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Molecular Characterization of Nipah Virus, a Newly Emergent Paramyxovirus

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Recently, a new paramyxovirus, now known as Nipah virus (NV), emerged in Malaysia and Singapore, causing fatal encephalitis in humans and a respiratory syndrome in pigs. Initial studies had indicated that NV is antigenically and genetically related to Hendra virus (HV). We generated the sequences of the N, P/C/V, M, F, and G genes of NV and compared these sequences with those of HV and other members of the family Paramyxoviridae. The intergenic regions of NV were identical to those of HV, and the gene start and stop sequences of NV were nearly identical to those of HV. The open reading frames (ORFs) for the V and C proteins within the P gene were found in NV, but the ORF encoding a potential short basic protein found in the P gene of HV was not conserved in NV. The N, P, C, V, M, F, and G ORFs in NV have nucleotide homologies ranging from 88% to 70% and predicted amino acid homologies ranging from 92% to 67% in comparison with HV. The predicted fusion cleavage sequence of the F protein of NV had a single amino acid substitution (K to R) in comparison with HV. Phylogenetic analysis demonstrated that although HV and NV are closely related, they are clearly distinct from any of the established genera within the Paramyxoviridae and should be considered a new genus.

INTRODUCTION

Nipah virus (NV) was isolated in March 1999 and subsequently identified as the etiological agent responsible for an outbreak of fatal viral encephalitis in Malaysia and Singapore. The outbreak, which began in the fall of 1998, reached its peak in the spring of 1999; overall, there were 265 human cases reported and 105 fatalities. NV also caused a respiratory illness and, to a lesser extent than in humans, a central nervous system disease in swine. Most human cases occurred in individuals who had occupational exposure to pigs (Anonymous, 1999a,b; Chua *et al.,* 1999, 2000; Paton *et al.,* 1999). The outbreak was controlled only after the new virus etiology was realized, swine were identified as the source of the virus infecting the humans, and more than 1 million swine in the affected areas were culled (Anonymous, 1999a). Therefore, this outbreak was devastating not only because of the loss of human life but also because of the severe economic loss suffered by the Malaysian swine industry. NV was isolated from both swine lung tissue and human brain tissue, and electron microscopy indicated that NV had structural features that were most consistent with those of the family Paramyxoviridae (anonymous, 1999a; Chua *et al.,* 2000). NV shared antigenic cross-reactivity with only Hendra virus (HV), and sequence obtained from NV had more homology with HV than with any other paramyxovirus (Anonymous, 1999a; Chua *et al.,* 2000).

HV, formerly known as equine morbillivirus, was first isolated during an outbreak of a severe respiratory illness that killed 14 horses and one human in Hendra, Queensland, Australia, in September 1994 (Murray, 1995; Selvey, 1994). The virus also caused the deaths of two horses and a human in the community of Mackay, 800 km north of Hendra (Rogers, 1996). Antibody to HV has been detected in four species of fruit bats (flying foxes) indigenous to Australia: the gray-headed (*Pteropus poliocephalus*), black (*P. alecto)*, little red (*P. scapulatus)*, and spectacled (*P. conspicillatus)* fruit bats (Mackenzie, 1999; Young *et al.,* 1996). In addition, a virus identical to HV has been isolated from two gray-headed flying foxes, one black flying fox, and a little red flying fox (Halpin, 1996). Thus the *Pteropus* species are currently thought to be the natural host of HV (Halpin, 1996; Mackenzie, 1999; Young *et al.,* 1996). The ultrastructural features and gene organization of HV indicate that it is a member of the family Paramyxoviridae (Hyatt, 1996; Murray, 1995). However, phylogenetic analysis has shown that HV does not belong to any of the known genera within the Paramyxoviridae (Gould, 1996; Murray, 1995; Wang *et al.,* 1998; Yu *et al.,* 1998a,b). Although HV is serologically cross-reactive with NV in immunofluorescence assays (Anonymous,

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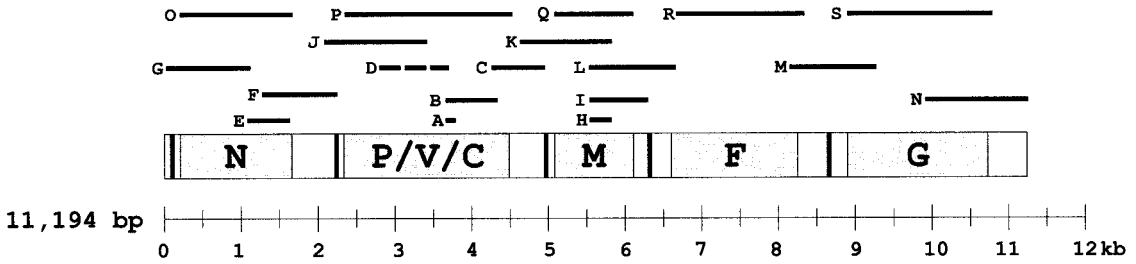


FIG. 1. PCR products used to obtain the sequence of the N, P, M, F, and G genes of NV. Schematic representation of the genome of NV and the location and size of the amplimers used to derive the sequence of the genes, including the nontranslated and intergenic regions. On the schematic genome, the shaded regions represent the predicted ORFs for the N, P, M, F, and G genes, the open (white) areas represent nontranslated regions, and the thick vertical bars represent the intergenic regions. Amplimers E, A, and H were used as starting points for genome walking. Fragment D was generated using the 5′ RACE method. Amplimers G, J, K, L, and M were used to identify the intergenic regions. Amplimers O, P, Q, R, and S are amplimers containing the complete ORFs of the N, P, M, F, and G genes.

1999a), it is not serologically cross-reactive with other members of the Paramyxoviridae (Murray, 1995).

The family Paramyxoviridae contains two subfamilies: the Paramyxovirinae and the Pneumovirinae. The subfamily Paramyxovirinae contains three genera: rubulaviruses, respiroviruses, and morbilliviruses (Bellini *et al.,* 1998; Pringle, 1998), and HV has been proposed to be a representative of a fourth genus within this subfamily (Wang *et al.,* 1998; Yu *et al.,* 1998b). In this study, we compared the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), and glycoprotein (G) genes of NV to HV and to other members of the subfamily Paramyxovirinae. We found that HV and NV form a group distinct from any of the other established genera within the Paramyxovirinae and, therefore, should be considered as members of a new genus.

RESULTS

Cloning and sequencing strategies

Primers specific for the N, P, and M genes of HV were used to amplify parts of the N, P, and M genes of NV (Fig. 1, amplimers E, A, and H, respectively). These three amplimers were then used as starting points to "walk up and down" the genome by using additional reverse transcription–polymerase chain reaction (RT–PCR) primers that were derived from HV sequences in regions of homology shared between most paramyxoviruses (Fig. 1, amplimers G, C, J, K, and M). 5′ Rapid amplification of cDNA ends (RACE) was used to clone a section of the P gene that has low homology to HV and other paramyxoviruses (Fig. 1, fragment D). A primer consisting of 35 deoxythymidines and a gene-specific primer were used to amplify the 3′ ends of the genes (Fig. 1, amplimers F, B, I, and N).

The strategy described above failed when we attempted to obtain the NV F and G gene sequences. To obtain the open reading frames (ORFs) of the F and G genes of NV, a cDNA library was constructed using mRNA derived from NV-infected Vero E6 cells. The cDNA library was screened using a probe consisting of either the F ORF or the G ORF from HV. Plasmid clones that contained the entire F or G ORFs were identified and sequenced.

Once a complete ORF was assembled, RT–PCR primers were constructed to amplify the entire ORF from RNA for additional sequencing (Fig. 1, amplimers O, P, Q, R, and S). All coding regions were sequenced a minimum of 10 times, and the nontranslated and intergenic regions were sequenced a minimum of 6 times. The complete nucleotide sequence of all of the genes reported here have been deposited in GenBank (accession numbers AF212302 for the N, P, and M; AF238466 for F; and AF238467 for G). We also sequenced the N, P, M, F, and G ORFs of HV and found 3 nucleotide (nt) differences in P, 2 nt differences in the M, 3 nt differences in F, and 4 differences in G between our sequence and the previously published sequence for HV (GenBank accession number AF017149).

