Short Communication

Newcastle disease virus nucleocapsid protein: self-assembly and length-determination domains

Chiew Ling Kho,[1] Wen Siang Tan,[1,2] Beng Ti Tey[3] and Khatijah Yusoff[1,2]

Department of Biochemistry and Microbiology, Faculty of Science and Environmental Studies[1], Institute of Bioscience[2] and Department of Chemical and Environmental Engineering, Faculty of Engineering[3], Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia

Correspondence
Khatijah Yusoff
(at Department of Biochemistry and Microbiology)
khatijah@fsas.upm.edu.my

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The nucleocapsid protein (NP) of Newcastle disease virus expressed in *E. coli* assembled as ring- and herringbone-like particles. In order to identify the contiguous NP sequence essential for assembly of these particles, 11 N- or C-terminally deleted NP mutants were constructed and their ability to self-assemble was tested. The results indicate that a large part of the NP N-terminal end, encompassing amino acids 1 to 375, is required for proper folding to form a herringbone-like structure. In contrast, the C-terminal end covering amino acids 376 to 489 was dispensable for the formation of herringbone-like particles. A region located between amino acids 375 to 439 may play a role in regulating the length of the herringbone-like particles. Mutants with amino acid deletions further from the C-terminal end (84, 98, 109 and 114 amino acids) tended to form longer particles compared to mutants with shorter deletions (25 and 49 amino acids).

Newcastle disease virus (NDV) is a member of the genus *Rubulavirus* of the family *Paramyxoviridae* (Rima *et al.*, 1995) and causes the highly contagious Newcastle disease (ND) in many avian species. The virus possesses a non-segmented negative-strand RNA genome of 15 186 nucleotides (Phillips *et al.*, 1998) which encodes six main structural proteins (Samson, 1988): nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large protein (L). The NP protein is the most abundant protein in the virus particles and together with genomic RNA makes up the core helical nucleocapsid structure of NDV. The genomic RNA is associated with the NP, P and L proteins to form the ribonucleoprotein complex (RNP), which serves as a template for RNA synthesis (Yusoff & Tan, 2001).

The NP protein of paramyxoviruses has been the focus of many studies for years due to its crucial functional role during replication of the genomic RNA (Horikami *et al.*, 1992; Curran *et al.*, 1993; Myers *et al.*, 1999). Much structural and functional information on this protein has been derived, mainly from other paramyxoviruses such as Sendai, measles and human parainfluenza type 2 (hPIV-2) viruses (Spehner *et al.*, 1991; Buchholz *et al.*, 1993, 1994; Bankamp *et al.*, 1996; Nishio *et al.*, 1999). Like many other paramyxoviruses, the nucleocapsid of NDV possesses a herringbone-like structure (Alexander, 1988). Recombinant NP protein of Newcastle disease virus expressed in *E. coli* (Kho *et al.*, 2001a) or the baculovirus expression system (Errington & Emmerson, 1997) is able to self-assemble to form ring- and herringbone-like structures which morphologically resemble the authentic nucleocapsid structures. Insertion of a 29 residue peptide containing the Myc epitope and His-tag sequences onto the C terminus of the NP protein inhibits assembly of the herringbone-like structures but does not impair formation of the ring-like particles (Kho *et al.*, 2001a). Furthermore, this foreign peptide was shown to be exposed on the surface of the ring-like particle (Kho *et al.*, 2001a; Rabu *et al.*, 2002). It is most likely that the herringbone-like particles are rather flexible and heterogeneous in both shape and size, making it difficult to obtain high-resolution analysis by X-ray crystallography. To date, no high-resolution structure is available for the NP of paramyxoviruses. Current structural models of the nucleocapsid proposed for this group of viruses were derived from the intermediate resolution studies of Sendai virus (Egelman *et al.*, 1989) and the N-RNA ring structure of rabies virus (Schoehn *et al.*, 2001), through a combination of electron microscopy and three-dimensional image reconstruction. Although the nucleocapsid-like particles formed by the NP protein of various paramyxoviruses are all helical in structure, significant structural differences have been reported depending on the virus genera (Bhella *et al.*, 2002).

