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Nipah Virus Edits Its P Gene at High Frequency To Express the V and W Proteins▽

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Nipah virus (NiV) is predicted to encode four proteins from its P gene (P, V, W, and C) via mRNA editing and an alternate open reading frame. By use of specific antibodies, the expression of the V, W, and C proteins in NiV-infected cells has now been confirmed. Analysis of the P-gene transcripts shows a ratio of P:V:W mRNA of 1:1:1, but this differs over time, with greater proportions of V and W transcripts observed as the infection progresses. Eighty-two percent of transcripts are edited, with up to 11 G insertions observed. This exceptionally high editing frequency ensures expression of the V and W proteins.

A characteristic feature of paramyxoviruses is the presence of an editing site in their P genes. This A_nG_n stretch of residues marks the position at which nontemplated G residues are added into a proportion of the P-gene mRNA transcripts in a process known as mRNA editing (10, 13). The addition of one or two extra G residues causes a frameshift such that the resulting proteins contain the same amino-terminal domain as that expressed from an unedited transcript but have a unique C-terminal domain that is expressed from either the +1 or +2 frame (13). Members of the *Morbillivirus*, *Respirovirus*, and *Avulavirus* genera express their P proteins from an unedited transcript, while the V protein is expressed from transcripts with one additional G residue and the W/D proteins are expressed from transcripts with two additional G residues (2, 13, 19, 28). Rubulaviruses have a different coding strategy, since their P proteins are expressed from the +2 transcript while the V protein is expressed from the unedited transcript and the W/I protein from the +1 transcript (5, 16, 18, 26, 27). Nipah virus (NiV) and Hendra virus, the two members of the *Henipavirus* genus, appear to conform to the same pattern as the morbilliviruses. Genome analysis and plasmid-based expression studies have shown that the cysteine-rich C-terminal domain, characteristic of all V proteins, is accessed via addition of one extra G nucleotide following the editing site (8, 9, 17, 20–23, 25). The addition of two G residues results in the expression of the W protein (17, 22, 23, 25). The W-encoding transcripts of other paramyxoviruses contain a stop codon shortly following the editing site, essentially producing a truncated protein representing the common N-terminal domain. In contrast, the henipavirus W proteins possess a substantial 43-residue unique C-terminal domain, and for NiV W, this domain has been shown to contain a nuclear localization signal (22). In this respect the henipavirus W protein seems analogous to the D protein of parainfluenza virus 3 (a respirovirus), whose 131-amino-acid C-terminal domain is also expressed from the +2 frame and which has also been reported to localize to the nucleus (19, 31). An additional P-gene product, the C protein, is expressed from an alternate open reading frame in paramyxoviruses of the *Respirovirus*, *Morbillivirus*, and *Henipavirus* genera (13).

The majority of these alternative P-gene products have been shown to function as inhibitors of the host innate immune response (7, 12). The NiV V and W proteins prevent interferon signaling by interacting with STAT1 and preventing its activation (20, 21, 23). NiV V, like other paramyxovirus V proteins, binds to mda-5 and thereby inhibits the downstream signaling events leading to beta interferon (IFN) synthesis (4). NiV W can also prevent IFN production through an unidentified mechanism that requires its nuclear localization (22). The NiV C protein has also been shown to inhibit the activation of an antiviral state, but the mechanism is unknown (17). All of this work, as well as experiments showing that the C, V, and W proteins can inhibit replication of a minigenome (25), has been performed using plasmid-based expression studies. Information regarding the expression of these proteins in NiV-infected cells is lacking, and given the roles of V and W as IFN antagonists, the extent of mRNA editing may play an important role in NiV pathogenesis.

