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Kinetic analysis of RNA editing of Newcastle disease virus P gene in the early period of infection

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Summary. – As a paramyxovirus, Newcastle disease virus (NDV) has the ability to edit its P (phosphoprotein) gene to synthesize three kinds of viral protein (P, V and W). It is technically very difficult to differentiate P, V and W mRNAs, and little was known about NDV regulation of RNA-editing frequency. To investigate the rules of NDV RNA editing, the ratio of the P gene-derived transcripts (P, V and W) was determined by sequencing at different time points post-infection. The results showed unstable ratio of V and W mRNA at different time points, and the frequency of NDV editing was significantly increased at the early period of infection ($P < 0.05$). Along with the increase in editing frequency, the levels of V and W transcripts were obviously increased at 8 and 10 hours post infection (hpi). In addition, it was shown that the number of inserted G residues in P-derived transcripts was not limited to one or two: +3G, +4G, +5G and even +9G transcripts were identified. Higher RNA editing frequency of NDV P gene occurred in the early period of infection, which may play a role in the process of viral infection.

Keywords: Newcastle disease virus; phosphoprotein; RNA editing; G insertion

Introduction

Newcastle disease virus (NDV) is a member of the family *Paramyxoviridae* (Mayo, 2002). The genome of NDV consists of a single negative-stranded non-segmented RNA that encodes six major structural proteins, namely nucleotide protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large protein (L) (Yusoff and Tan, 2001). The NP, P and L proteins are associated with the formation of viral RNA-dependent RNA polymerase (vRdRp), which is responsible for the synthesis of all viral mRNAs as well as viral genomic and antigenomic RNAs (Kolakofsky *et al.*, 2005). NDV initiates its replication and transcription at the moment when vRdRp recognizes and interacts with the cis-acting sequences located at the 3' end of the viral genome or antigenome. The genomic RNA is used as a template to synthesize viral mRNAs and also antigenomic RNAs; correspondingly, antigenomic RNA is the template for the synthesis of genomic RNA (Lamb and Kolakofsky, 2002).

Like other paramyxoviruses, the vRdRp of NDV has the ability to edit P gene-derived mRNAs to synthesize more viral proteins in the process of "NDV transcription" (Fearns *et al.*, 2000; Butcher *et al.*, 2001). P gene was inserted with one or two Guanines (G) following the conserved editing locus (UUUUUCCC), therefore, three kinds of mRNA, the P mRNA (with no frameshift), the V mRNA (with +1 frameshift) and the W mRNA (with +2 frameshifts) are transcribed (Steward *et al.*, 1993). These P gene-encoded proteins share a common amino

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Abbreviations: G = Guanine; hpi = hours post infection; IFN-α = interferon alpha; NDV = Newcastle disease virus; NP = nucleotide protein; P = phosphoprotein; NiV = Nipah virus; vRdRp = viral RNA-dependent RNA polymerase

terminus but differ in the carboxyl terminus (Horvath, 2004). The molecular weights of V and W proteins are about 36 K and 33 K in SDS-PAGE, much smaller than that of P protein (Chambers and Samson, 1982). Of the three P gene-derived products, the P protein is known as an indispensible subunit for the formation and functioning of NDV RdRp (Smallwood *et al.*, 1994; Curran *et al.*, 1995; Tarbouriech *et al.*, 2000; Smallwood and Moyer, 2004). It has also been shown that the P protein acts as a chaperon to prevent uncontrolled encapsidation of non-viral RNA by NP proteins (Errington and Emmerson, 1997). Another product of the NDV P gene, V protein, has a zinc-binding cysteine-rich region at its carboxyl terminus, which is conserved in paramyxoviruses (Steward *et al.*, 1995). The NDV V protein functions as an alpha interferon (IFN-α) antagonist and is associated with viral pathogenesis and host range restriction (Mebatsion *et al.*, 2001; Huang *et al.*, 2003; Park *et al.*, 2003a). Siba K. Samal showed that the V protein of NDV mediated degradation of STAT1 (a critical signaling factor in the IFN pathway) to block further cellular antiviral activities induced by IFN-α (Huang *et al.*, 2003). The W protein is the shortest product encoded by the P gene of NDV, and its function is unknown (Park *et al.*, 2003b). In the case of Nipah virus (NiV), the W protein can sequester STAT1 in the nucleus, blocking both cytoplasmic and nuclear forms of STAT1 (Shaw *et al.*, 2004). It has also been shown that NiV W proteins inhibit signaling pathways triggered by both virus and Toll-like receptor 3 (TLR3) (Shaw *et al.*, 2005).

