The Journal of Microbiology, December 2001, p.293-299 Vol. 39, No. 4
Copyright © 2001, The Microbiological Society of Korea

Production of the Nucleocapsid Protein of Newcastle Disease Virus in *Escherichia coli* and its Assembly into Ring- and Nucleocapsid-like Particles

Chiew Ling Kho, Wen Siang Tan and Khatijah Yusoff*

Department of Biochemistry and Microbiology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.

(Received July 11, 2001 / Accepted September 21, 2001)

The nucleocapsid (NP) protein of Newcastle disease virus (NDV) and its derivative (NP_{cfus}) containing the *myc* region and six histidine residues fused to its C-terminus were expressed abundantly in *Escherichia coli*. The proteins were purified by sucrose gradient centrifugation. Both the NP and NP_{cfus} proteins self-assembled into ring-like particles with a diameter of 24 ± 2 nm around a central hole of 7 ± 1 nm. Some of these ring-like particles stacked together to form nucleocapsid-like structures which are heterogeneous in length with a diameter of 20 ± 2 nm and a central hollow of 5 ± 1 nm. Only a very small amount of the monomers in the particles was linked by inter-molecular disulfide bonds. Fusion of the C-terminal end to 29 amino acids inclusive of the *myc* epitope and His-tag did not impair ring assembly but inhibited the formation of the long herringbone structures. Immunogold labeling of the particles with the anti-*myc* antibody showed that the C-terminus of the NP_{cfus} protein is exposed on the surface of these ring-like particles.

Key words: protein assembly, fusion protein, NDV, nucleocapsid

Newcastle disease virus (NDV) is the prototype of avian paramyxovirus (2), which causes the highly contagious Newcastle disease (ND) in many avian species, resulting in substantial losses in the poultry industry worldwide. Its genome is a linear, non-segmented, single-stranded negative sense RNA with a molecular weight of $5.2\text{-}5.7 \times 10^3$ kDa, or approximately 15,186 nucleotides (19, 22) which encodes six main structural proteins (23). The genomic RNA is associated with the nucleocapsid (NP), phospho- (P) and large (L) proteins. These macromolecules form the transcriptive complex of the virus, which in turn is surrounded by a lipid bilayer membrane derived from the plasma membrane of its host cell (21). Embedded in the membrane are the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. Beneath the lipid bilayer is a shell of protein known as the matrix (M) protein, which is believed to interact with the transcriptive complex (20). Both the HN and F glycoproteins are thought to work together synergistically in a rather complex manner in their association with a host cell receptor that has yet to be identified (25).

Electron microscopic investigations of the isolated NP of NDV (8) and other paramyxoviruses [Sendai virus (16), simian virus 5 (9), mumps virus (17), and measles virus (10)] revealed that they are of closely similar appearance resembling classical herringbone morphology. In virtually all cases, flexible helical structures of about 18 nm in diameter and 1 μm in length were observed. The essential subunit of the NP is a single polypeptide of about 500 amino acids with a molecular weight of about 60 kDa (18): NDV (53 kDa), simian virus 5 (56 kDa), Sendai virus (57 kDa), measles virus (58 kDa), and mumps virus (61 kDa). It has been suggested that around 2200-2600 NP subunits encapsulate the viral RNA (vRNA) and protect it against ribonuclease attack (6).

The NP protein of Sendai virus expressed in mammalian cells, in the absence of the vRNA and any other viral proteins, were shown to assemble into structures morphologically equivalent to those isolated from the intact virions (5). This observation has also been reported for the NP protein of measles virus when it is expressed alone in insect cells (11). However, there is no information available on the assembly of the NP protein although its sequence has been reported (18). Therefore, the purpose of this research was to study the assembly of the full-length and C-terminal fusion NP proteins expressed in *E. coli*.

Materials and Methods

Virus propagation and purification

NDV strain AF2240 was grown in the allantoic cavity of

* To whom correspondence should be addressed.
(Tel) 603-89486101, 3559; (Fax) 603-89430913
(E-mail) khatijah@fsas.upm.edu.my

8 to 9-day-old chicken embryonated eggs according to the procedures of Blaskovic and Styk (4). The allantoic fluid was then clarified by centrifugation at 6000 × g for 20 min, at 4°C. For virus purification, NDV particles were first concentrated by spinning at 40,000 × g for 3 h and dissolved in NTE buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM EDTA). The concentrated virus particles were layered on top of a 10 to 50% (w/v) linear sucrose gradient in NTE buffer and centrifuged at 100,000 × g for 4 h. The virus band was collected, diluted 5 × with NTE buffer, pelleted by centrifugating at 100,000 × g for 2 h, and resuspended in a small volume of NTE buffer.

