (MeV) (Gombart *et al.,* 1992), *Phocine Distemper virus* (PDV) (Blixenkrone-Moller *et al.,* 1992), NDV (Steward *et al.,* 1993), hPIV4a and hPIV4b (Kondo *et al.,* 1990), TiV (Chua *et al.,* 2001), and MuV, in which it is designated I (Paterson and Lamb, 1990). Of the rubulaviruses with the potential to encode W, only TiV has been shown to do so by the insertion of a single G at the editing site. In contrast, MuV, hPIV4a, and hPIV4b do so by the insertion of four Gs. It appears that the P/V gene of MenV thus has the potential to code for three proteins, all of which share identical N-termini 151 aa in length (Table 2).

As for other viruses in the subfamily *Paramxyovirinae,* the proteins encoded by the MenV P/V gene were among the least conserved. The greatest degree of conservation in MenV P was in the C-terminal region of the protein after the mRNA editing site (Fig. 3B). For most paramyxoviruses this region also corresponds to the more highly conserved Cys-rich domain of the V protein, which is accessed via an alternative reading frame. As evident in Fig. 3A, MenV V contains all seven Cys residues that are conserved in V proteins of all *Paramyxovirinae* which have a functional V gene coding region. This Cys-rich region resembles a zinc finger domain (Thomas *et al.,* 1988) and the V proteins of MeV, SV5, and NDV have all been shown to bind zinc (Liston and Briedis, 1994; Paterson *et al.,* 1995; Steward *et al.,* 1995).

The cleavage sequence of MenV F_0 is multibasic as for other fusion proteins of the *Rubulavirus* and *Morbillivirus* genera and conforms to the sequence specificity R-X-K/R-R, required for cleavage by endoproteases located in the trans-Golgi network such as furin (Hosaka *et al.,* 1991). The cleavage activation site is identical to that of SV5 and contains four Arg residues (Fig. 4). The fusion peptide, situated at the new N-terminus of F_1 following cleavage of F_0, is believed to insert into target membranes to initiate the fusion process. An adjacent heptad repeat domain, HRA (Fig. 4), is thought to play a key role in this process by means of a conformational change which results in repositioning of the fusion peptide toward the target cell membrane in a more optimal position for fusion (reviewed by Lamb, 1993). The presence of a second heptad repeat, HRB, adjacent to the putative transmembrane domain, in addition to the conservation of nine Cys residues in the extracellular domain of F_1 and the similarity in location of Gly and Pro residues, suggest that MenV F is likely to be structurally and functionally similar to other *Paramyxovirinae* F proteins. However, in contrast to other rubulaviruses, in which both F_1 and F_2 subunits are N-glycosylated (Lamb and Kolakofsky, 1996), MenV F contained six potential sites for the addition of N-linked sugars, all of which were on the F_1 subunit.

Comparison of the MenV HN protein with attachment proteins from other *Paramyxovirinae* members unexpectedly revealed the lowest sequence conservation of the six MenV putative proteins examined (Table 3). Alignment of the globular head region of MenV HN with representative respirovirus and rubulavirus attachment proteins (Fig. 5) revealed several significant observations, as follows: (1) MenV HN shared 14 conserved Cys residues with other respirovirus and rubulavirus HN proteins. In addition, the majority of the structurally important Pro and Gly residues were conserved, suggesting the overall structure of the MenV HN is likely to be similar. (2) Of the seven active site residues considered important determinants of neuraminidase activity (Langedijk *et al.,* 1997), only two or three are conserved