The existence of an NP–NP interaction that gives rise to the organized nucleocapsid herringbone-like features is evident in vesicular stomatitis (Sprague *et al.*, 1983), Sendai (Buchholz *et al.*, 1993) and measles viruses (Spehner *et al.*, 1991; Fooks *et al.*, 1993). The NP–NP interacting domains have been mapped to the N-terminal two-thirds of the NP protein for both Sendai and measles viruses (Buchholz *et al.*, 1993; Liston *et al.*, 1997) whereas in the case of hPIV-2, the N-terminal 294 amino acids of NP have been shown to be necessary for this interaction (Nishio *et al.*, 1999). In many cases, these self-assembled nucleocapsid-like particles appeared to enwrap nonspecific RNA of different lengths.

However, the detailed *in vivo* assembly pathway for this group of negative-strand RNA viruses is still far from complete. For NDV, the NP–NP self-assembly domain has yet to be identified. Therefore, this study was performed to define the contiguous sequence of the NP protein which is involved in self-assembly by deletion mutagenesis. In addition, the effect of deletions on the NP protein relative to its nucleocapsid-like phenotypes was also examined.

For NP self-assembly study purposes, a total of 11 deletion mutants with successive deletions from either the 5′- or the 3′-end of NP gene were constructed (Fig. 1a). C-terminal deletion constructs $NP_{\Delta C464}$, $NP_{\Delta C440}$, $NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$, $NP_{\Delta C375}$, $NP_{\Delta C366}$ and $NP_{\Delta C245}$ differed from the full-length NP by deletion of 25, 49, 84, 98, 109, 114, 123 and 244 amino acids from the C terminus of the NP protein, respectively. N-terminal deletion mutants $NP_{\Delta N26}$, $NP_{\Delta N122}$ and $NP_{\Delta N245}$ were the result of 25, 121 and 244 amino acid deletions from the N terminus of the NP protein, respectively. Both the N- and C-terminally deleted cDNA fragments were generated by PCR of the plasmid DNA pTrcHis2-NP (Kho *et al.*, 2001a), which encodes the full-length NP gene, using the respective primer pairs. Each of the deleted cDNA fragments was ligated to the pTricHis2 expression vector (Invitrogen) and then introduced into *E. coli* strain TOP 10. Recombinant plasmids were isolated from the putative clones and the mutants were confirmed by DNA sequencing as described in Kho *et al.* (2001a).

All of the truncated proteins were successfully expressed in *E. coli* TOP 10 cells by induction with IPTG (1 mM) for 5 h, as analysed by Western blotting using NDV antiserum as the probe (Fig. 1b). The truncated proteins were expressed to their expected sizes, ranging from approximately 27 to 50 kDa, and displaying a stepwise decrease in size, corresponding to the sizes of the deletions (Fig. 1b). Most of the mutated proteins were produced in readily detectable amounts except for $NP_{\Delta C366}$, $NP_{\Delta C245}$, and $NP_{\Delta N122}$, which were expressed at rather low levels as compared to other proteins (data not shown). It was speculated that the low expression level of these mutated proteins was most probably due to their instability when expressed *in vivo*. The same phenomenon was observed in certain NP deletion mutants of Sendai virus when an attempt to map the domains on NP protein involved in nucleocapsid formation was made (Buchholz *et al.*, 1993).

For production of purified protein, *E. coli* cell pellets (from 500 ml culture) were harvested, resuspended in lysis buffer [50 mM Tris (pH 7·9), 0·1 % Triton X-100, 0·2 mg lysozyme ml^{-1} and 4 mM $MgCl_2$; 25 ml], and then lysed by sonication. The lysate was then treated with RNase (5 μg ml^{-1}) and DNase I (5 μg ml^{-1}) for 1 h at room temperature (25 °C) and finally the cell extract was recovered after centrifugation at 10 000 r.p.m. (JA 14 rotor, Beckman) for 20 min at 4 °C. The mutated proteins were then precipitated by increasing the saturation of ammonium sulphate to 40 %. Results showed that proteins $NP_{\Delta C464}$, $NP_{\Delta C440}$, $NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$, $NP_{\Delta C375}$ and