Genomic RNA extraction, RT-PCR and cloning

Total RNA was extracted from 250 µl of the NDV infected allantoic fluid using the Trizol LS reagent (Gibco BRL, USA). Three primers: NPf1 (5'CCT TCT GCC AAC ATG TCT TC 3'), NPr1 (5'TCA ATA CCC CCA GTC GGT GT 3'), and NPr2 (5' ATA CCC CCA GTC GGT GTC 3') were used to amplify the NP gene. Incorporation of primers NPf1 and NPr1 produced a PCR product containing a stop codon at its 3-end, while primers NPf1 and NPr2 gave rise to a gene product which had no stop codon. Synthesis of the first strand cDNA was carried out in 30 µl of reaction mixture containing 0.4 µM each of the primer pairs, 0.2 mM of each deoxynucleoside triphosphate (MBI Fermentas, Inc., USA), 5 U of AMV reverse transcriptase (Promega, USA), 8 U of RNase inhibitor (Gibco BRL, USA), 1.5 mM of $MgCl_2$ and 1 × of reaction buffer [50 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, and 0.1% Triton X-100]. The mixture was incubated at 42°C for 30 min and then heated at 94°C for 3 min. A total of 20 µl PCR mixture containing 1 U DyNAzyme EXT DNA polymerase (FINNZYMES, Finland), 1.5 mM of $MgCl_2$ and 1 × of reaction buffer was then added to the above cDNA mixture and subjected to a 30-cycle PCR profile of 94°C/30s, 55°C/50s and 72°C/1min, followed by a final extension step of 72°C/7 min.

The PCR products were analyzed on 1% TAE agarose gels; DNA bands were recovered from the gels and ligated with the pTrcHis2 vector (Invitrogen, USA) at 25°C for 5 min. The ligation mixture was transformed into *E. coli* strain TOP10 (Invitrogen, USA). Recombinant plasmids were isolated from the putative clones which were screened by PCR and sequencing.

Protein expression, SDS-PAGE and Western blotting

The positively identified clones were cultured in LB medium containing 50 µg/ml ampicillin at 37°C. When the cultures reached A_{600} around 0.6 to 0.8, 1 mM of IPTG was added into the cultures and shaking was continued for 3-5 h. One ml of the cells was pelleted by centrifugation and resuspended in 100 µl of 1 × SDS-PAGE sample buffer and boiled for 10 min. Five µl of the samples were then loaded onto an SDS-12% polyacrylamide gel and electrophoresed at 32 mA. The proteins on the gel were then transferred onto a nitrocellulose membrane and blocked with 1:10 dilution of skim milk (KPL, USA) for 1 h. The diluted anti-NDV chicken serum or anti-*myc* monoclonal antibody (for fusion protein) was added to the membrane and shaken for 1 h. The membrane was then thoroughly washed with TTBS washing solution (TBS containing 0.5 % Tween 20) and exposed to peroxidase-labeled antibody for another 1 h. The membrane was again washed thoroughly before BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) solution was added as substrate for the peroxidase.

Purification of NP protein

E. coli harboring plasmids pTrcHis2-NP or pTrcHis2-NP-c, respectively encoding either the wild-type NP protein or its fusion derivative (NP_{cfus}) were grown at 37°C in LB medium supplemented with 50 µg/ml ampicillin. Protein expression was induced by the addition of 1 mM IPTG when the cultures reached A_{600} around 0.6 to 0.8. After overnight shaking, the cells were harvested by centrifugation at 10,000 × g for 15 min at 4°C, resuspended in lysis buffer [50 mM Tris (pH7.9), 0.1% Triton X-100, 0.2 mg/ml lysozyme, and 4 mM $MgCl_2$]. The lysate was then treated with 5 µg/ml RNase and DNase for 30 min at 25°C. The cell extract was recovered by centrifugation at 20,000 × g for 20 min at 4°C and precipitated by ammonium sulphate (0 to 60% saturation). The precipitate was pelleted by centrifugation under the same conditions and dialyzed extensively in dialysis buffer (50 mM Tris, 100 mM NaCl, pH 7.8). The dialyzed solution was applied to 10-50% sucrose gradient and centrifuged at 110,000 × g for 5 h at 4°C. Fractions containing the NP protein were pooled, concentrated with a 100 kDa cut-off Centricon centrifugal filter (Millipore, USA) and dialyzed.