in MenV HN (Fig. 5, residues 1, 7, and possibly 5), whereas six are conserved in respirovirus and rubulavirus attachment proteins (all but residue 2). The second active site residue, Asp 151 in influenza viruses, has no direct homolog in paramyxovirus HN proteins, although residues equivalent to MuV Asp 204 and Asp 267 in Fig. 5 have been suggested as alternative counterparts (Colman *et al.,* 1993; Langedijk *et al.,* 1997). Asp 204 is conserved in all rubulavirus and respirovirus HN proteins and has since been shown to be critical for neuraminidase activity in NDV (Deng *et al.,* 1999), making this residue the more likely counterpart of influenza virus Asp 151. Nevertheless, neither of the suggested counterparts is conserved in MenV HN. (3) MenV HN contains only the last two amino acid residues in the sequence motif NRKSCS, which is conserved in all known rubulavirus and respirovirus HN proteins and is thought to form part of the sialic acid binding site (Jorgensen *et al.,* 1987; Mirza *et al.,* 1994). Morbillivirus H proteins, which are generally considered to lack neuraminidase activity, have four of the active site residues conserved (residues 1, 5, 6, and 7) and only the second to last residue in the hexapeptide NRKSCS (Langedijk *et al.,* 1997). These observations suggest that the MenV HN protein may not possess the same degree of neuraminidase activity characteristic of rubulavirus and respirovirus attachment proteins.

Phylogenetic relationships of MenV with viruses in the subfamily *Paramyxovirinae* were estimated using the complete sequences of the deduced NP, P, M, F, and HN proteins. When comparing the NP, P, M, and F proteins, MenV was consistently placed within the cluster defined by the *Rubulavirus* genus, as illustrated in Fig. 6A for the NP protein. Trees for the P, M, and F proteins are not shown due to their similarity with that derived for NP. The close relationship between the NP proteins of MenV and TiV was evident by their placement on the same branch within the rubulavirus lineage. In contrast, the tree derived from analysis of attachment proteins placed MenV outside the rubulavirus cluster between NDV, which de Leeuw and Peeters (1999) argue is only distantly related to members of the *Rubulavirus* genus, and the cluster that defines the *Respirovirus* genus (Fig. 6B). Phylogenetic analyses thus provided strong support for the inclusion of MenV as a new member of the genus *Rubulavirus*, even though an obvious explanation for the apparent difference in the evolutionary development of MenV HN compared with the NP, P, M, and F proteins is lacking.

In summary, partial characterization of the MenV genome and analysis of the putative protein products from five genes have provided sufficient evidence for the classification of MenV as a novel member of the genus *Rubulavirus* in the subfamily *Paramyxovirinae*. In doing so, the advantages of using a cDNA subtraction strategy to facilitate sequencing of an unknown virus were established, demonstrating the utility of this approach to the rapid characterization of any unknown viral isolate.