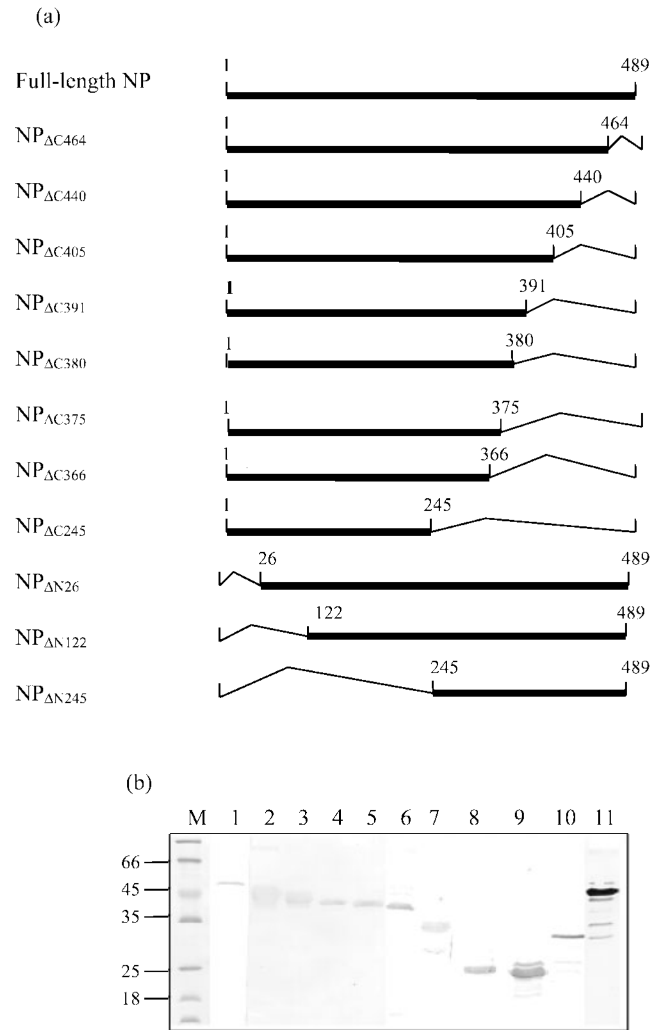

Fig. 1. (a) Schematic representation of the deletion mutants of NP protein. Thick lines represent the protein product of each truncated NP gene and the amino acid positions are indicated on top of these lines. Angled lines indicate deleted regions. (b) Western blot analysis of the expressed truncated NP proteins. All the truncated NP proteins were fractionated on SDS-12 % polyacrylamide gels, electrotransferred to nitrocellulose membranes and probed with anti-NDV chicken serum. The sizes of the expressed truncated proteins correspond to the expected sizes ranging from approximately 27 to 50 kDa. Lanes 1 to 8 represent C-terminally truncated NP proteins: $NP_{\Delta C464}$, $NP_{\Delta C440}$, $NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$, $NP_{\Delta C375}$, $NP_{\Delta C366}$ and $NP_{\Delta C245}$. Lanes 9 to 11 denote N-terminally truncated NP proteins: $NP_{\Delta N245}$, $NP_{\Delta N122}$ and $NP_{\Delta N26}$. Lane M: molecular mass markers in kDa.

$NP_{\Delta N26}$ were efficiently precipitated at different percentages of saturation (data not shown). In contrast, proteins $NP_{\Delta C366}$, $NP_{\Delta C245}$, $NP_{\Delta N122}$ and $NP_{\Delta N245}$ were scarcely precipitated by ammonium sulphate and were almost invisible when analysed by SDS-PAGE after precipitation (data not shown). The difficulty in precipitating these

truncated proteins was most probably due to the drastic decrease in protein solubility which resulted in the retention of the insoluble proteins in cell pellets. For subsequent protein purification, all mutated proteins except $NP_{\Delta C366}$ and $NP_{\Delta N245}$ were subjected to 10 to 50 % sucrose gradient centrifugation at 38 000 r.p.m. (SW 41 Ti rotor, Sorvall) for 5 h at 4 °C. These two mutated proteins were excluded due to the difficulty in getting sufficient soluble protein for any further studies. After centrifugation, fractions (0·5 ml) were collected by punching a hole at the bottom of the tube, and samples from each fraction were analysed by SDS-12 % PAGE to confirm the presence of the required proteins.