Immunogold labeling and electron microscopy

About 15 µl of the purified NP_{cfus} proteins were absorbed to carbon coated grids and incubated with the anti-*myc* antibody with a 1 : 50 dilution in blocking buffer [1% (w/v) BSA in PBS, pH 7.4] at room temperature for 1 h. The grids were then washed four times with blocking buffer for 1 min each and followed by incubation with 5 nm gold particles conjugated with protein A (BBI, UK) at a 1 : 10 dilution in blocking buffer. After incubation for 1 h, the grids were washed four times with blocking buffer and another three times with dH_2O before finally being stained with 2% uranyl acetate. The grids were viewed under the Hitachi H-7100 transmission electron microscope (TEM). The diameters of the ring-like particles were measured as described (10).

Virus disruption assay

Purified virus particles were disrupted by adding an equal volume of disruption buffer (1% Triton X-100, 2 mM

DTT, 150 mM NaCl, 10 mM Tris-HCl, pH7.4) to the purified virus and incubated for 5 min at room temperature. Fifteen µl of the disrupted viral particles were then absorbed to carbon-coated grids, negatively stained with methylamine tungstate, and examined with the electron microscope.

Results

Expression of NP and its C-terminal fusion proteins in E. coli

Plasmids pTrcHis2-NP and pTrcHis2-NP-c, respectively encoding the NP protein and its derivative (NP_{cfus}) which has a C-terminal extension of 29 amino acids inclusive of the *myc* epitope (10 residues) and six His residues were constructed and introduced into *E. coli* strain TOP10 cells. Expression of these proteins was under the control of the inducible *trc* promoter with IPTG. Western blot analysis of cell lysates (Fig. 1a) with the anti-NDV chicken serum gave rise to protein bands of approximately 53 kDa (lane 3) and approximately 58 kDa (lane 2), which respectively, correspond to the molecular mass of the NP and NP_{cfus} proteins. In addition, three to four protein bands with molecular masses smaller than the NP protein and its derivative were also observed. These smaller fragments could have been generated by proteolytic degradation of the full-length NP protein which were also observed in protein preparations from NDV grown in chick embryos (12). In addition, the NP_{cfus} protein carrying the *myc* epitope which migrated as a single band at about 58 kDa also gave positive signals with the anti-*myc* monoclonal antibody (Fig. 1b, lane 1).

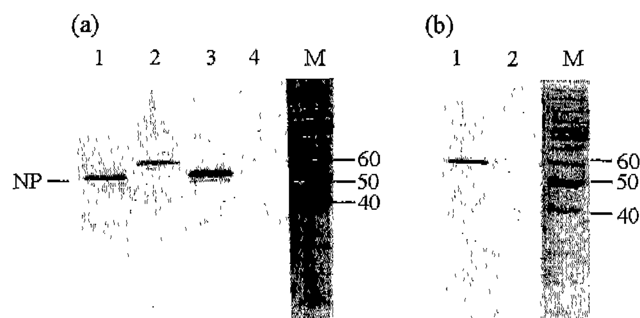

Fig. 1. Western blot analysis of the expressed NP and NP_{cfus} proteins. (a) NP and NP_{cfus} proteins were fractionated on an SDS-12% polyacrylamide gel, electrotransferred to a nitrocellulose membrane and probed with the anti-NDV chicken serum. The size of the recombinant NP protein (lane 3) corresponds to the NP protein of the purified NDV (lane 1) which is about 53 kDa. The NP_{cfus} protein appeared as a sharp band of about 58 kDa (lane 2). No band was detected in the control cell lysate from *E. coli* cell harboring pTrcHis2 vector alone (lane 4). The NP_{cfus} protein was also probed with the anti-*myc* monoclonal antibody (b) which detects the *myc* epitope fused to it. Again, a sharp band of about 58 kDa was detected; no band was observed in the cell lysates of the controls (lane 2). Lane M, molecular mass markers are indicated in kDa.

Purification of NP particles

Salting out of the recombinant proteins from the cell extracts by increasing the concentration of ammonium sulphate gradually showed that most of the NP and NP_{cfus} proteins precipitated between 0 to 10% and 10 to 25% saturation, respectively. These precipitated fractions were then purified by sucrose gradient centrifugation. The sedimentation profiles of each of these proteins showed a peak which contained reasonably pure NP (Fig. 2a; fractions 7 to 18) and NP_{cfus} proteins (Fig. 2b; fractions 1 to 15) as indicated by SDS-PAGE (Fig. 2c and d). This observation suggests that both the NP and its derivative C-terminal extension proteins could self-assemble into particles of different sizes.