To address these issues, 293T cells were infected with NiV (isolate UMMC1 [3]; GenBank accession no. AY029767) under biosafety level 4 conditions. The infection was performed in duplicate at a multiplicity of 0.4, and the NiV- or mock-infected cells were collected at 2.5, 6, 9.5, 24, and 30 h postinfection. The cells were lysed and processed for RNA extraction according to the protocol for the RNAeasy and Qiashredder kits (Qiagen). Proteins were precipitated from the eluate with 20% trichloroacetic acid and dissolved in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. These proteins were subjected to Western blot analysis

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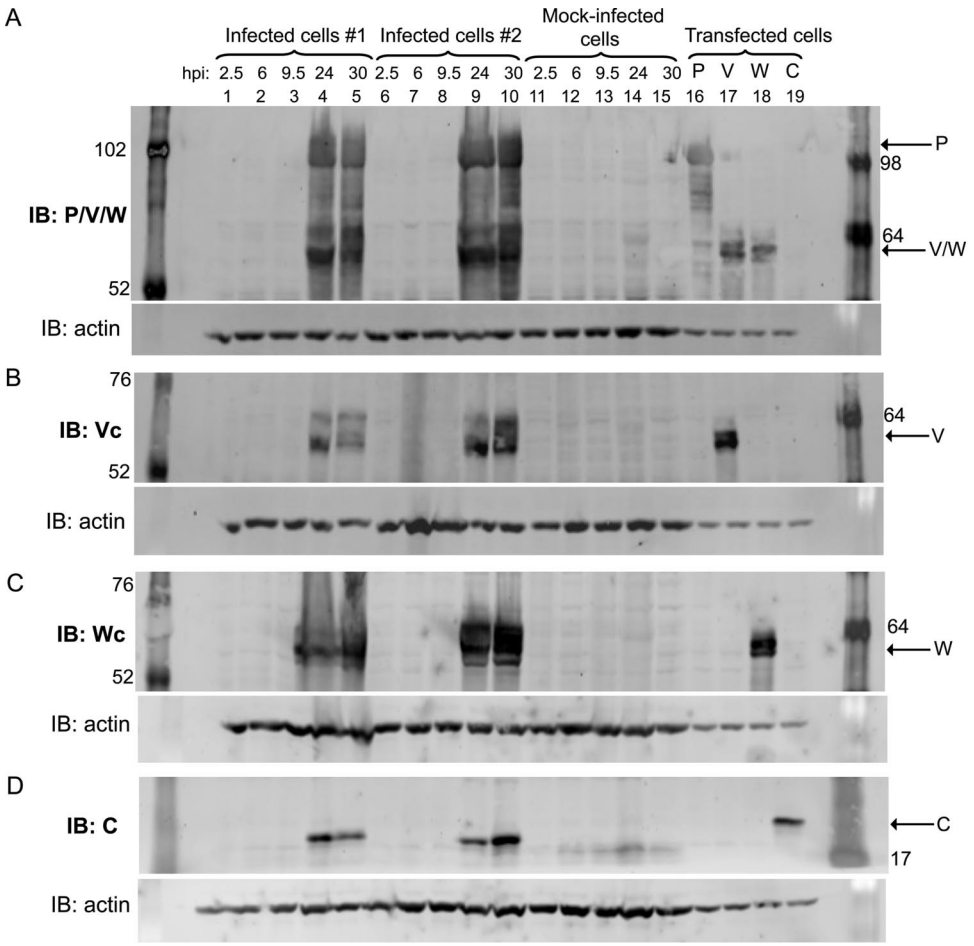

FIG. 1. Western blot analysis of P, V, W, and C expression in NiV-infected cells. Protein from NiV-infected 293T cells (lanes 1 to 10), mock-infected cells (lanes 11 to 15), or cells transfected with expression plasmids for hemagglutinin-tagged P, V, W, and FLAG-tagged C (lanes 16 to 19) was subjected to Western blot analysis with antibodies specific for the common N-terminal domain of NiV P/V/W (A), the C-terminal domain of NiV V (B), the C-terminal domain of NiV W (C), the NiV C protein (D), or actin (A, B, C, and D). For the infected samples, the number of hours postinfection is indicated above each lane. The positions of the P, V, W, and C proteins are indicated with arrows, and the numbers to the left and right indicate the molecular weight markers. The P/V/W antibody is a rabbit polyclonal raised against amino acids 1 to 407 of P/V/W, which was expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli*. The Wc rabbit polyclonal antibody was raised against the C-terminal portion of the W protein (amino acids 408 to 450), which was expressed as a GST fusion protein in *E. coli*. The Vc and C rabbit polyclonal antibodies were both raised against keyhole limpet hemocyanin-conjugated peptides corresponding to amino acids 428 to 443 of the V protein and amino acids 15 to 36 of the C protein, respectively. IB, immunoblot.