Fig. 2 shows the protein sedimentation profiles of the truncated NP proteins. The profile, which appeared as a major peak located around fractions containing herringbone-like particles, as demonstrated in the full-length NP protein (Fig. 2) (preparation as described in Kho *et al.*, 2001a) was observed for mutated proteins $NP_{\Delta C464}$, $NP_{\Delta C440}$, $NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$, $NP_{\Delta C375}$ and $NP_{\Delta N26}$, indicating the ability of these mutants to form particles. However, it was obvious that these particles had different sizes or shapes judging from the respective positions of the major peak. In addition, for $NP_{\Delta C464}$ and $NP_{\Delta C440}$, there was an additional peak at fractions 2 to 4. Nevertheless, SDS-PAGE analysis showed that the peak consisted mainly of other *E. coli* proteins. The position of the peak for C-terminal deletion mutants shifted towards the denser fractions, and this was clearly demonstrated in $NP_{\Delta C405}$ and $NP_{\Delta C391}$ (Fig. 2). Furthermore, a small portion of these two mutated proteins was sedimented at the bottom of the centrifuge tubes, indicating the presence of particles with higher density (data not shown). On the other hand, the tendency for peak shifting to denser fractions was not obvious in the N-terminally deleted $NP_{\Delta N26}$. For $NP_{\Delta N122}$ and $NP_{\Delta C245}$ (Fig. 2) however, no clearly resolvable peak was observed. Thus, it was anticipated that these two mutants did not retain the ring- and herringbone-like structures.

In order to determine the contiguous amino acid sequence involved in NP nucleocapsid assembly, protein samples that formed the major peak of the purified truncated NP proteins were pooled, dialysed and negatively stained with uranyl acetate (2 %) before being viewed under a Hitachi H-7100 or the Phillip CM 12 electron microscope. As expected, $NP_{\Delta C464}$ (fractions 6 to 8), $NP_{\Delta C440}$ (fractions 8 to 9), $NP_{\Delta C405}$ (fractions 4 to 6), $NP_{\Delta C391}$ (fractions 3 to 5), $NP_{\Delta C380}$ (fractions 6 to 8) and $NP_{\Delta C375}$ (fractions 4 to 6) formed highly organized herringbone-like particles, but with notably different structural lengths (Fig. 3).

The N-terminally deleted protein $NP_{\Delta N26}$, which has a deletion of 25 residues from the N terminus though displaying two peaks on the protein sedimentation profile (Fig. 2), failed to self-assemble to form any herringbone-like structure. This mutated protein, however, did form almost homogeneous aggregates (Fig. 3). Obviously, $NP_{\Delta N26}$ protein has an abnormal NP–NP interaction which readily explains its inability to fold properly. The deleted 25 amino acids are predicted to form an α-helical structure which seems to be necessary for proper folding of NP. Furthermore, with longer deletions up to 121 amino acids (Fig. 3, $NP_{\Delta N122}$) and beyond, the formation of any regular structure was completely abrogated, as no regular, ordered structures were seen. Similar irregular structures were also observed in $NP_{\Delta C245}$, which has 244 amino acids deleted from the C-terminal end (data not shown). Further study of $NP_{\Delta C366}$ was precluded because of the insolubility of the protein, which might result from improper folding. In view of this, it is predicted that $NP_{\Delta C366}$ would be unable to form ring- or herringbone-like particles. $NP_{\Delta C366}$ has a further 9 amino acids deleted compared to $NP_{\Delta C375}$, the last C-terminal deletion mutant with self-assembly capability. Amino acids at position 366 to 375 may therefore be critical in ensuring successful NP–NP contact for self-assembly.

This study shows that the entire N-terminal region up to residue 375 (77 %) is essential for assembly of the nucleocapsid while the C-terminal 114 amino acids, from positions 376 to 489 (23 %), are dispensable for assembly. Sequence analysis of NP proteins of paramyxoviruses revealed that 77 % of the N-terminal region contains the four strongly conserved segments (Kho *et al.*, 2001b). The biological functions of these segments are still unclear but they have been predicted to be involved in NP–NP contacts during assembly (Morgan *et al.*, 1984).

An interesting observation in this NP self-assembly study was that further amino acid deletions from the C-terminal end of the NP protein ($NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$ and $NP_{\Delta C375}$) resulted in the formation of longer herringbone-like structures as compared to mutated proteins with shorter deletions ($NP_{\Delta C464}$ and $NP_{\Delta C440}$) (Fig. 3). This suggests that the tendency of these mutated proteins to form a relatively long structure was the result of amino acid deletions covering positions 406 to 489, which somehow enhance the polymerization of NP monomers. This might be achieved via some secondary structural changes of these NP mutant polypeptides in an unknown manner that permits a stronger contact between the truncated NP monomers. Subsequently, upon sucrose gradient centrifugation, these herringbone-like particles which have a higher stability could withstand the centrifugal forces better. Thus, the monomeric ring was not easily shed from the helices of the herringbone-like particles in mutated proteins $NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$ and $NP_{\Delta C375}$ compared to $NP_{\Delta C464}$ and $NP_{\Delta C440}$, which formed rather short herringbone-like particles, presumably due to stronger NP–NP or NP–RNA interactions.