Given that the P, V and W transcripts of a paramyxovirus are very similar, it is very difficult to differentiate their mRNAs. Direct sequencing of these mRNAs has been used for several viruses to study RNA editing (Galinski *et al.*, 1992; Steward *et al.*, 1993; Jacques *et al.*, 1994; Hausmann *et al.*, 1996; Mebatsion *et al.*, 2001; Bankamp *et al.*, 2008). Measles viruses showed 60% of transcripts encoding P, 35% of transcripts encoding V, and the remaining 5% encoded the hypothetical W protein (Bankamp *et al.*, 2008). For bovine parainfluenza virus 3 (bPIV3), the editing ratio of P:V:W was 65%:52%:25%, while the human parainfluenza virus 3 (hPIV3) had insertions of 1 to 6 G residues at equal frequencies (Galinski *et al.*, 1992). Teshome Mebatstion and colleagues sequenced several P gene-derived transcripts in NDV-infected cells and found that 68% were P-encoding mRNAs, 29% were V-encoding mRNAs, and 2% were W-encoding mRNAs (Mebatsion *et al.*, 2001). Expression of NiV P, V, and W proteins was proved to be varying over time in a recent study (Kulkarni *et al.*, 2009). To better understand the NDV RNA editing, three different genotypes of NDV strain LaSota, JS10 and ZJ1 were used to infect DF-1 cells, a chicken embryo fibroblast cell line, and the ratio kinetics of P, V and W transcripts were determined at different time points post infection.

Materials and Methods

Cells and viruses. DF-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C and 5% CO_2. The NDV strain LaSota was obtained from China Institute of Veterinary Drug Control, JS10 was isolated by our lab in Jiansu province, China (Meng *et al.*, 2012) and ZJ1 was a gift from Professor Xiufan Liu (Huang *et al.*, 2004). Each virus strain was injected into 9- to 11-day-old specific-pathogen-free (SPF) embryonated eggs to produce fresh allantoic fluid. The 50% tissue culture infective dose ($TCID_{50}$) of each virus in the allantoic fluid was determined by indirect immunofluorescence assay (IFA) as described previously (Sun *et al.*, 2012).

Detection of the RNA editing frequency. The DF1 cells in a monolayer, at 80% to 90% confluence, were washed with PBS three times, followed by infection with respective NDV at a multiplicity of infection (MOI) of 3 as described before (Sun *et al.*, 2013). The infected cells were collected at 2 hr intervals from 2 hr post infection (hpi) to 24 hpi for detection of the P gene-derived mRNAs. Specifically, the total RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen, CA, USA). The RNA pellet was resolved in 50 μl RNase-free water after washing with 70% ethanol. To remove DNA contamination, DNase I was added to the RNA extract at a final concentration of 1 U/μl at 37°C for 30 min, followed by inactivation at 65°C for 10 min in the presence of 5 mmol/l EDTA. Subsequently, reverse transcription reaction of RNA fragments was performed with the M-MLV reverse transcriptase (Promega, Madison, WI, USA) and the primer P18TV (5'-TTT TTT TTT TTT TTT TTT A/G/C-3') according to the manufacturer's protocols; in this way, the viral mRNA rather than viral genomic RNA was selectively reverse transcribed. The sequences surrounding the RNA editing site of the NDV P gene-derived mRNA were determined by a standard amplification of PCR with Platinum® Taq DNA Polymerase High Fidelity. The reaction parameters were: 94°C for 2 min followed by 25 cycles of 94°C for 30 sec, 58°C for 30 sec and 68°C for 1 min. The P gene in strains ZJ1, LaSota and JS10 was amplified using primers PZPU (5'-TTA CGG CCC AGG ACA AAT CAG C-3')/PZPD (5'-ACT GGG TGG GAT CGG GCT ACT GC-3'), PLPU (5'-GCA ATC CCA CAA GGC AAG ACC A-3')/PLPD (5'-CGG GTG AGA TCG GGC AAC TG-3') and PJSPU (5'-ACA GCT CAG GGC AAA CCA GTG-3')/PJSPD (5'-TCC TAG GTT TGC TTC CAT CAC TGC-3'), respectively. The RT-PCR product in the agarose gel was subjected to gel extraction (TaKaRa), then cloned into the TA cloning system (Promega, Madison, WI), and subsequently transformed into *Escherichia coli* DH5α for sequence analysis. At least 50 positive clones from each time point were collected for sequencing (Sangon Biotechnology, Shanghai, China). Alignment of sequences and prediction of amino acid sequences were conducted with the Lasergene package (DNASTAR Inc. Madison, WI 53715, USA). The experiment was repeated three times. The percentage of RNA editing of NDV at different time points were compared with chi-squared (χ^2) test using

GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

Western blot. At 4, 8, 12, 24 and 36 hpi, the DF-1 cells were washed thoroughly and lysed in RIPA lysis buffer (Beyotime, China). The lysates were denatured with an equal volume of 2× loading buffer (Beyotime, China) at 95°C for 5 min. Treated samples were electrophoresed on a 10% SDS-PAGE gel and transferred to Immobilon®-P Polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After transfer, the membranes were incubated at room temperature for 1hr with mouse anti-P/NDV or anti-V/NDV antibodies (Qiang *et al.*, 2013), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1hr. Protein blots were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and imaged by an EU-88 image scanner (Seiko Epson Corp.). The gray intensity of proteins of interest was quantified by the Quantity One 1-D software version 4.4.0 (Bio-Rad Laboratories, Inc.).

Results

In total, 1,350 individual clones were collected at different time points post infection for sequence analysis. Of them, 1,240 clones were successfully sequenced. When compared to the reference sequences available in GenBank, the nucleotide sequences of all clones were identical to the P gene of the parental virus, except for the one or more G residues insertion in the RNA-editing site.

The time-related changes in RNA editing of NDV P/V/W mRNAs are displayed in Fig. 1. It shows that the proportion of P:V:W apparently differed over time. In the case of the strain ZJ1, the ratios of P:V:W observed at 4 and 24 hpi were P63.9%:V27.8%:W8.3% and P65.3%:V26.5%:W8.2%, respectively, which was similar to the ratio observed previously (P68.3%:V29.3%:W2.4%) (Mebatsion *et al.*, 2001). However, a greater percentage of edited transcripts was observed at 6–12 hpi and the lowest percentage of unedited P transcripts occurred at 10 hpi (Fig. 1a). A chi-squared (χ^2) test was used to compare the percentage of RNA editing of NDV at different time points. The P value was greater than 0.05 due to small sample number, although the trend was obvious that more edited transcripts were observed at 8 and 10 hpi.

A similar phenomenon was observed in LaSota- and JS10-infected DF1 cells (Fig. 1b and c). The proportions of P:V:W at 4 and 24 hpi were close to the widely accepted pattern of 68.3% P, 29.3% V and 2.4% W (Mebatsion *et al.*, 2001). An increased RNA-editing percentage was observed at 8-10 hpi. Combining the data from different NDV strains, a paired-samples *t*-test was used to compare the percentages detected at each time point, and it revealed that the editing frequency at 8 hr and 10 hr was significantly higher than that at 24 hpi ($P < 0.05$). Along with the increase in editing frequency, the V and W transcripts were obviously increased at 8 and 10 hpi, and the peak number of V transcripts appeared at 8 hpi (Fig. 2).