using antibodies raised against the common N-terminal domain of the P, V, and W proteins (Fig. 1A), the unique C-terminal domain of the V protein (Fig. 1B), the unique C-terminal domain of the W protein (Fig. 1C), and the C protein (Fig. 1D). Equal loading of the samples was monitored with an antibody against actin (A4700; Sigma). As controls for the mobility of P, V, W, and C and for antibody specificity, whole-cell extracts from 293T cells transfected with expression constructs for hemagglutinin-tagged P, V, W, and FLAG-tagged C were included in each blot. All NiV antibodies detected specific proteins in the 24- and 30-h samples for both infections, indicating that all four of the predicted protein products of the NiV P gene are expressed in an infected cell. In Fig. 1A, bands consistent with the sizes of P and V/W can be observed. V and W differ by only six amino acids in length and so cannot be distinguished from one another on the basis of molecular weight. The expression of these proteins can be better observed using antibodies specific for V or W (Fig. 1B and C), and it is clear that in infected cells, a higher-molecular-weight species of both V and W is seen that is not present in transfected cells. This is most likely due to a modification that occurs only in the context of the virus infection, and we can speculate that differential phosphorylation or acetylation may be the cause since these modifications have been described for the NiV P protein in the virion (24). In contrast, the NiV C protein migrates as a single species corresponding to its predicted size of 18 kDa (Fig. 1D). The slower migration of the plasmid-expressed C protein is most likely due to the presence of a tag on the C terminus.

To assess the extent of editing that occurs in NiV-infected cells and to calculate the proportion of P:V:W transcripts, total RNA extracted from the infected cells at each time point was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using an oligo(dT) primer. A 341-bp region span-