The phenomenon of monomeric ring structures being shed continuously from helical structures during sucrose gradient centrifugation was suggested by Bhella *et al.* (2002) in their study on the nucleocapsid morphology of measles virus, simian virus 5 (SV5) and respiratory syncytial virus. The prediction that the C terminus of NP (amino acid positions 376 to 439) may enhance the NP

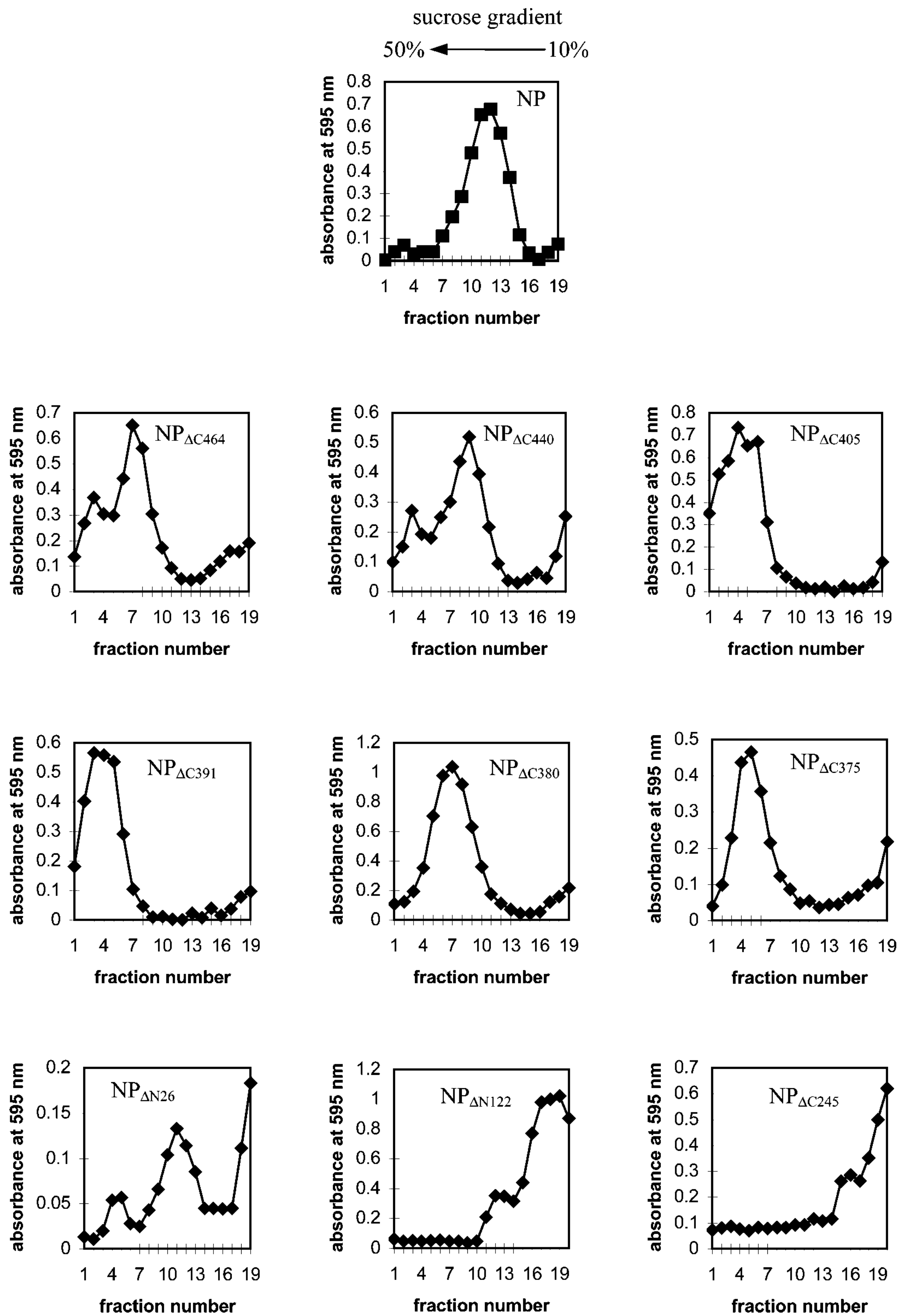

Fig. 2. Protein sedimentation profiles of truncated NP proteins. After ammonium sulphate precipitation, the proteins were subjected to 10 to 50% sucrose gradient centrifugation at 38 000 r.p.m. (rotor SW 41 Ti) for 5 h. The respective purified proteins were fractionated (0·5 ml each) and the total protein content was determined by the Bradford assay. The sedimentation profile of full-length NP was also included for comparison purposes.