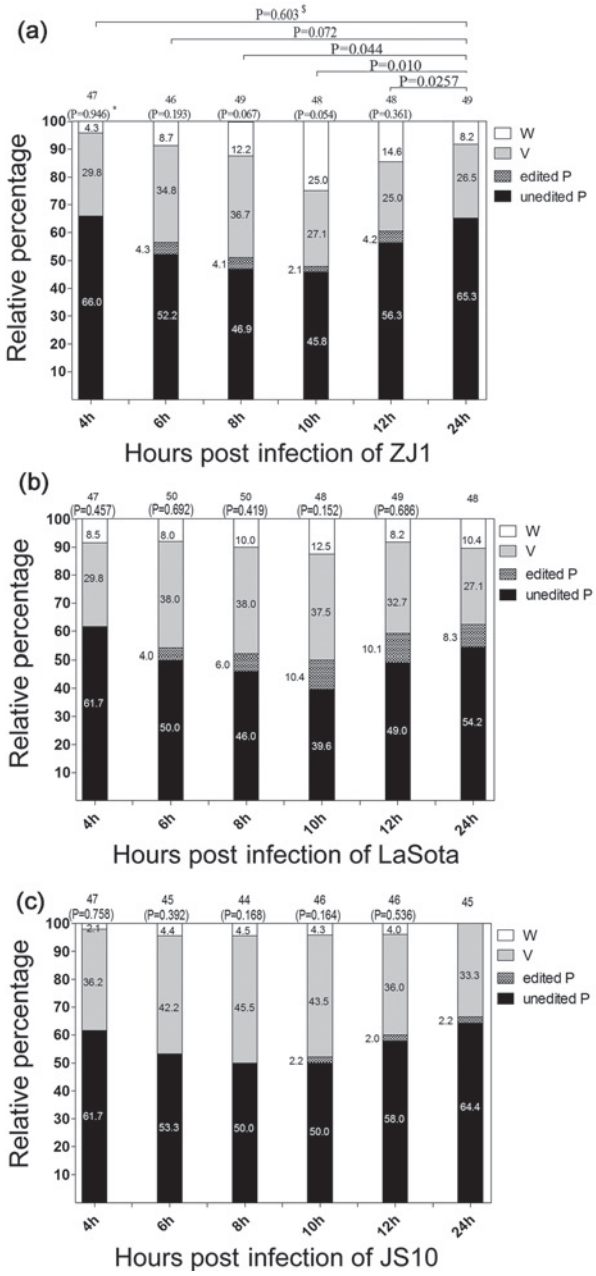

Fig. 1

The relative percentage of P gene-derived transcripts in NDV-infected DF1 cells at different time points post infection

Panels (a), (b), and (c) show data from ZJ1, LaSota and JS10 infected cells, respectively. * The P values of chi-squared (χ^2) test are marked in front of each item of data. The RNA-editing frequency of P gene at 4, 6, 8, 10 and 12 hpi was compared with that at 24 hpi. $ The P-value of a paired-sample *t*-test was marked. The percentages of RNA editing of three NDV stains at different time points were compared statistically.

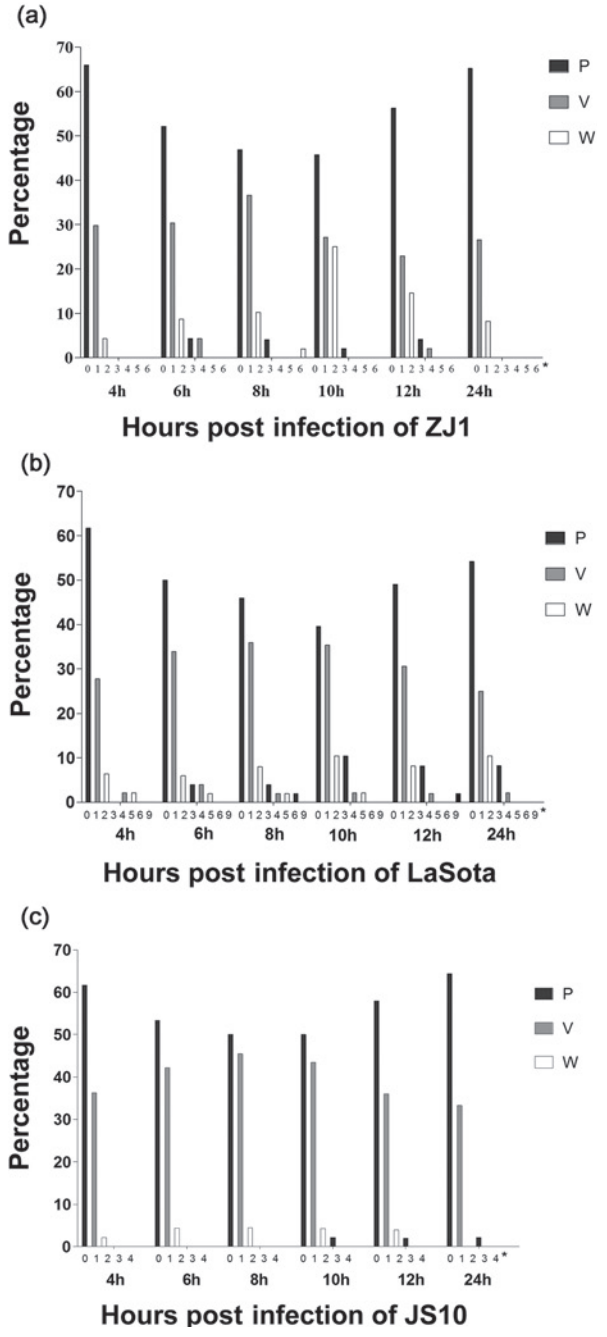

Fig. 2

The kinetic changes of the NDV P gene-derived P, V and W transcripts in the early period of infection