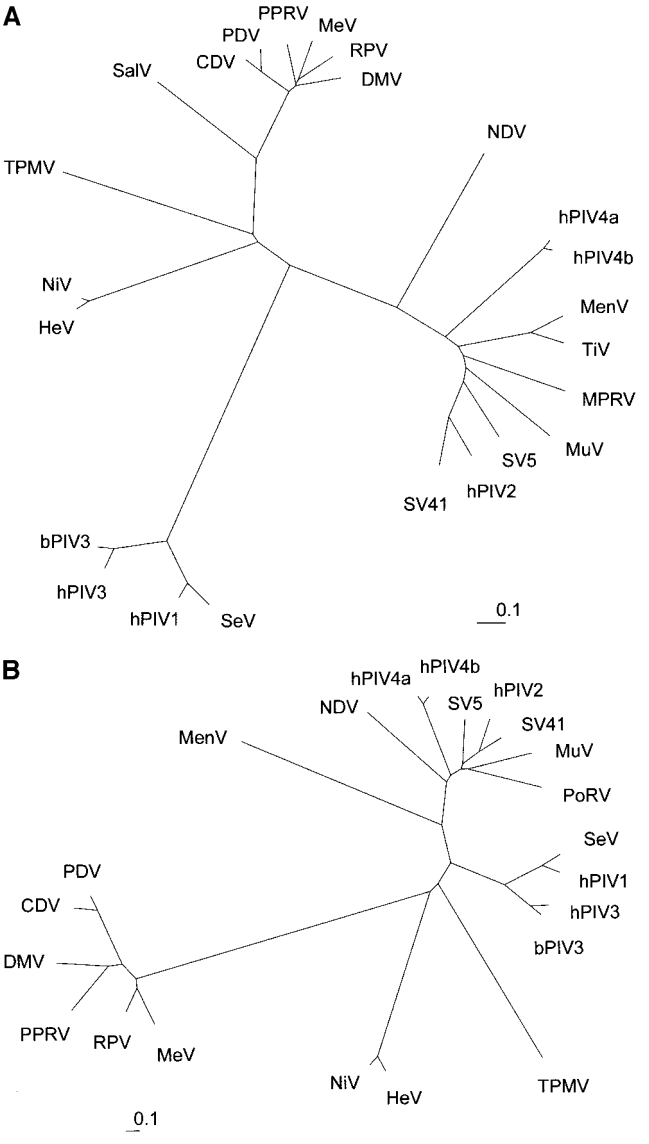

FIG. 6. Phylogenetic trees based on the complete sequences of the nucleoprotein (A) and attachment (B) proteins from members of the subfamily *Paramyxovirinae*. The trees shown were generated using distance matrix methods (Neighbor) in the PHYLIP software package. Branch lengths represent relative genetic distances. Trees generated using maximum parsimony methods (Protdist) in the same software package were almost identical (data not shown).

MATERIALS AND METHODS

Virus culture and isolation of mRNA

Virus was isolated from clinical samples provided by the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales and plaque purified 3 times in Vero cells. The virus name was abbreviated to MenV to avoid confusion with measles virus, commonly abbreviated to MV, but also listed as MeV in the Seventh Report of the

International Committee on Taxonomy of Viruses (Lamb *et al.,* 2000).

Vero cell monolayers were infected with plaque-purified MenV at a multiplicity of 0.05 $TCID_{50}$/cell and at 48 h postinfection (hpi) cytoplasmic RNA was isolated from infected and uninfected cells using the RNeasy Midi Kit (Qiagen, Germany). Poly(A)-containing mRNA was purified from cytoplasmic RNA using the Oligotex mRNA Midi Kit (Qiagen) and RNA concentrations were determined using the GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, USA).

Isolation and cloning of viral cDNA using the Clontech PCR Select cDNA Subtraction Kit

The selection and amplification processes were performed as described in the manual accompanying the kit. Briefly, double-stranded (ds) cDNA was made from mRNA of uninfected Vero cells (driver cDNA) and MenV-infected Vero cells (tester cDNA) and digested with *Rsa*I. In separate reactions tester cDNA was ligated to kit-provided adaptors 1 and 2R before mixing with excess driver cDNA. After heating and hybridization the products included driver ds cDNA and hybrids containing driver cDNA and adaptor-linked cellular cDNA from the tester populations. Tester cDNA derived from viral mRNA and virus-induced cellular mRNA remained single-stranded. Primary hybridization mixes were combined without denaturing and a second hybridization step permitted the formation of differentially expressed ds cDNA containing one strand with adaptor 1 and a second with adaptor 2R. Differentially expressed molecules were amplified in a primary and a secondary PCR, using primers specific to adaptors 1 and 2R, and the products analyzed by electrophoresis in agarose gels. The predominant products in the secondary PCR were gel purified using the QIAquick PCR Gel Extraction Kit (Qiagen) and together with an unfractionated sample containing all products, were digested with *Rsa*I and cloned into the *Eco*RV cloning site of the vector pZErO-2 (Invitrogen, USA). Electrocompetent *Escherichia coli* strain TOP10 was mixed with 2 μl of each ligation reaction and transformed using a Gene Pulser apparatus (Bio-Rad Life Sciences Group, USA) and plated out on Luria Broth (LB) agar containing 50 μg/ml kanamycin to produce a cDNA library comprising cDNAs derived from differentially expressed cellular and viral mRNAs. Plasmid DNA was purified from 40 clones (10 from each of the four cloning events) using the QIAprep Spin Miniprep Kit (Qiagen) in preparation for sequencing.