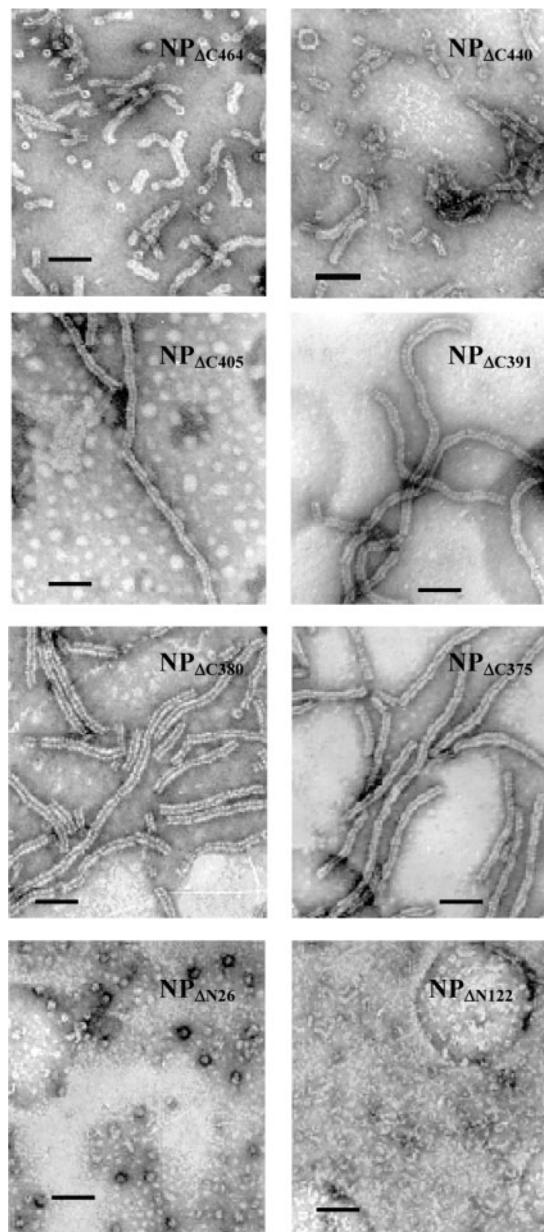

Fig. 3. Electron micrographs of N- and C-terminally truncated NP proteins. Samples of each truncated protein were pooled from fractions that constitute the tip of the peak based on the protein sedimentation profile and further confirmed by SDS-12 % polyacrylamide gel analysis. The pooled proteins were dialysed and stained with 2 % uranyl acetate. $NP_{\Delta C464}$ showed the presence of ring and short herringbone-like particles. Similar structures were observed in $NP_{\Delta C440}$. Relatively longer herringbone-like particles were observed for $NP_{\Delta C405}$, $NP_{\Delta C391}$, $NP_{\Delta C380}$ and $NP_{\Delta C375}$. $NP_{\Delta N26}$ protein formed nearly homogeneous aggregates. No regular, ordered structures were seen in $NP_{\Delta N122}$, indicating complete loss of NP–NP interaction in this mutant. Bars represent 100 nm.

polymerization activity remains a brave assumption since there is no precedent for this observation in other non-segmented single-stranded RNA viruses. In addition, it has been reported that nucleocapsids formed by tail-less NP protein of SV5 appeared to be more rigid (Mountcastle *et al.*, 1974). On the other hand, removal of the C-terminal 23 amino acids of the nucleoprotein of influenza virus, a segmented single-stranded RNA virus, increased the protein oligomerization activity (Elton *et al.*, 1999). This supports the notion that deletion of the C terminus of NDV NP may allow a stronger NP–NP contact, resulting in the formation of long herringbone-like structures. Nevertheless, further investigation along these lines should prove worthwhile.

This NP protein self-assembly study demonstrates that the NP–NP binding sites are located in the first 375 amino acids at the N terminus. The C terminus, on the other hand, is not required for nucleocapsid assembly but may play a regulatory role in NP polymerization. The important role of this part, as well as other NP functional domains, remains to be explored further. In this regard, targeting conserved amino acids for site-directed mutagenesis coupled with function analysis could be a fruitful approach to further delineate the multiple important regions of the NP protein.

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