Intergenic regions and transcriptional start and stop signals

The N, P, M, F, and G genes of NV are flanked by the intergenic sequence, GAA, which is identical to that found in HV, the morbilliviruses, and the respiroviruses (Table 1). The predicted transcriptional initiation signal for the N, P, M, and F genes of NV are identical to those in HV, whereas there is a single nucleotide change in the gene start of the G gene. The gene start signals of HV and NV are more closely related to those of the morbilliviruses and the respiroviruses than to those of the rubulaviruses (Table 1). Except for the M and F genes, which have 2 nt changes in each, the transcriptional termination signals of NV are identical to those of HV (Table 1). The termination signal for the N gene of NV differs by only 1 nt from those of the respiroviruses, Sendai virus, and human parainfluenza virus 3 (HPIV-3), and the termination signal for the P

TABLE 1

Comparison of the Intergenic Sequences and Transcriptional Start and Stop Signals of NV and HV and Other Members of the Subfamily Paramyxovirinae

Genes	Virus	Gene stop	Junction	Gene start
1-N				
	HV		GAA	UCCUUGGUUCU
	NV		GAA	UCCUUGGUUCU
	MV		GAA	UCCUAAGUUCU
	CDV		GAA	UCCCAGUUACU
	Sendai		GAA	UCCCAGUUUC
	HPIV-3		GAA	UCCUAAUUUC
	Mumps			UUCGGUCCUUCACC
N-P				
	HV	AAUUCUUUUU	GAA	UCCUAGGUUCU
	NV	AAUUCUUUUU	GAA	UCCUAGGUUCU
	MV	AAUAUUUUUU	GAA	UCCUUGGUCCA
	CDV	AAUAUUUUUU	GAA	UCCUGGGUCCA
	Sendai	CAUUCUUUUU	GAA	UCCCACUUUC
	HPIV-3	CAUUCUUUUU	GAA	UCCUAAUUGC
	Mumps	UCAGAAAUUCUUUUUU	AA	UCCGGGCCUUUCUU
P-M				
	HV	AAUUCUUUUU	GAA	UCCUCUGUCCA
	NV	AAUUCUUUUU	GAA	UCCUCUGUCCA
	MV	AAUAUUUUUU	GAA	UCCUCGUUUCA
	CDV	AAUAUUUUUU	GAA	UCCUGUUCUCG
	Sendai	AAUUCUUUUU	GAA	UCCCACUUUC
	HPIV-3	AAUUCUUUUU	GAA	UCCUAUUUUC
	Mumps	GGAUAAUUUAUUUUUU	A	UUCGUGCUUGUGUU
M-F				
	HV	AAUUCUUUUU	GAA	UCCUCGGUUC
	NV	AAUGUUUUUU	GAA	UCCUCGGUUC
	MV	UUUGUUUU	GAA	UCCCGGUUCC
	CDV	AAUUAGUUUU	GAA	UCCCAGGUCC
	Sendai	UAUUCUUUUU	GAA	UCCCUAUUUC
	HPIV-3	UUUAGUUUUU	GAA	UCCUAGUUUC
	Mumps	CUUUAAUAUCUUUUUU	A	UUCGGAUCUUCCUA
F-G (H, HN, SH)				
	HV	AAAUGUUUUUU	GAA	UCCUGGGUUCA
	NV	AAUUAUUUUUU	GAA	UCCUGGGUCCA
	MV	UUAAUUUU	GAA	UCCCACGUUCU
	CDV	UAAUUUCUUUU	GAA	UCCCGAGUCCA
	Sendai	UAUUCUUUUU	GAA	UCCCACUUUCA
	HPIV-3	CAUGUUUUUU	GAA	UCCUUGUUUCA
	Mumps	UUUCUAAAUCUUUUUUU	GAUUUA	UUCUUACUUAGAGG
G (H, HN)				
	HV	UAAUUCUUUUU		
	NV	UAAUUCUUUUU		
	MV	AUUCUUUUU		
	CDV	AAUAUUUUUUU		
	Sendai	AAUUCUUUUU		
	HPIV-3	UAAUGUUUUUU		
	Mumps	CUAAUAAUUCUUUUUU		

Note. Genomic sequences are shown. Abbreviations: 1-N, 3′ leader sequence to the N gene; N-P, nucleocapsid gene to the phosphoprotein gene; P-M, phosphoprotein gene to the matrix gene; M-F, matrix gene to the fusion gene; F-G (H, HN, SH) fusion gene to the glycoprotein (G) of NV and HV, the hemagglutinin (H) gene of MV and CDV, the hemagglutinin-neuraminidase (HN) gene of Sendai and HPIV-3, or the short-hydrophobic (SH) gene of mumps virus; and the G (H, HN) transcriptional stop for the G, H or HN gene. The sequences for MV, Sendai, and mumps were obtained from Bellini *et al.* (1998). The accession numbers for HV, CDV, and HPIV-3 are AF017149, AF014953, and D84095, respectively.

gene of NV is identical to those found in the respiroviruses (Table 1). Thus the NV termination signals of N, P, and M are more closely related to the respiroviruses than to the morbilliviruses. However, the gene termination signals of the F and G genes and the gene start signals of HV and NV for all of the genes described

TABLE 2

Comparison of the Sequences of the N, P/C/V, M, F, and G Genes of HV and NV

Gene	Virus	ORF			5′ Nontranslated		3′ Nontranslated	
		Length[a]	% Amino acid identity[b]	% Nucleotide homology[b]	Length[c]	% Homology[b]	Length[c]	% Homology[b]
N	Hendra	532	92.1	78.4	57	66.7	568	41.1
	Nipah	532			57		586	
P	Hendra	707	67.6	70.5	105	41.9	469	40.9
	Nipah	709			105		469	
V	Hendra	55	81.1	88.5				
	Nipah	52						
C	Hendra	166	83.2	85.0				
	Nipah	166						
M	Hendra	352	89.0	77.1	100	40.0	200	40.0
	Nipah	352			100		200	
F	Hendra	546	88.1	74.2	272	44.1	418	41.4
	Nipah	546			284		412	
G	Hendra	604	83.3	70.8	233	43.8	516	45.6
	Nipah	602			233		504	

[a] Length in amino acids.

[b] Percent identity or homology after sequences were aligned using GAP from GCG.

[c] Length in nucleotides. HV gene lengths and 5′ and 3′ nontranslated sequences and gene lengths were obtained from GenBank accession number AF017149. Primers derived from the previously published genomic sequence were used to amplify and sequence the N, P, M, F, and G ORFs of HV and that sequence is used in comparison shown in the table. The percent amino acid identity does not take into consideration conserved amino acid changes.

here appear to be equidistant from the morbilliviruses and respiroviruses.

The nucleoprotein gene

The mRNA for the N gene of NV is predicted to be 2242 nt long, and two ORFs of substantial size were found. The largest ORF is 1599 nt in length and is predicted to encode a protein of 532 amino acids (calculated M_r of 57993), which is identical to the size of the N gene of HV (Yu *et al.,* 1998b) (Table 2). The larger ORF begins with the first available AUG codon from the 5′ end of the mRNA. The sequences flanking the AUG codon are not in a favorable translational initiation context (Kozak, 1991); however, based on homology to HV and the lack of another in-frame AUG codon within 207 nt, it is predicted that this AUG codon is used for translation initiation of the N protein. A second ORF begins 1483 nt downstream from the start site of the N protein and potentially encodes a 72-amino-acid protein that is not found in HV and has no significant homologies with any known protein. The initiating AUG for this potential ORF is not in a favorable translational initiation context.

The homology between the coding sequences for the N proteins of NV and HV is 78.4%; however, the level of nucleotide homology within the 5′ and 3′ nontranslated regions of the N gene mRNA of NV and HV decreases to 66.7% and 41.1%, respectively (Table 2). Overall, the N proteins of NV and HV are quite conserved (Table 2), but the amino acid changes are not distributed evenly across the protein (Fig. 2). The identity of the first 400 amino acids of the proteins is 98% between HV and NV, with 6 of the 8 substitutions being conservative. However, 22 of the 42 amino acid differences between the N proteins of NV and HV are located in a 28-amino-acid region in the carboxyl-terminal portion of the protein, and only 5 of these are conservative.

The P/V/C gene

The mRNA for the P/V/C gene is predicted to be 2704 nt long with an ORF of 2130 nt that is predicted to encode P (Table 2). As observed for the N gene, the sequence coding for the P of NV has higher levels of homology to HV than the sequences of the 5′ and 3′ nontranslated regions (Table 2). Translation of NV P is predicted to begin at the first AUG available in the reading frame, and the predicted P of NV is 2 amino acids longer than the P protein of HV (Wang *et al.,* 1998). Overall, the NV and HV P proteins share 67.6% identity, whereas the amino-terminal 137 amino acids and the carboxyl-terminal 321 amino acids have identities of approximately 80% (Fig. 3A). However, the region of the NV P protein between amino acids 138–389 has only 43% identity to HV, and only 17% of these substitutions are conservative.