The various kinds of mRNA transcribed from P gene were identified from NDV-infected cells and sorted according to its G insertions number. Panels (a), (b), and (c) show data from ZJ1, LaSota and JS10 infected cells, respectively. *The numbers of G insertions identified in each type of P gene-derived transcripts were displayed under each bar graph.

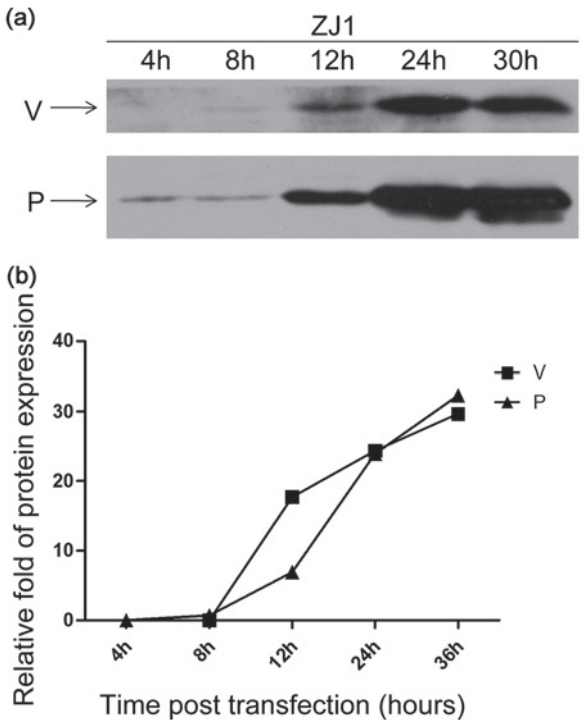

Fig. 3

The P and V proteins expression in NDV-infected DF1 cells

(a) The P and V protein of ZJ1 were detected in WB assay at 4, 8, 12, 24 and 36 hpi. (b) The expression growth curves of P and V protein were constructed based on the gray intensity of protein blots. Each time point of the P curve represents the relative ratio of expression level compared to that at 4 hpi; each time point of the V curve represents the relative ratio of V protein compared to that at 8 hpi.

The amount of P and V protein in ZJ-1-infected DF1 cells were detected in the WB assay at different time points. As shown in Fig. 3a, P protein was observed at 4 hpi and V protein was not detectable until 8 hpi. The expression level of P protein was always higher than that of V protein; nevertheless, the amount of V protein increased by 17 times from 8 hpi to 12 hpi. By contrast, the amounts of P protein increased by only 6 times at the same time, thus the increase rate of P protein was much lower than that of V protein (Fig. 3b).

There were differences in the RNA-editing frequency among three NDV strains used in this study. The lowest percentage of un-edited transcripts (39.6%) was observed in LaSota, while the highest was observed in ZJ1 (66.0%). The percentage of V transcripts ranged from 25% to 36.7% in ZJ1, 27.1% to 45.8% in LaSota, and 33.3% to 40.8% in JS10. In comparison, the yield of V transcripts in ZJ1-infected cells was lowest. In the case of W transcripts, ZJ1 showed the highest percentages at 10 hpi (25%), while few W transcripts were observed in JS10.