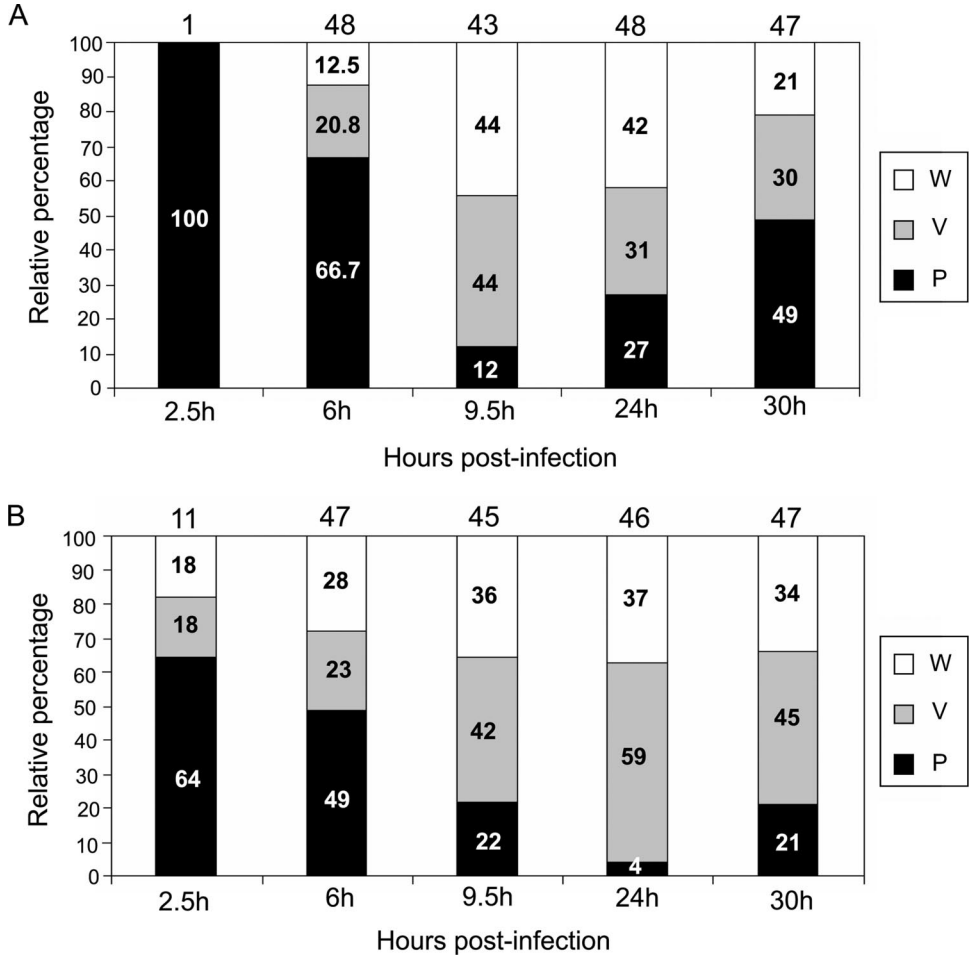

FIG. 2. The relative percentages of P, V, and W transcripts in NiV-infected cells at different times postinfection. The region spanning the P-gene editing site was amplified from RNA extracted from NiV-infected 293T cells at the indicated times postinfection. The number of clones sequenced per time point is indicated above the graph, and the relative percentages of P-, V-, and W-encoding transcripts, based on the number of G-residue insertions, are illustrated in the graphic. Panels A and B show data from duplicate infections.

ning the editing site in the NiV P gene was amplified by PCR using *Taq* DNA polymerase (Invitrogen) and the following primers: NiV-P_{1032} (5′-CAGCAAGGGAAAGATGCTCAG CCT) and NiV-P_{1373} (5′-TTAACCGCAGTGGAAGCATTC AGTTG). The product was cloned into pGEMT (Promega), and where available, more than 40 individual clones for each sample were sequenced. No visible PCR product was observed for any of the mock-infected samples, and no NiV-positive clones were obtained. For the 2.5-h infection time point, only 1 positive clone was obtained from the first infection and 11 positive clones were obtained from the second infection. The sequences of all positive clones were analyzed, and the number of G insertions that follow the editing site was determined. Based on this information, the protein product encoded by each clone was determined. Figure 2 shows the relative percentages of P, V, and W transcripts detected in NiV-infected cells at each time point for the duplicate infections. Although the pattern is not identical for the two infections, the trend is similar, with a greater proportion of P transcripts present at early times postinfection. As the infection progresses, there is a marked increase in the percentages of V and W transcripts, which at times equal or outnumber the P transcripts. The small increase in the number of P transcripts seen at the last time point is probably reflective of a second cycle of infection. No difference was observed in the levels of V and W transcripts, indicating equal expression levels. To our knowledge, this is the first kinetic analysis of paramyxovirus mRNA editing, and the data suggest that there may be temporal regulation of P, V, and W expression at the level of transcription and editing. Further exploration of this possibility using nonpathogenic paramyxoviruses, which allow for more-rigorous experimental conditions, will be necessary. Of course, the relative stability of the mRNA transcripts and of the protein may also affect the final protein levels in the infected cells.

A striking observation was that the G-residue insertions were not restricted to +1 or +2 but instead ranged from +1 to +11 (Fig. 3). In other words P transcripts include those with 0, 3, 6, and 9 insertions, V transcripts include those with 1, 4, 7, and 10 insertions, and W transcripts include those with 2, 5, 8, and 11 insertions. Transcripts with these extra G residues are predicted to encode proteins with one, two, or three additional glycine amino acids at the end of the N-terminal domain. It