Like the P gene of HV, the respiroviruses, and the morbilliviruses (Bellini, 1998), a second ORF encoding the C protein of NV begins with consecutive AUG codons

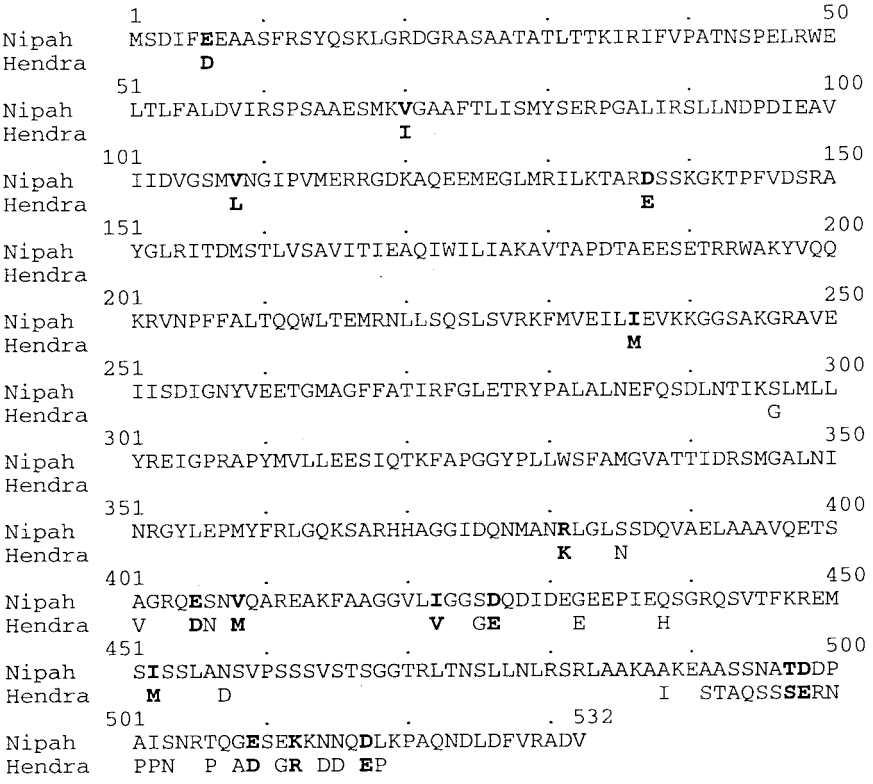


FIG. 2. Comparison of the N proteins of NV and HV. The alignment shows the predicted amino acid sequence of the N protein of NV and indicates positions that differ in the N proteins of NV and HV. Conservative amino acid changes are shown in bold. Numbers correspond to amino acid positions in the NV N protein; dots mark lengths of 10 amino acids.

located 23 and 26 nt downstream of the P protein initiation codon. It has not been established which AUG codon is the actual initiator of translation. If translation is initiated at the first AUG codon, the predicted C protein of NV is 166 amino acids in length and has an M_r of 19735. It is identical in size to the C protein of HV and has a homology of 85.0% at the nucleotide level and an identity of 83.2% at the amino acid level (Table 2). Except for the region between amino acids 31 and 45, which contains 11 nonconserved amino acid substitutions, the C proteins of NV and HV differ at 17 amino acid positions, and 7 of these are conservative (Fig. 3B). HV contains another ORF in the same frame as C that putatively encodes a short basic protein (SB) of 65 amino acids (Wang *et al.,* 1998), but this ORF is not found in the NV P gene.

Like most members of the subfamily Paramyxovirinae (Bellini, 1998), the P gene of NV contains a conserved AG-rich region (edit site) in which the viral polymerase adds nontemplated Gs to the mRNA (Paterson and Lamb, 1990). The identification of plasmid clones of NV P containing 0, 1, or 2 G insertions at this site indicated that RNA editing occurs in NV. The RNA editing site of NV is identical to the editing site found in both HV and in some of the morbilliviruses (Table 3). As in HV and the morbilliviruses, the unedited message is predicted to code for P. The addition of a single nontemplated G allows access to a cysteine-rich reading frame, producing a predicted V protein containing the amino-terminal part of P and 52 amino acids unique to the V reading frame. The predicted V protein of NV is 3 amino acids shorter than the V in HV (Wang *et al.,* 1998) and has an identity of 81.1% with the HV V (Table 2). All of the cysteine residues except one are conserved between NV and HV (Fig. 3C). The addition of two nontemplated G residues to the P mRNA allows access to a 47-amino-acid ORF and potentially encodes a protein that would be analogous to the W protein that has been described for Sendai virus (Steward *et al.,* 1993; Vidal *et al.,* 1990). This potential ORF is also conserved in HV and is predicted to be of the same size as NV.

The matrix gene

The mRNA for the M gene is predicted to be 1359 nt in length, with an ORF of 1059 nt encoding the predicted M protein (Table 2). HV and NV have a homology of 77.1% in the ORF, but the 3′ and 5′ noncoding regions have an homology of only 40% (Table 2). Based on homology to HV, the first available AUG codon is predicted to initiate translation, and this codon is in a favorable context for translation. There is another in-frame initiation codon 36 nt downstream from the first

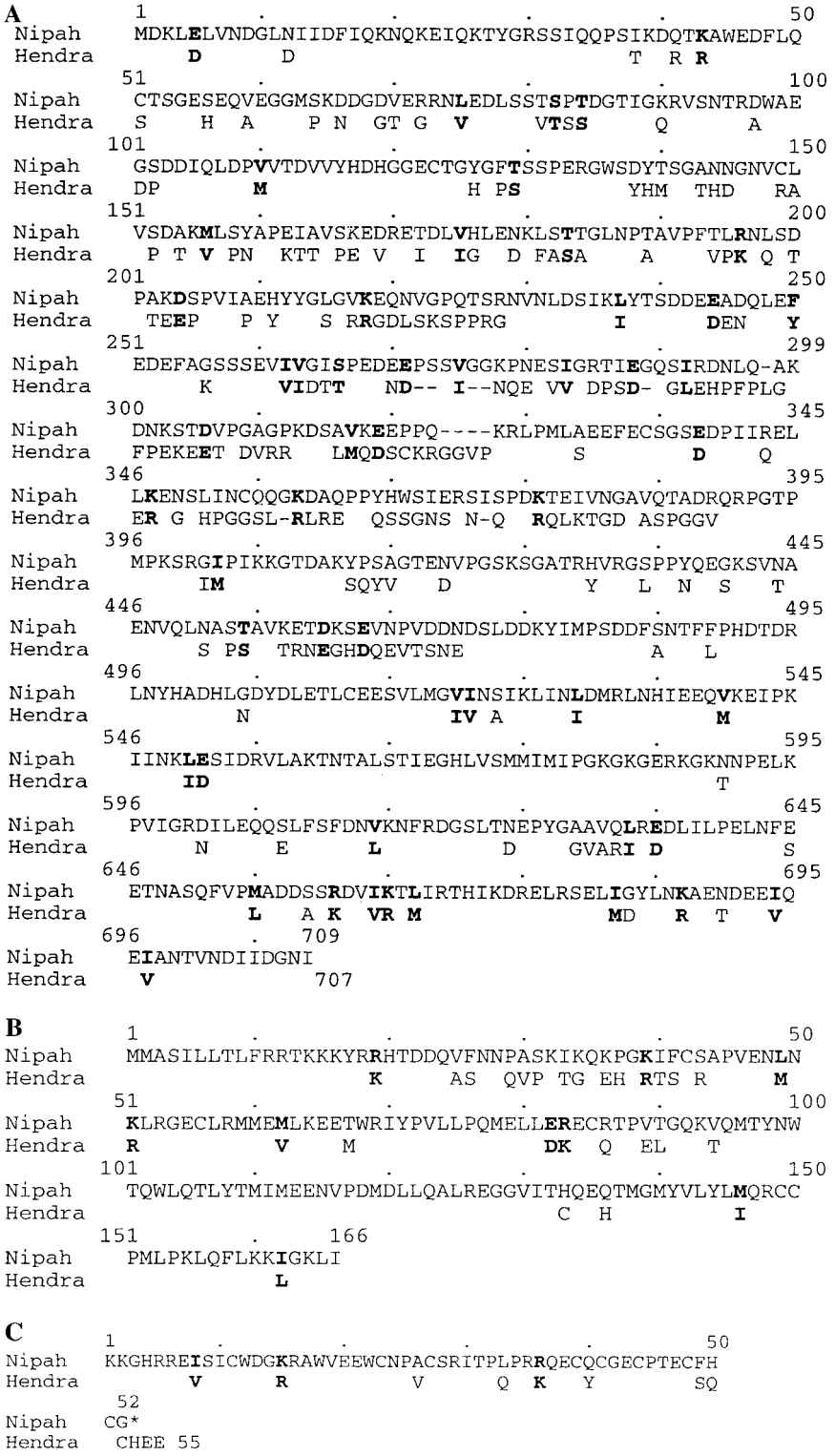


FIG. 3. Comparison of the P, V, and C proteins of NV and HV. Alignments show the predicted amino acid sequence of the NV P (panel A), C (panel B), and V (panel C) proteins and the amino acids that differ in the P, C, and V proteins of HV. Numbers correspond to amino acid positions in the NV P, C, or V protein; dots mark lengths of 10 amino acids. Gaps (indicated by dashes) were made in the alignment of the P to maximize the regions of homology. Conservative amino acid changes are shown in bold. Asterisk indicates the COOH terminus of the protein. During sequencing of the P gene of HV, we found that an additional A nucleotide was inserted at position 1526 of the published P sequence and a T nucleotide was deleted at position 1559 (Wang *et al.,* 1998). These nucleotide changes result in 12 amino acid changes.