It was remarkable that the number of G residues inserted in P-derived transcripts was not limited to one or two (Table 1). Of all the clones sequenced in this study, 36 clones contained three extra G residues (predicted to

Table 1. The number of G residues inserted in the P gene-transcribed mRNAs in NDV-infected cells

Strain	P					V			W		
	Unedited[1]	+3G	+6G	+9G	%[2]	+1G	+4G	%	+2G	+5G	%
ZJ1	322	11	0	0	58.01	156	6	28.22	78	1	13.76
LaSota	165	20	1	1	55.00	114	7	35.59	28	4	9.41
JS10	180	5	0	0	56.75	129	0	39.57	12	0	3.68
Total	667	36	1	1	56.85	399	13	33.23	118	5	9.92

[1]Represents no G was inserted. [2]Represents the proportion of P, V or W transcripts in the P gene-transcribed products.

encode a P protein analog), 13 clones contained four extra G residues (predicted to encode a V protein analog) and 5 clones contained five extra G residues (predicted to encode a W protein analog). One clone had 9 extra G residues in the RNA editing site, while +7G or +8G transcripts were not found, presumably because of their extremely low frequency of occurrence.

Discussion

Quantitation of paramyxovirus RNA-editing frequency is technically hard to perform (Kolakofsky *et al.*, 2005; Kulkarni *et al.*, 2009). The only difference among the P gene-derived transcripts was the number of G residues inserted. Therefore, the typical nucleic acid detection techniques, such as PCR and real-time PCR, cannot be used for the quantitation, whereas the genetic sequencing is the only way to differentiate these transcripts, which has limited the research on RNA editing (Galinski *et al.*, 1992; Ghosh *et al.*, 1996; Hausmann *et al.*, 1996; Kolakofsky *et al.*, 2005; Kulkarni *et al.*, 2009; Lo *et al.*, 2009, 2012). Up to date, little was known about NDV regulation of RNA-editing frequency. In this study, more than one thousand P gene-derived transcripts were cloned, sequenced and analyzed at different time points post NDV infection. The experiments were performed in three different genotypes of NDV strains, LaSota (with a genomic RNA of 15,186 nt), ZJ1 (with a genomic RNA of 15,192 nt) and JS10 (with a genomic RNA of 15,198 nt). The results showed that all three NDV strains regulated their P:V:W ratio over time. In the early period of infection, NDV significantly increased the RNA editing frequency; as a result, the level of V and W transcripts was obviously elevated, which presumably increased the level of V and W protein expression. Similar results were observation of the WB assay for P and V protein in NDV-infected DF1 cells (Fig. 3). The amount of V protein increased by 17 times from 8 hpi to 12 hpi; by contrast, the amounts of P protein increased by only 6 times at the same time, the increase rate of P protein was thus much lower than of V protein. It suggests that NDV tended to produce more V protein at 8–12 hpi, which was consistent with the results of RNA-editing frequency.

It has been proposed that paramyxovirus RNA editing results from vRdRp stuttering. In the process of transcription, vRdRp could pause at the insertion site, causing the base pairing between the 3' end of the nascent mRNA and its template to be broken transiently, and slippage and insertion to occur (Vidal *et al.*, 1990; Hausmann *et al.*, 1999; Kolakofsky *et al.*, 2005). NDV V protein is an interferon type I (IFN-I) antagonist, which is involved in virulence and host restriction (Mebatsion *et al.*, 2001; Huang *et al.*, 2003; Park *et al.*, 2003a). The cysteine-rich carboxyl terminus of the V protein targets the STAT1 protein for degradation, and consequently blocks IFN signaling to facilitate the propagation of NDV offspring (Huang *et al.*, 2003). Our results indicated that higher frequency of RNA editing occurs in the early period of NDV infection, encoding more V mRNAs. It is reasonable to assume that the up-regulation of V transcripts observed in the early phase of infection assists in virus propagation. The detailed mechanism requires further exploration.

In addition, more insertions of G residues than +1 or +2 were found in the 1,240 transcripts. Of them, 36 clones contained 3 extra G insertions, 13 clones contained 4 extra G insertions, 5 clones contained 5 G insertions and 1 had 9 G insertions; these were predicted to encode the analogs of P, V and W proteins (Table 1). The predominant insertion patterns, however, were +1G and +2G. The hyper-edited transcripts comprised 4.52% of the P-derived products. The proteins encoded by these transcripts were definitely rare. This implies that the hyper-edited transcripts are byproducts of RNA editing.

As a paramyxovirus, NDV has the ability to edit its P gene to synthesize three kinds of viral protein (P, V and W). Our results showed that NDV regulated its RNA editing mechanism to change the proportion of P:V:W in the course of infection. Higher RNA editing frequency of NDV P gene occurred in the early period of infection, which may be conductive to the interferon antagonism.

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