Isolation and amplification of viral genomic RNA

Monolayers of Vero cells were infected with MenV at a multiplicity of 0.01 $TCID_{50}$/ml. At 48 hpi cells were washed with PBS and resuspended in TNM (10 mM Tris, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$) at 4°C and lysed by addition of Nonidet-P40 to 1% (v/v). Nuclei and the associated cytoskeletons were pelleted and the cytoplasmic fraction removed. Cytoskeletons were sheared off the nuclei by Dounce homogenization and the nuclei pelleted. The cytoplasmic and cytoskeletal fractions were pooled and EDTA and DOC added to 10 mM and 1% (w/v), respectively. After clarification at 10,000 rpm in a SW55 rotor for 10 min at 4°C, the lysate was layered over 20–40% CsCl gradients in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA) with 0.2% sodium lauroyl sarcosinate. Following centrifugation in a SW41 rotor for 24 h at 25,000 rpm the visible ribonucleoprotein (RNP) band at 1.31 g/cm^3 was collected, diluted, and pelleted at 35,000 rpm for 1.5 h in a SW41 rotor. Purified genomic RNA was prepared from the pelleted RNP using the RNeasy Mini Kit (Qiagen) and quantified using the GeneQuant II RNA/DNA Calculator (Pharmacia Biotech). Viral cDNA was synthesized using the TimeSaver cDNA Synthesis Kit (Amersham Pharmacia Biotech, USA) from 1 μg of genomic RNA using random hexamer primers diluted 1 in 200 as recommended by the supplier to maximize the generation of long cDNA products.

MenV-specific primers and primers designed from highly conserved regions of selected rubulavirus genome sequences were synthesized by a commercial provider (GeneWorks, Australia). PCR was performed using the Platinum PCR SuperMix kit (Life Technologies Inc., USA) in 25 μl volumes, containing 20 pmol of each oligonucleotide primer and 1 μl of cDNA template synthesized from MenV genomic RNA as described above. PCRs were incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA) under cycling conditions of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1–2 min depending on the size of the expected PCR product, and a further 5 min extension at 72°C. PCR products were routinely visualized with ethidium bromide in 1.0–2.0% agarose gels and were purified using the QIAquick PCR Purification Kit (Qiagen) in preparation for sequencing. PCRs were always conducted in duplicate and both strands of each product sequenced and compared to identify random sequence errors introduced by the recombinant *Taq* DNA polymerase, which lacked a proofreading capability.

The RT-PCR primers used to generate amplimers G–L (Fig. 1) to complete initial characterization of genes NP, P/V, M, F, and HN are as follows (primers designed from highly conserved regions of selected rubulavirus genome sequences are underlined): (G) 1066 bp, 5′-TGATGAGGAACTATACATTTGC-3′ and 5′-CACATCAATGGGACGGACTTTC-3′; (H) 1090 bp, 5′-GGCGGAATCCGAAATGAGCAAG-3′ and 5′-AGTGTGGGACCCCACATATC-3′; (I) 1317 bp, 5′-GGAATCGTGGATATGTGGGG-3′ and 5′-AGTTGTGTCTGGAAGACTGTAG-3′; (J) 1059 bp, 5′-CTGGCTAAAAGTCAGTGGGTGG-3′ and 5′-AGCAGTGAACAC-CAAAGGATAC-

3′; (K) 701 bp, 5′-CGCGGATCCACCAAGGGGARAA-3′ (a rubulavirus-specific primer complementary to the highly conserved 13 nt genome termini) and 5′-AGTGGGCTGATCATGAGCTGTC-3′ and 5′-TGACCCAGACTTGAACTAGTAC-3′ (nested primers specific to clone A); and (L) 611 bp, 5′-AAGTAACGAGCACTACAAGACC-3′ and 5′-GGTGAGTTTARATGKACTTCRGG-3′.