TABLE 3

Comparison of the RNA Editing Sites of HV and NV with Other Members of the Subfamily Paramyxovirinae

Virus	Editing site		Insertions	Protein
HV	GGGUAAUUUUUCCC	GUGUC	+1G	V
NV	GGGUAAUUUUUCCC	GUGUC	+1G	V?
Genus *Morbillivirus*				
MV	GGGUAAUUUUUCCC	GUGUC	+1G	V
PDV	GGGUAAUUUUUCCC	GUGUC	+1G	V
CDV	AGGUAAUUUUUCCC	GUGUC	+1G	V
Genus *Respirovirus*				
HPIV-3	CUUAAUUUUUUCCC	CCUUU	+1G	V
Sendai	AGUUGUUUUUUCCC	GUAUC	+1G	V
Genus *Rubulavirus*				
NDV	UUACGAUUUUUCCC	GGGUA	+1G	V
HPIV-2	GUUGAAAUUCUCCC	CCCCU	+2G	P
SV5	GCUAAAAUUCUCCC	CGUCC	+2G	P
HPIV-4	CUAUAAAUUCUCCC	CCCUU	+2G	P
Mumps	UCUUAAAUUCUCCC	CCCGG	+2G	P

Note. Genomic sequences (3′ to 5′) are shown. The nontemplated G nucleotide or nucleotides are added to the mRNA either within or after the run of genomic C residues. It has recently been demonstrated that in Sendai virus, the G residue is added after the second C residue (Hausmann *et al.,* 1999). Also shown are the G insertions required to produce the V protein for HV, NV, the morbilliviruses, and the respiroviruses and the P protein for the rubulaviruses. Abbreviations: Newcastle disease virus (NDV), phocine distemper virus (PDV), and Simian virus 5 (SV5). The editing sites for MV, HPIV-3, Sendai, NDV, HPIV-2, SV5, HPIV-4, and mumps were obtained from Collins *et al.* (1996). The sequence of the editing sites for HV and CDV were obtained from sources described previously. PDV sequence was obtained from GenBank accession number D10371.

AUG that could be used to initiate translation, but it is in a less favorable context for translation. The M ORF is predicted to encode a 352-amino-acid protein with an M_r of 39928. Of the 39 predicted amino acid changes between the M proteins of NV and HV, 12 occur within the first 13 amino acids and 16 of these 39 substitutions are conservative (Fig. 4).

The fusion gene

The mRNA for the F gene is predicted to be 2337 nt in length, with an ORF of 1641 nt encoding the F protein (Table 2). HV and NV have an homology of 74.2% in the ORF, but the 3′ and 5′ noncoding regions have homologies of only 41.4% and 44.1%, respectively (Table 2). There are two in-frame AUG codons available for initiation within the first 22 amino acids; however, neither of them is in an optimal context (Kozak, 1991). If the first AUG is used to initiate translation, the ORF is predicted to encode a 546-amino-acid protein that is the same size as the HV F (Gould, 1996) and the NV F protein would have a predicted M_r of 60233.

Like the F proteins of other Paramyxoviridae (Lamb and Kolakofsky, 1996), the NV F protein is predicted to be a type I transmembrane protein with a membrane spanning domain located at amino acids 489–518 (Fig. 5) and a short cytoplasmic region of approximately 30 amino acids. The first 28 amino acids of the NV F protein have only 6 amino acids in common with the same region of the HV F (13 if conservative amino acid changes are allowed). Despite these amino acid substitutions, this region is hydrophobic in both viruses and is predicted to

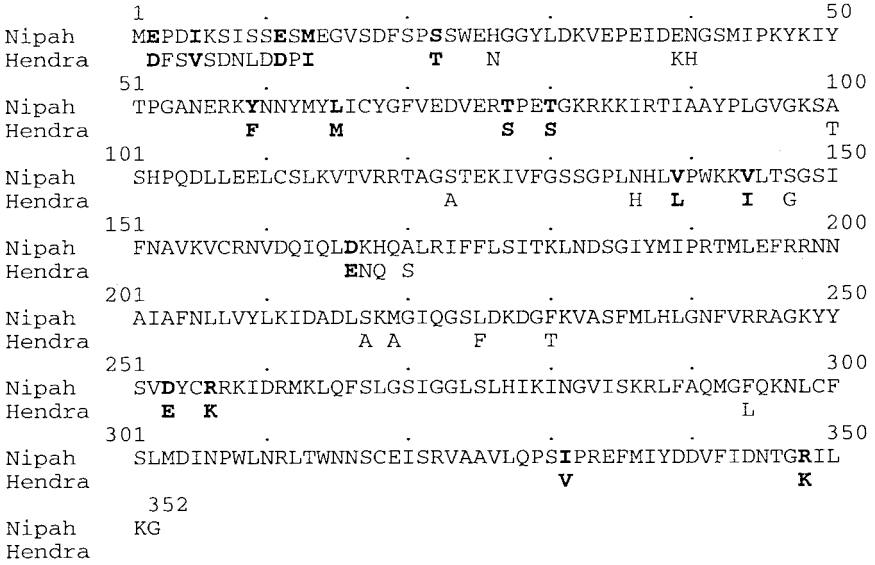


FIG. 4. Comparison of the M proteins of NV and HV. The predicted amino acid sequence of the NV M open reading frame is shown along with the amino acids that differ in the M protein of HV. Conservative amino acid changes are shown in bold. Numbers correspond to amino acid positions in the NV M protein; dots mark lengths of 10 amino acids.

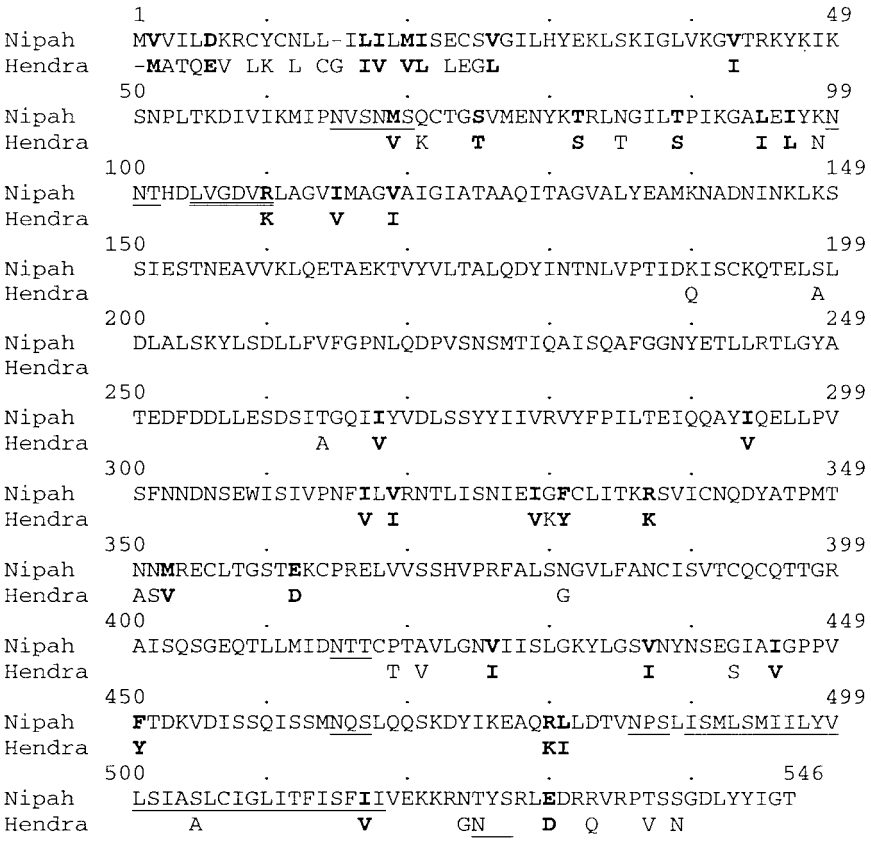


FIG. 5. Comparison of the F proteins of NV and HV. The alignment shows the predicted amino acid sequence of the F protein of NV and indicates positions that differ in the F proteins of NV and HV. Conservative amino acid changes are shown in bold. Numbers correspond to amino acid positions in the NV F protein; dots mark lengths of 10 amino acids. Double underline indicates the position of the F_1–F_2 cleavage site. Single underlined triplets indicate potential N-linked glycosylation sites. Longer single underlined region at the COOH-terminus of the F protein indicates the membrane anchor sequence.

be the signal sequence. If the second AUG were used to initiate translation in NV, this signal sequence would not be present in the protein.