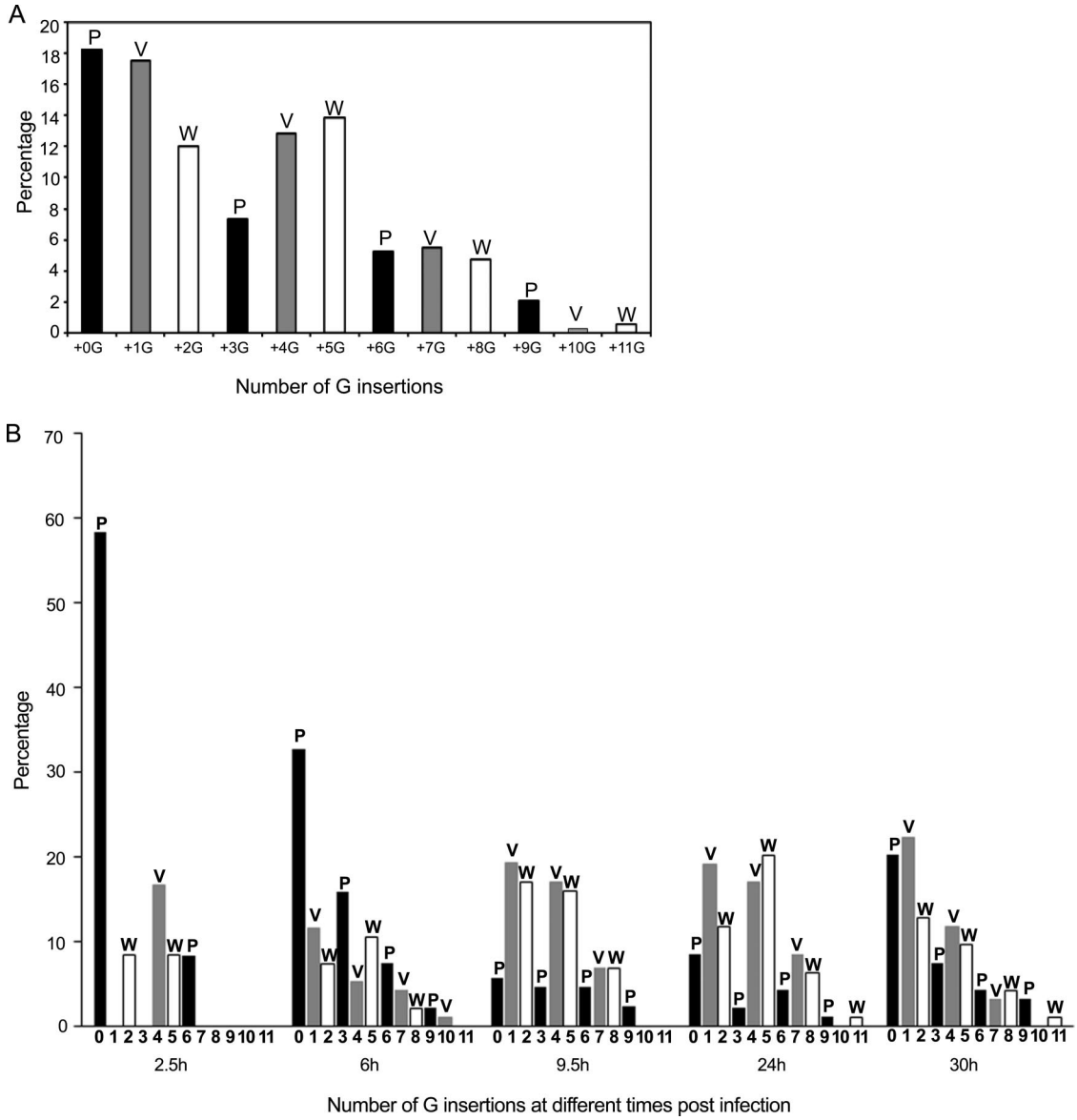

FIG. 3. The editing frequency of the NiV P gene and the pattern of G insertions. The percentages of unedited (+0G) and edited (+1G to +11G) NiV P-gene transcripts are illustrated either over the entire infection course (A) or at the different times postinfection (B). The encoded protein is indicated above each bar. These data encompass the duplicate infections shown in Fig. 2 (383 clones in total), and for the 2.5-, 6-, 9.5-, 24-, and 30-h time points, the data are compiled from 12, 95, 88, 94, and 94 clones, respectively.