Rapid amplification of cDNA ends

The protocol for 5′ RACE, used to determine transcriptional start sequences, was adapted from a published method (Tillett *et al.,* 2000). Briefly, Vero cell monolayers were infected with plaque-purified MenV at a multiplicity of 0.05 $TCID_{50}$/cell and at 72 hpi mRNA was isolated from infected cells using the Oligotex Direct mRNA Mini Kit (Qiagen) and quantitated as described earlier. First-strand synthesis of cDNA from each of the viral NP, P, M, F, and HN mRNAs was performed in separate reactions using the ThermoScript RT-PCR System (Life Technologies) from 250 ng of mRNA, using specific primers resulting in RT products of approximately 500 bp in length (NP, 5′-ATCCGTCAATCTCTAGCCTC-3′; P, 5′-CACATCAATGGGACGGACTTTC-3′; M, 5′-TTGGTGCTTGTTGAAGATGG-3′; F, 5′-AACCCTATAGCCACACCTGC-3′; and HN, 5′-TGATCAGTGACCGACTTACC-3′). RT reactions were incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) at 65°C for 1 min, 60°C for 1 min, 55°C for 1 min, 50°C for 30 min, followed by five cycles at 50°C for 1 min, 55°C for 1 min, and 60°C for 1 min. A final incubation at 85°C for 5 min was performed to inactivate the reverse transcriptase enzyme. Purification of first-strand cDNAs and ligation of a 3′-end cordecypin-blocked anchor oligonucleotide (5′-GAAGAGAAGGTGGAAATGGCGTTTTGG-3′) to the 3′ end of purified cDNAs was performed as described by Tillett *et al.* (2000). PCR amplifications using an anchor-specific primer (5′-CCAAAACGCCATTTCCACCTTCTCTTC-3′) and hemi-nested primers specific to each viral mRNA (NP, 5′-GCACGGGCAGAATTACTAGC-3′ and 5′-GTCACAGGAGGCAATTCAATGTC-3′; P, 5′-TCTTTGCATTGTCTCTGTCC-3′ and 5′-ACGCTCAAGAAATGCTCCAC-3′; M, 5′-TTGCACTCTTTCTGACGATG-3′ and 5′-AAAGGAAAGCGGAAGCTCGG-3′; F, 5′-CTGAACTAAGGAGGTTACAG-3′ and 5′-ACCTGTGTATCAGGCCAAT-C-3′; and HN, 5′-CTATTAGTTGCACAACCTCC-3′ and 5′-GGAATAAAGTCCTGCATGTG-3′) were conducted with the Platinum PCR SuperMix kit (Life Technologies) to generate secondary amplimers 200–250 bp in size. PCR products were purified as described earlier, treated with T4 DNA polymerase (New England Biolabs Inc., USA) to generate blunt ends, and cloned into the vector pZErO-2 (Invitrogen) cut with *Eco*RV in preparation for sequencing.

This method was also successfully employed to determine the 13 nt 5′ terminal sequence of the antigenome, which is complementary to the 13 nt 3′ terminal sequence of the genome. MenV genomic RNA prepared as described earlier, but without CsCl gradient purification, was used for first-strand cDNA synthesis. Using the same set of NP mRNA specific primers listed above, MenV antigenomic RNA was selectively reverse transcribed and amplified resulting in products 55 nt larger than those derived from NP mRNA.

An abbreviated 3′ RACE protocol was developed for determination of transcriptional termination signals. Using mRNA isolated as for the 5′ RACE protocol, first-strand cDNA synthesis was performed using the ThermoScript RT-PCR System (Life Technologies) from 500 ng of mRNA using the kit provided oligo$(dT)_{20}$ primer. Using mRNA specific primers (NP, 5′-TGATGAGGAACTATACATTTGC-3′; P, 5′-ACACGAGCAACATGCAATC-3′; M, 5′-TTACTGTGTGGTCATCCTGC-3′; F, 5′-GATGCAATTGTCTTTCACCG-3′; and HN, 5′-AAGTAACGAGCACTACAAGACC-3′) and an oligo(dT) primer with a GC-rich tail (5′-CGCGTCGACTAGTACT$_{15}$-3′) to improve amplification efficiency, primary PCR amplimers were generated from each of the NP, P, M, F, and HN cDNAs and purified using the QIAquick PCR Purification Kit (Qiagen) in preparation for sequencing.

Analysis of P mRNA editing

To examine the number of G insertions at the P mRNA editing site, mRNA was isolated from MenV-infected Vero cells and quantitated as for the 5′ RACE protocol. First-strand cDNA synthesis was performed using the ThermoScript RT-PCR System (Life Technologies) from 1 μg of mRNA using the random hexamer primers supplied. A 1/5 aliquot was used as template for PCR with 5 pmol of each of two primers (5′-AAAGAAGTCACCACCGCAGC-3′ and 5′-GGTAATAACAGACTGCTGAG-3′) to amplify a 520-bp product containing the editing site centrally within. PCR was carried out using the Platinum PCR SuperMix kit (Life Technologies) in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a further incubation at 72°C for 5 min. The products were then purified and cloned into *Eco*RV cut pZErO-2 (Invitrogen), transformed into *E. coli* strain TOP10, and plated onto LB agar containing kanamycin as described earlier, to produce a library containing P mRNA-specific cDNAs. Colonies were picked at random for sequencing and the number of G insertions at the editing site of each clone was determined by comparison with the corresponding viral genomic sequence.