Paramyxoviridae F proteins are synthesized as an inactive precursor (F_0) that is cleaved by host cell proteases to release the new amino-terminus of F_1, thus forming the biologically active protein consisting of the F_1 peptide disulfide-linked to the F_2 peptide (Lamb and Kolakofsky, 1996). The predicted cleavage site for NV is shown in Table 4. A lysine-to-arginine change is the only difference between HV and NV in the cleavage site (Table 4). The amino-terminal 20 amino acids of the F_1 peptide of NV are hydrophobic and share extensive homology with the rubulaviruses, morbilliviruses, and respiroviruses (Table 4). Interestingly, the amino-terminal amino acid of the F_1 peptide of both HV and NV is a leucine, whereas in every other member of the Paramyxoviridae, except for some avirulent strains of NDV, it is a phenylalanine.

Apart from the amino-terminal 28 amino acids, the F proteins of HV and NV have only 19 nonconservative and 27 conservative amino acid changes throughout the rest of the molecule. The 200 amino acids that comprise the amino-terminal portion of the F_1 proteins share more than 96% homology. The F protein of NV is predicted to have six, N-linked glycosylation sites, with three in the F_1 peptide and three in the F_2 peptide. These sites are also conserved in HV, but HV also has one additional site in the cytoplasmic domain.

The glycoprotein (G) gene and antigenic analysis

The gene coding for the putative attachment glycoprotein of NV virus is 2543 nt in length and contains an ORF encoding a protein of 602 amino acids (M_r = 67038) that has 83.3% identity to the G protein of HV. The predicted G protein of NV is two amino acids smaller than the G protein of HV (Table 2). Only two of the three in-frame initiation codons at the start of the reading frame for the G protein of HV are conserved in the G protein of NV (Yu *et al.,* 1998a). Because the amino-termini of these glycoproteins have not yet been sequenced, it is not known whether the predicted 50% amino acid variation observed over the first 30 amino

TABLE 4

Comparison of the Fusion Protein Cleavage Sequences of HV and NV with Those of the Paramyxoviridae

Virus	Cleavage peptide[a]	NH_2 terminus of F1[a]
Hendra	L-D-V-G-V-K	L-A-G-V-V-M-A-G-I-A-I-G-I-A-T-A-A-Q-I-T
Nipah	L-D-V-G-V-R	L-A-G-V-I-M-A-G-V-A-I-G-I-A-T-A-A-Q-I-T
Respiroviruses		
HPIV-1	D-N-P-Q-S-R	F-F-G-A-V-I-G-T-I-A-L-G-V-A-T-A-A-Q-I-T
HPIV-3	D-P-R-T-K-R	F-F-G-G-V-I-G-T-I-A-L-G-V-A-T-S-A-Q-I-T
Sendai	G-V-P-Q-S-R	F-F-G-A-V-I-G-T-I-A-L-G-V-A-T-S-A-Q-I-T
Morbilliviruses		
Measles	S-R-R-H-K-R	F-A-G-V-V-L-A-G-A-A-L-G-V-A-T-A-A-Q-I-T
CDV	S-R-R-K-K-R	F-A-G-V-V-L-A-G-A-A-L-G-V-A-T-A-A-Q-I-T
Rinderpest	S-R-R-H-K-R	F-A-G-V-A-L-A-G-A-A-L-G-V-A-T-A-A-Q-I-T
Rubulaviruses		
Mumps	S-R-R-H-K-R	F-A-G-I-A-I-G-I-A-A-L-G-V-A-T-A-A-Q-V-T
SV5	T-R-R-R-R-R	F-A-G-V-V-I-G-L-A-A-L-G-V-A-T-A-A-Q-V-T
NDV	G-R-R-Q-K-R	F-I-G-A-V-I-G-S-V-A-L-G-V-A-T-A-A-Q-I-T
NDV (avirulent)	G-G-R-Q-G-R	L-I-G-A-I-I-G-G-V-A-L-G-V-A-T-A-A-Q-V-T
Pneumoviruses		
RSV	K-K-R-K-R-R	F-L-G-F-L-L-G-V-G-S-A-I-A-S-G-V-A-V-S-K
BRSV	K-K-R-K-R-R	F-L-G-F-L-L-G-I-G-S-A-I-A-S-G-V-A-G-S-K
PVM	K-R-K-K-R	F-L-G-L-I-L-G-L-G-A-A-V-T-A-G-V-A-L-A-K

[a] Sequences from Bellini *et al.* (1998) except for sequence for NDV (accession number AF048763) and avirulent NDV (Glickman *et al.,* 1988).

acids for these proteins is expressed (Fig. 6). Although the lengths of the 5′ nontranslated regions of the mRNA are the same for NV and HV, the 3′ untranslated region of the HV mRNA is 12 nt longer (Table 2).

Overall, there are 128 predicted amino acid substitutions between the G proteins of NV and HV. However, all 17 cysteines, all 8 N-linked glycosylation sites, and 31 of 36 proline residues in NV are conserved. The G proteins of HV and NV are predicted to be type II membrane glycoproteins. There are only two amino acid substitutions in the predicted hydrophobic transmembrane domains of the G proteins of HV and NV and only one of these is nonconservative (Fig. 6). In both G proteins, the first 100 amino acids of the extracellular domain are relatively conserved with approximately 90% homology, whereas the level of homology for the remainder of the extracellular domain decreases to approximately 75%.

Neutralizing antibodies against the Paramyxoviridae are directed against the surface glycoproteins. Although the G and F proteins of HV and NV have over 80% amino acid homology, neutralization tests using hyperimmune mouse ascitic fluids to HV or NV detected significant antigenic differences (Table 5) between these viruses. There was an 8- to 16-fold difference in neutralization titers between the homologous and heterologous ascitic fluids.

Phylogenetic analyses

Phylogenetic analyses of the nucleotide sequences of the N, P, C, M, F, and G ORFs from representatives of the subfamily Paramyxovirinae showed that HV and NV consistently formed a unique cluster that was more closely related to the morbilliviruses and the respiroviruses than to the rubulaviruses (Fig. 7). Bootstrap confidence levels of 100% for the N, P, C, F, and G sequences supported these conclusions. The recently described Tupaia paramyxovirus (Tidona *et al.,* 1999) was also not included within any of the known genera but was more closely related to HV and NV than to any of the other viruses (Figs. 7A–7C). These observations suggested that HV and NV should be considered as a fourth new genus within the Paramyxovirinae.

DISCUSSION

In this report, we describe the molecular characterization of the N, P, M, F, and G genes of NV, a newly described and highly pathogenic member of the Paramyxoviridae. Sequence analysis indicated that NV is more closely related to HV than to any other member of the Paramyxoviridae. Although the viruses are close relatives, they are clearly different viruses. The levels of nucleotide sequence homology between the N, P, M, F, and G ORFs of NV and HV are similar to the levels of homology observed between related viruses within the other genera of the subfamily Paramyxovirinae. These two viruses form a group that is distinct from any of the established genera and should be considered as a new genus. Until the isolation of NV, HV had been the sole member of this proposed new genus.

It is difficult to discern from the sequence data whether HV and NV are more closely related to the