remains to be seen whether this has any functional consequences for the proteins. Combining the data from the different time points and the duplicate infections (383 clones), 18% of NiV P-gene transcripts are unedited (0 insertions) and 82% are edited (+1 to +11). The most dominant insertions (>10%) are +1, +2, +4, and +5, and this can be seen particularly at the 9.5- and 24-h time points, when 94% and 91% of the transcripts, respectively, are edited (Fig. 3B). Overall, the ratio of P:V:W transcripts is about 1:1:1 (33%:36%:31%), and of the P transcripts, 44% are edited (+3, +6, and +9 insertions). This editing frequency is higher than that described for any other paramyxovirus to date, and the abundance of NiV V and W transcripts relative to P transcripts is much higher (Table 1). By comparison, a recent report on four strains of measles virus shows a combined editing frequency of 42%, with 60% of transcripts encoding P, 35% of transcripts encoding V, and the remaining 5% encoding the hypothetical W protein (1). Similar numbers have been reported for Newcastle disease virus (15). The only other paramyxoviruses known to hyperedit their P genes are human and bovine parainfluenza virus 3 (PIV3) viruses, which predominantly insert 1 to 6 G residues at equal frequency, although up to 12 insertions have been documented (6, 19). For bovine PIV3, the editing frequency has been calculated as 65%, with 52% P transcripts, 25% V transcripts, and 23% D transcripts (19). It is striking that NiV and PIV3, which both encode a unique protein (W/D) from the +2 open reading frame in their P genes, both also display a significantly higher editing frequency than other paramyxoviruses whose P

TABLE 1. Paramyxovirus P-gene mRNA editing in members of the *Henipavirus*, *Morbillivirus*, *Avulavirus*, and *Respirovirus* genera

Virus[a]	Editing frequency (%)	% P transcripts	% V transcripts	% W/D transcripts	Dominant insertions	Reference
NiV	82	33	36	31	+1, +2, +4, +5	This study
MeV	42	60	35	5	+1	1
NDV	32	68	28	4	+1	15
bPIV3	65	52	25	23	+1 to + 6	19
SeV	31	69	30	1	+1	10

[a] NiV, *Nipah virus*; *Henipavirus* genus. MeV, *measles virus*; *Morbillivirus* genus. NDV, *Newcastle disease virus*; *Avulavirus* genus. bPIV3, *bovine parainfluenza virus 3*; *Respirovirus* genus. SeV, *Sendai virus*; *Respirovirus* genus.

proteins are encoded by faithful P-gene transcripts. We speculate that this is to ensure adequate expression of the W/D proteins, and for NiV W at least, we know that it plays an important role in allowing the virus to evade the innate immune response (22, 23). Of note, transcriptional RNA editing is also required for expression of the Ebola virus full-length glycoprotein, and here too, a random editing pattern of the GP gene is observed (29, 30). Therefore, different negative-strand RNA viruses use similar strategies to express important replication or pathogenicity factors.

A comparison of the editing sites of NiV and PIV3 does not reveal any common feature that may account for the increased editing rate observed in these two paramyxoviruses. The henipavirus editing sites are actually very similar to that of measles virus, differing only by two nucleotides at positions −7 and −8 relative to the A_nG_n run. The nucleotides at the −1 and −2 positions, which have been identified as the reason for the differing editing frequencies of Sendai virus and PIV3 (11), are identical in PIV3, henipaviruses, and morbilliviruses. Additional features, such as the processivity of the viral polymerase and its ability to pause at the editing site, may also play a role in determining the editing rate and perhaps in the observed temporal expression ratio of P:V/W mRNA in NiV-infected cells.

All experiments involving live Nipah virus were carried out in the INSERM BSL-4 Laboratory Jean Merieux in Lyon, France. We thank the biosafety team members for their support and assistance in conducting experiments.

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ADDENDUM

Following submission of this paper, similar results were published by Lo et al. (14).

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