Nucleotide and amino acid sequence analyses

Purified PCR products and cloned DNA were sequenced using fluorescence-based dideoxy BigDye ter-

minator chemistry (Applied Biosystems) and analysis of the sequencing products was performed using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The derived nucleotide sequences were analyzed and managed using the program SeqMan 4.00 in the Lasergene software package (DNASTAR Inc., Madison, WI). Sequence similarity comparisons using the GenBank/EMBL and SWISSPROT databases were undertaken using the BLAST service (Altschul *et al.,* 1997) of the National Centre for Biotechnology Information (NCBI). Deduced MenV amino acid sequences were aligned with cognate proteins in the *Paramyxoviridae* family using the programs Clone Manager 5 and Align Plus 4 in the Sci Ed Central software package (Scientific and Educational Software, USA), and amino acid sequence identities were determined by pairwise alignment (Clustal method) using MegAlign 4.00 in the Lasergene software package (DNASTAR). Phylogenetic analyses of full-length NP, P, M, F, and HN protein sequences were undertaken using programs available through the Bionavigator web interface (eBioinformatics Pty Ltd, http://www.eBioinformatics.com). Multiple sequence alignments were generated using Clustal W (Thompson *et al*., 1994) and evolutionary relationships were estimated using programs in the PHYLIP software package (Version 3.2; Felsenstein, 1989), including distance matrix (Neighbor, Fitch) and maximum parsimony (Protdist) methods. The significance of the derived trees was evaluated by bootstrap resampling of 100 replicate data sets and comparison with consensus trees generated using the additional programs Seqboot and Consense. Phylogenetic trees were viewed using TreeView software (Page, 1996).

Database Accession Nos. of sequences used for comparison are as follows: (NP) bPIV3 (*Bovine Parainfluenza virus type 3*) (AF178654); CDV (*Canine distemper virus*) (AF014953); DMV (Z36978); HeV (AF017149); hPIV1 (D01070); hPIV2 (X57559); hPIV3 (*Human Parainfluenza virus type 3*) (AB012132); hPIV4a (M32982); hPIV4b (M32983); MeV (K01711); MPRV (X85128); MuV (AB040874); NDV (AF064091); NiV (AF212302); PDV (X75717); PPRV (*Peste-des-Petits-Ruminants virus*) (X74443); RPV (AF132934); SaIV (AF237881); SeV (M30202); SV5 (AF052755); SV41 (X64275); TiV (AF298895); TPMV (AF079780); (V) hPIV2 (X57559); hPIV4a (M55975); hPIV4b (M55976); MuV (AB040874); NDV (Q06428); PoRV (228836); SV5 (AF052755); SV41 (X64275); TiV (AF298895); (P) as for V except NDV (AF064091) and PoRV (228838); (F) CDV (AF014953); HeV (AF017149); hPIV3 (AB012132); MeV (K01711); MuV (AB040874); NDV (AF048763); NiV (AF238466); SeV (M30202); SV5 (AF052755); (HN) bPIV3 (AF178654); CDV (AF014953); DMV (Z36978); HeV (AF017149); hPIV1 (AF016280); hPIV2 (X57559); hPIV3 (AB012132); hPIV4a (M34033); hPIV4b (AB006958); MeV (K01711); MuV (AB040874); NDV (AF204872); NiV (AF238467); PDV (Z36979); PoRV (S77541); PPRV (Z81358); RPV (AF132934); SeV (M30202); SV5 (AF052755); SV41 (X64275); and TPMV (AF079780).

Nucleotide sequence accession number

The sequences reported in this paper have been deposited in the GenBank database (Accession No. AF326114).

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