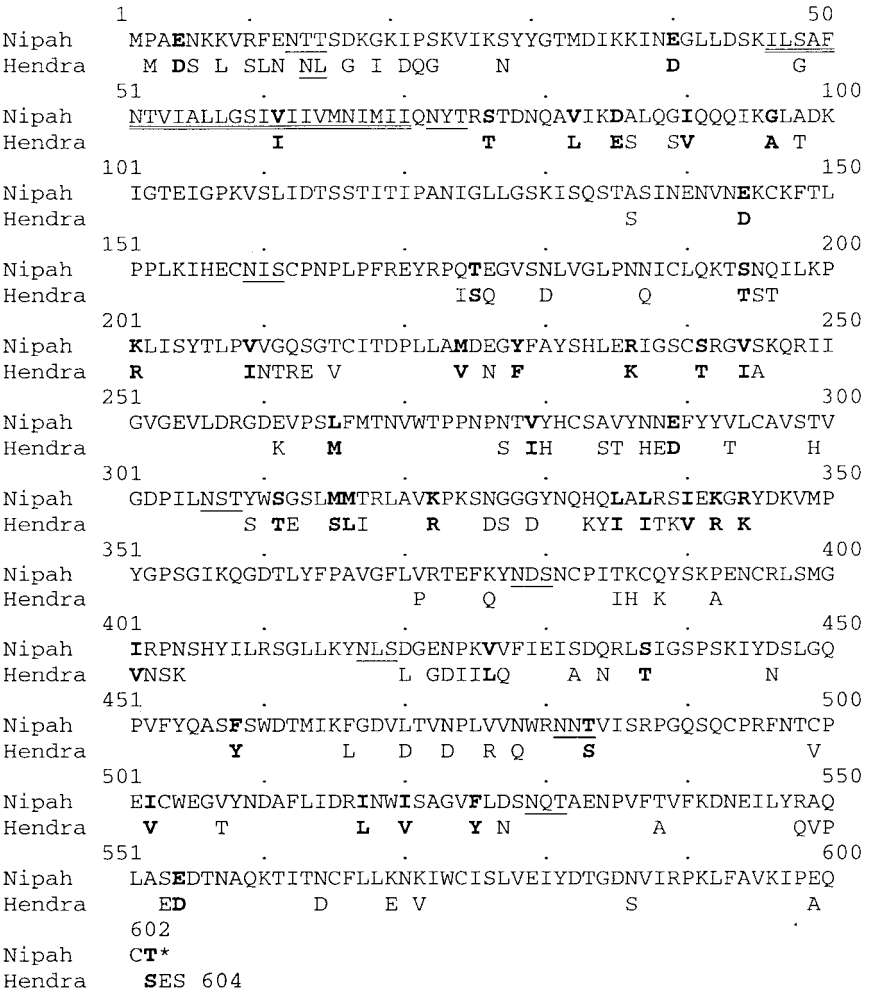


FIG. 6. Comparison of the G proteins of NV and HV. The alignment shows the predicted amino acid sequence of the G protein of NV and indicates positions that differ in the G proteins of NV and HV. Conservative amino acid changes are shown in bold. Numbers correspond to amino acid positions in the NV G protein; dots mark lengths of 10 amino acids. Double underlining indicates the position the membrane anchor domain. Single underlined triplets indicate potential N-linked glycosylation sites. Asterisk indicates the COOH-terminus of the protein.

respiroviruses or to the morbilliviruses. In fact, HV and NV contain genetic characteristics from both of these genera. For example, the RNA editing sites of HV and NV are identical to those of many morbilliviruses, whereas the transcriptional termination signals are more closely related to those of the respiroviruses. The intergenic sequences from HV and NV are identical to those in both the morbilliviruses and respiroviruses, and the transcription initiation signals of HV and NV are closely related to both of these two genera. It is interesting to note that the transcriptional initiation signals of HV, NV, the respiroviruses, and the morbilliviruses are identical at positions 1, 2, 3, and 10, and each has a pyrimidine residue at position 4.

TABLE 5

Cross-Neutralization of HV and NV

	Neutralization[a] titer versus	
Antibody	Hendra	Nipah
Hendra-HMAF[b]	640	80
Nipah-HMAF[b]	20	320

[a] Determined by plaque neutralization as described in Materials and Methods.

[b] Hyperimmune mouse ascites fluid was prepared as described in Materials and Methods.

Like the morbilliviruses, the respiroviruses, and HV, NV is predicted to encode multiple proteins from the P gene (Bellini, 1998; Griffin and Bellini, 1996; Patterson, *et al.,* 1989; Thomas *et al.,* 1988; Wang *et al.,* 1998). The C and V proteins are predicted to be made by NV and have been identified in HV-infected cells (Wang *et al.,* 1998). The cysteine-rich region of the V protein forms a zinc finger domain, and the V protein of MV has been shown to bind zinc (Liston and Briedis, 1994). The NV V would

be predicted to have a similar domain because all of the cysteine residues that are conserved in the V proteins of the morbilliviruses and the respiroviruses are conserved in NV (Baron *et al.,* 1993). In addition, only one cysteine residue is not conserved between NV and HV, and this cysteine is not found in any of the other paramyxoviruses. Insertion of 2 Gs at the editing site of both NV and HV is predicted to produce a protein that would be analogous to the W proteins that have been described for other Paramyxovirinae (Delenda *et al.,* 1998; Steward *et al.,* 1993; Vidal, *et al.,* 1990).

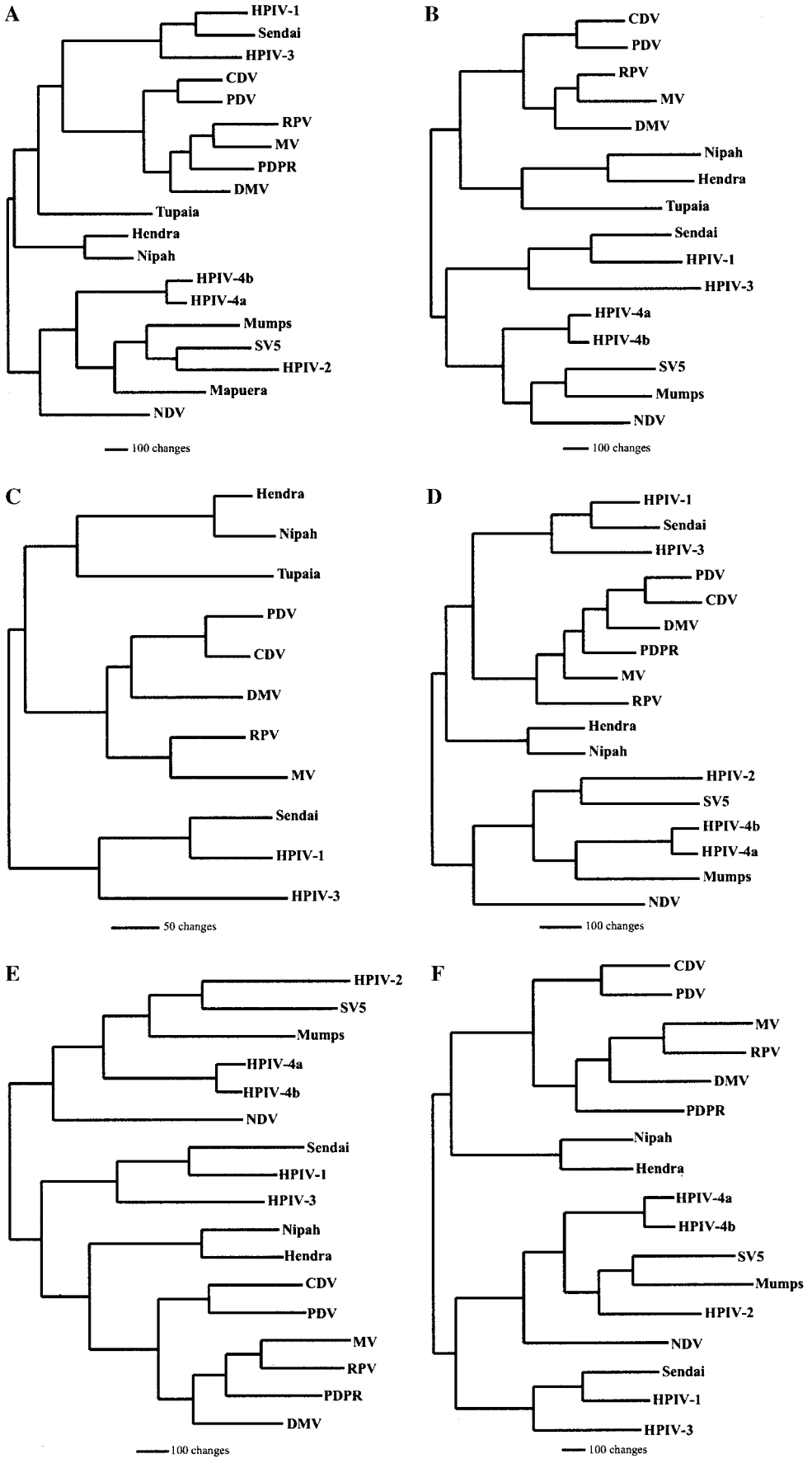
The fusion cleavage site of the morbilliviruses, rubulaviruses, and pneumoviruses are highly basic and contain the R-X-R/K-R consensus sequence that is required for cleavage by furin (Hosaka, 1991). However, neither HV nor NV contains this sequence in their respective cleavage sites, so these viruses must use other host cell proteases. The respiroviruses also do not have furin cleavage sites and must have exogenous proteases added to grow in tissue culture (Scheid and Choppin, 1974). Unlike Sendai, neither HV nor NV requires the addition of exogenous proteases for growth in tissue culture, so the proteases responsible for HV and NV F_0 cleavage must differ from those used by the respiroviruses. In addition, the lysine-to-arginine change at position six of the cleavage site in NV may affect the range of proteases able to cleave the protein and/or the efficiency of cleavage.

The amino-terminal 20 amino acids of the F_1 peptide are highly conserved between HV and NV and the other Paramyxovirinae. This region is thought to initiate the fusion process by intercalating into membranes of host cells (Lamb and Kolakofsky, 1996). Except for some avirulent strains of NDV, HV and NV are unique within the Paramyxoviridae in having a leucine at the amino-terminus of the F_1 peptide rather than a phenylalanine. The biological consequences of this amino acid substitution in HV and NV are unknown. For the avirulent strain of NDV, amino acid substitutions that destroy the furin recognition sequence may also contribute to the loss of virulence.

The F proteins of the Paramyxoviridae contain heptad repeat regions immediately after the 20-amino-acid hydrophobic fusion region and adjacent to the transmembrane region (Buchholz *et al.,* 1993; Chambers *et al.,* 1990). The heptad repeats are composed of leucine/isoleucine zipper domains that can form a triple-stranded coil composed of three α-helices (O'Shea *et al.,* 1989; Harbury *et al.,* 1994). In the regions of the F protein that would be expected to contain the heptad repeats, both HV and NV share a high level of amino acid homology. In addition, all cysteine residues outside of the signal sequence are conserved between HV and NV and the other Paramyxovirinae, suggesting that the HV and NV F proteins are structurally similar to the F proteins of other Paramyxovirinae.

HV is unique among the Paramyxovirinae in having an attachment glycoprotein lacking detectable erythrocyte binding and neuraminidase activities (Yu *et al.,* 1998a). The sequence of the G protein of HV was recently compared with the structural alignment of the globular head regions of the attachment proteins of the Paramyxovirinae (Langedijk *et al.,* 1997; Yu *et al.,* 1998a). Whereas the sequence of the HV G protein had only minimal sequence homology with the attachment proteins of other members of the Paramyxovirinae, several important structural features were conserved. The locations of all seven of the proposed disulfide bonds between HV and the respiroviruses are conserved, and five of these bonds are also shared between HV and the rubulaviruses and the morbilliviruses. There also is conservation of structurally important amino acids, but the amino acids residues that are necessary for neuraminidase activity are not conserved in HV (Yu *et al.,* 1998a). Most of these important amino acid residues are conserved in the G proteins of both HV and NV, including the eight residues that are highly conserved among the attach-

FIG. 7. Phylogenetic analyses of the N, P, C, M, F, and G ORFs from members of the Paramyxovirinae subfamily. Phylogenetic analysis using PAUP 4.02 was performed on viral sequences from the following genes: N (panel A), P (panel B), C (panel C), M (panel D), F (panel E), and G (panel F). The trees shown were based on maximum parsimony; however, analysis of the same data using maximum likelihood gave TREES with nearly identical topologies (data not shown). DMV, dolphin morbillivirus; PDPR, peste-des-petits-ruminants virus; and RPV, rinderpest virus. Scale representing the number of nucleotide changes is shown for each phenogram. accession numbers used: (panel A) CDV, AF014953; DMV, X75961; HPIV-1, D01070; HPIV-2, M55320; HPIV-3, D10025; Mapuera, X85128; Mumps, D86172; MV, K01711; NDV, AF064091; PDPR, X74443; PDV, X75717; RPV, X68311; Sendai, X00087; SV5, M81442; and Tupaia, AF079780; (panel B) CDV, X51869; DMV, Z47758; HPIV-1, M74081; HPIV-3, X04721; HPIV-4a, M55975; HPIV-4b, M55976; Mumps, D86173; MV, M89920; NDV, M20302; PDV, X75960; RPV, X68311; Sendai, M30202; SV5, AF052755; and Tupaia, AF079780; (panel C) CDV, AF014953; DMV, Z47758; HPIV-1, M74081; HPIV-3, D00047; MV, AB016162; RPV, X68311; Sendai, AB005796; and Tupaia, AF079780; (panel D) CDV, M12669; DMV, Z30087; HPIV-1, S38067; HPIV-2, M62734; HPIV-3, D00130; HPIV-4a, D10241; HPIV-4b, D10242; Mumps, D86171; MV, AB012948; NDV, AF089819; PDPR, Z47977; PDV, X75717; RPV, M34018; Sendai, U31956; and SV5, M32248; (panel E) CDV, M21849; DMV, AJ224704; HPIV-1, M22347; HPIV-2, M60182; HPIV-3, X05303, HPIV-4a, D49821; HPIV-4b, D49822; Mumps, D86169; MV, AB003178, NDV, AF048763; PDPR, Z37017; PDV, AJ224706; RPV, M21514; Sendai, D17334; SV5AB021962; (panel F) CDV, AF112189; DMV, AJ224705; HPIV-1, U709498; HPIV-2, D000865; HPIV-3, AB012132; HPIV-4A, M34033; HPIV-4B, AB006954; Mumps, X99040; MV, K01711; NDV, AF204872; PDPR, Z81358; PDV, Z36979; RPV, AF132934; Sendai, U06433; SV-5, S76876.


A
HPIV-1
Sendai
HPIV-3
CDV
PDV
RPV
MV
PDPR
DMV
Tupaia
Hendra
Nipah
HPIV-4b
HPIV-4a
Mumps
SV5
HPIV-2
Mapuera
NDV
100 changes
B
CDV
PDV
RPV
MV
DMV
Nipah
Hendra
Tupaia
Sendai
HPIV-1
HPIV-3
HPIV-4a
HPIV-4b
SV5
Mumps
NDV
100 changes
C
Hendra
Nipah
Tupaia
PDV
CDV
DMV
RPV
MV
Sendai
HPIV-1
HPIV-3
50 changes
D
HPIV-1
Sendai
HPIV-3
PDV
CDV
DMV
PDPR
MV
RPV
Hendra
Nipah
HPIV-2
SV5
HPIV-4b
HPIV-4a
Mumps
NDV
100 changes
E
HPIV-2
SV5
Mumps
HPIV-4a
HPIV-4b
NDV
Sendai
HPIV-1
HPIV-3
Nipah
Hendra
CDV
PDV
MV
RPV
PDPR
DMV
100 changes
F
CDV
PDV
MV
RPV
DMV
PDPR
Nipah
Hendra
HPIV-4a
HPIV-4b
SV5
Mumps
HPIV-2
NDV
Sendai
HPIV-1
HPIV-3
100 changes

ment proteins of viruses in the subfamily Paramyxovirinae (Yu *et al.,* 1998a).

Despite the conservation of important structural features of the G and F proteins of HV and NV, amino acid substitutions in the extracellular domains of these proteins lead to significant antigenic differences in neutralization assays. HV and NV do not react with antiserum to any of the other paramyxoviruses (Chua *et al.,* 1999, 2000) but do share cross-reactive epitopes with each other. Enzyme immunoassays that were developed to support the investigation of the outbreak of NV in Malaysia initially used HV as the source of antigen (Anonymous, 1999a).

As described above, HV and NV share characteristics with other members of the Paramyxovirinae, such as gene order, conserved intergenic, transcriptional initiation, and transcriptional termination sequences, a conserved mRNA editing site, the presence of the C and V ORFs in the P gene, and the overall structure of the surface glycoproteins required for attachment and membrane fusion. The amino acid heterogeneity in the carboxyl-terminal portion of the N protein and the central region of the P protein along with the relative conservation of the M protein have been described for other members of the Paramyxovirinae (Baczko *et al.,* 1992; Taylor *et al.,* 1991). However, HV and NV have several characteristics that clearly separate them from the established genera. Members of the Paramyxoviridae typically have genomes that are approximately 15,500 nt in length (Bellini *et al.,* 1998), whereas the HV genome is composed of 18,234 nt (GenBank accession number AF017149). Presumably, the NV genome will be of a similar length based on the degree of homology observed for the first five genes of these two viruses. With the exception of the M gene, the genes of HV and NV have 3′ nontranslated regions that are much longer than those found in any other member of the subfamily Paramyxovirinae and would partially explain the longer length of the HV and NV genomes. In the order Mononegavirales, the only other viruses that contain such large 3′ nontranslated regions are the filoviruses (Feldmann *et al.,* 1992; Sanchez *et al.,* 1993). It is interesting to note that although the sizes of the 3′ nontranslated regions are conserved between NV and HV, the level of sequence homology is quite low. It is possible that despite the sequence differences, RNA secondary structures are conserved that could be important in mRNA stability and/or "translatability." In addition, both HV and NV have P proteins that are more than 100 amino acids larger than any other P protein found in other members of the family. Finally, the attachment protein of HV, and presumably that of NV, is unique among the Paramyxovirinae in having no detectable neuraminidase or erythrocyte binding activities.

In addition to the unique features of the genomes of HV and NV described above, both HV and NV are unique among the Paramyxovirinae in that they have unusually broad host ranges. HV has been shown to infect horses, humans, cats, rabbits, fruit bats (Wang *et al.,* 1998; Westbury, 1995), and a wide array of cell lines (Murray, 1995). HV is pathogenic in humans, horses, cats, and guinea pigs (Hooper, 1997; Westbury *et al.,* 1996). During the NV outbreak in Malaysia, humans, pigs, cats, and dogs were infected and had disease. NV N gene sequences obtained from tissue samples from humans, pigs, cats, and dogs were identical to each other and to the sequence obtained from viral isolates (Anonymous, 1999b; and unpublished observations). In addition, HV and NV contain cross-reactive antigenic domains that are not shared with any other members of the Paramyxovirinae.

In summary, the phylogenetic analysis of the N, P, C, M, F, and G ORFs of HV and NV illustrate that these viruses do not group within any of the established genera of the subfamily Paramyxovirinae. Furthermore, the unique features of the HV and NV genomes, their broad host range, and antigenic cross-reactivity clearly indicate that NV and HV should be considered members of a new genus within the subfamily Paramyxovirinae.

MATERIALS AND METHODS

Virus culture and RNA isolation

The NV strain used in this study was isolated directly from brain tissue in Vero E6 cells. The virus was passaged one time in Vero E6 cells and harvested when the cytopathic effect was maximal. All work with live virus was performed under BSL-4 conditions. RNA was extracted from the infected cells by using the guanidinium acid–phenol technique (Chomczynski and Sacchi, 1987).

Amplification of viral sequences

RT–PCR was performed as previously described (Rota *et al.,* 1994) except that the cycling conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The reaction was then incubated at 72°C for an additional 5 min. PCR products were visualized using agarose gel electrophoresis and were purified by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Figure 1 shows the RT–PCR products used to construct the nucleotide sequence of the NV N, P, M, F, and G genes. The following primers were used for the indicated RT–PCR products (restriction enzyme sites are underlined): (A) 141 bp, 5′-CATTAAAAAGGGCACAGACGC-3′ and 5′-TGGACATTCTCCGCAGT-3′; (B) 726 bp, 5′-CATTAAAAAGGGCACAGACGC-3′ and 5′-TCTGCCAAAGGAACAAATTGTGATG-3′; (C) 827 bp, 5′-TCCTGAGCTTAAACCAGTGAT-3′ and 5′-CCGGTACC(T_{35})-3′ [oligo(dT)]; (D) see 5′ RACE below; (E) 552 bp; 5′-ATCAATCGTGGTTATCTTGAA-3′ and 5′-

GATATCTAGATCACACGTCTGCTCTAAC-3′ (*Xba*I); (F) 1005 bp, 5′-GCTGCAGTTCAGGAAACATCAG-3′ and oligo(dT); (G) 1201 bp, 5′-ACCGAACAAGGGGAAATATGG-3′ (this primer represents the 3′ end of the HV genome) and 5′-GGCCTAGTCTGAAATACATAG-3′; (H) 319 bp, 5′-ACTATCACAAAGATCAATGATTC-3′ and 5′-CACCATTGATCTTAATGTGGAG-3′; (I) 730 bp, 5′-ACTATCACAAAGATCAATGATTC-3′ and oligo(dT); (J) 1356 bp, 5′-AGGTAATGCTCGCACAAGTGA-3′ and 5′-TGCACAGACAGTTGACGATCT-3′; (K) 1295 bp, 5′-TCAGACGATAACACAATACTT-3′ and 5′-CACCATTGATCTTAATGTGGAG-3′; (L) 1094 bp, 5′- ACTATCACAAAGATCAATGATTC-3′ and 5′-CAACACTACACTCCGAGATCA-3′; (M) 5′-CTCTACTACATTGGGACATAG-3′ and 5′-TAGTAATGATCTAGATCTCCA-3′; (N) 1370 bp 5′-ATGATGACCCGTCTAGCTGTG-3′ and oligo(dT); (O) 1599 bp, 5′-GATCGTCGACATGAGTGATATCTTTGAAGAG-3′ (*Sal*I) and 5′-GATATCT AGATCACACATCAGCTCTGACGAA-3′ (*Xba*I) (ORF for N); (P), 2130 bp, 5′-ATGGATAAATTGGAACTAGTC-3′ and 5′-TCAAATATTACCGTCAATGAT (ORF for P); (Q) 1059 bp, 5′-ATGGAGCCGGACATCAAGAGT-3′ and 5′-TTAGAAGAATTCTGTTTAGCC-3′ (ORF for M); (R) 1641 bp, 5′-GATCGAATTCGACATGGTAGTTATACTTGAC-3′ (*Eco*RI) and 5′-GATCGGATCCGAATACACTATGTCCCAATGTAG-3′ (*Bam*HI) (ORF for F); and (S) 1809 bp, 5′-GATCGGATCCATGCCGGCAGAAAACAAGAA-3′ (*Bam*HI) and 5′-GATCCTCGAGTAGATCAATCCATTAAATTATGAG-3′ (*Xho*I).

The oligonucleotide primers used to synthesize cDNA and amplify the ORFs of the Hendra N, P, M, F, and G genes are as follows: N, 5′-GATCGAATTCATGAGTGATATATTTGAC-3′ (*Eco*RI) and 5′-GATATCTAGATCACACGTCTGCTCTAAC-3′ (*Xba*I); P, 5′-GATCGAGCTCATGGACAAGTTGGATCTAGTC-3′ (*Sac*I) and 5′-GATCCTCGAGTTAGATGTTCCCATCAATAAT-3′ (*Xho*I); M, 5′-GATCGAGCTCACCTGA GTGGCCAGAACC-3′ (*Sac*I) and 5′-GATCCTCGAGAGTGAATGTTTTATTTCA-3′ (*Xho*I); F, 5′-GATCCCATGGATTATTTATCTTTAAGCA-3′ (*Nco*I) and 5′-GATCCTGCAGTGATTGTAGTGTATTTTA-3′ (*Pst*I); and G, 5′-GATCGAATTCATGATGGCTGATTCC-3′ (*Eco*RI) and 5′-GATACTCGAGTCAACTCTCTGAACA-3′ (*Xho*I).

5′ RACE and cloning

The protocol for 5′ RACE was carried out according to the recommendations of the manufacturer (Life Technologies, Gaithersburg, MD). First-strand synthesis of cDNA was primed using a NV P gene-specific primer (NVPER: 5′-ACACTCTTGCCTTCTTGGTAG-3′) and 1 μg of infected cell RNA. PCR amplification of the tailed first-strand cDNAs was done by using the primer NVPER and the Abridged Anchor primer. PCR products were purified (see above) and cloned into a plasmid vector, pCR2.1 (InVitrogen, San Diego, CA).

Construction of cDNA library for NV

A cDNA library of NV-infected cells was prepared by using a commercial library construction kit (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocols. Briefly, 3 μg of infected cell RNA was used as template for first-strand synthesis of cDNA using a *Not*I primer–adapter that binds to the poly(A) tail of mRNA and Superscript II (Life Technologies) reverse transcriptase. After second-strand synthesis by DNA polymerase I, *Sal*I adapters were added using T4 DNA ligase. cDNA was then digested with *Not*I and size-fractionated by column chromatography before being ligated to *Not*I–*Sal*I cut pSPORT I.

To screen for plasmid clones that containing NV F and G gene inserts, colonies were transferred onto nylon membranes and hybridized to digoxygenin-labeled probes (Boehringer-Mannheim, Indianapolis IN) prepared from plasmid clones of the G and F genes of HV (unpublished data). Plasmid DNA from colonies that hybridized to the HV F and G gene probes was further analyzed by Southern blotting and DNA sequencing.

Sequence analysis

Plasmid DNA or purified PCR products were sequenced using a cycle sequencing reaction with fluorescent dye terminators (Perkin–Elmer, Applied Biosystems Division, Foster City, CA), and the reaction products were analyzed by using an ABI 373 automatic sequencer (Perkin–Elmer, Applied Biosystems Division). Sequencing primers were provided with the plasmid cloning vectors or were based either on the previously published sequence of HV (GenBank accession number AF017149) or on the sequence of NV. Sequence data were analyzed using version 10.0 of the Genetics Computer Group package (Devereaux, 1984), PHYLIP version 3.4 (Felenstein, 1988), and PAUP (Swofford and Olsen, 1990). For the analysis of conservative amino acid changes, amino acids were grouped into families as follows: (F, Y), (M, L, I, V), (A, G), (T, S), (E, D), (K, R), and any changes involving an H, a P, a W, or a C were not considered conservative.

Neutralization tests

Mouse hyperimmune ascitic fluids (HMAF) for HV and NV were prepared as previously described (Brandt *et al.,* 1967). Plaque reduction neutralization assays were performed in the BSL-4 laboratory. Dilutions of HMAF to NV or HV were incubated with approximately 100 pfu of HV or NV before being transferred to Vero E6 cell monolayers. Plaques were visualized after 6 days, and the neutralizing antibody titer was recorded as the reciprocal of the highest dilution of HMAF producing at least a 90% reduction in plaque formation.

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