NP protein self-assembles into ring-like particles and nucleocapsid-like structures

Electron microscopic examination of negatively stained samples from the tip of the peaks (NP: fractions 12 to 14; NP_{cfus}: fractions 11 to 13) revealed that the NP and NP_{cfus} proteins associated to form ring particles with a diameter of 24 ± 2 nm (n=50), and a central hole of 7 ± 1 nm (n=50) (Fig. 3a and b). As the sedimentation rate is dependent upon particle mass, the early fractions of the peak should be enriched in larger particles and later fractions in smaller particles. Fractions from the edge of the peaks (NP: fractions 7 to 8 and 17 to 18; NP_{cfus}: 2 to 6 and 14 to 15) were therefore pooled, dialyzed and concentrated separately. Examination of the first two fractions of the NP protein under a TEM revealed structures (Fig. 3c) that are morphologically very similar to fractured nucleocapsids released from ruptured NDV particles (Fig. 3e). The nucleocapsid-like structures have a stacked-ring structure (Fig. 3c, indicated by arrows) with a width of 20 ± 2 nm (n=10), but are heterogeneous in length. They also have a central channel with a diameter of approximately 5 ± 1 nm (n=10). A very small amount of rather short nucleocapsid-like structures were also observed in the first few fractions of the NP_{cfus} protein (Fig. 3d), suggesting that fusion with the extra peptide sequences at the C-terminal end of the NP protein did, to a certain extent, disrupt the assembly of nucleocapsid-like structures. The last two fractions of the peaks were found to contain predominantly incomplete ring particles (Fig. 3f and g). The above structures were not found in protein extracts from *E. coli* harboring the expression vector without the NP coding region (data not shown).

To study whether the C-terminal portion of the NP_{cfus} protein is exposed on the surface of the ring-like particles, the purified samples (Fig. 2b, fractions 11 to 13) from the sucrose gradient were treated with the anti-*myc* monoclonal antibody, followed by protein A conjugated with 5

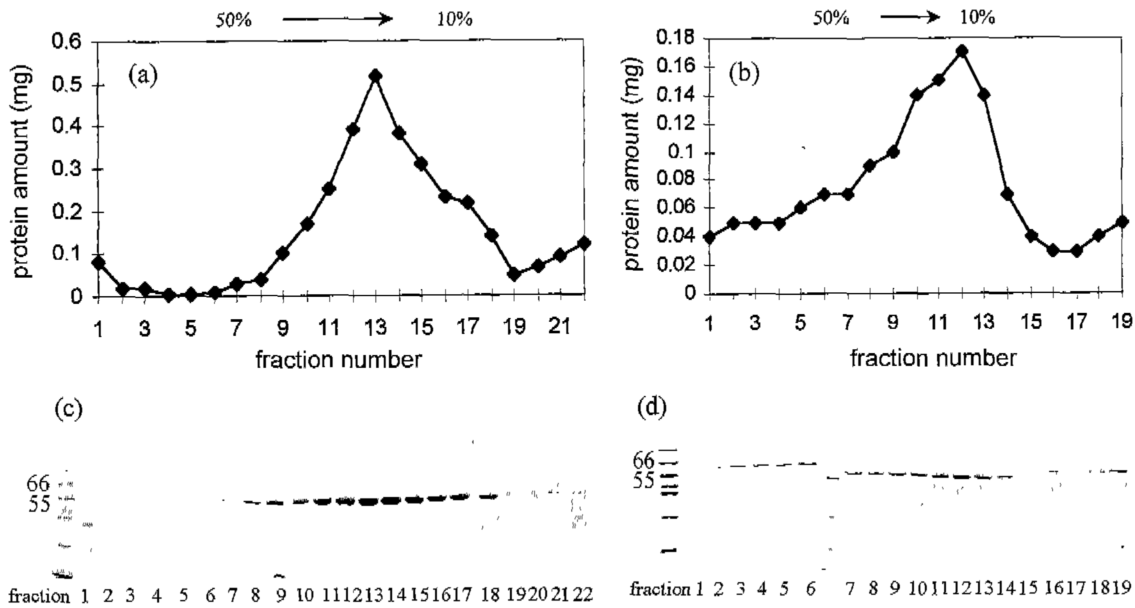

Fig. 2. Protein sedimentation profiles of NP and NP_{cfus} proteins. After ammonium sulphate precipitation, both the NP and NP_{cfus} proteins were subjected to 10-50% sucrose gradient centrifugation at 110,000 × g for 5 h. The respective purified proteins were fractionated (0.5 ml each) and the total protein content in each fraction was determined by the Bradford assay. (a) and (b) represent the sedimentation profiles for NP and NP_{cfus} proteins, respectively. The fractionated NP (c) and NP_{cfus} (d) proteins were separated by SDS-12% PAGE, and stained with Coomassie brilliant blue.

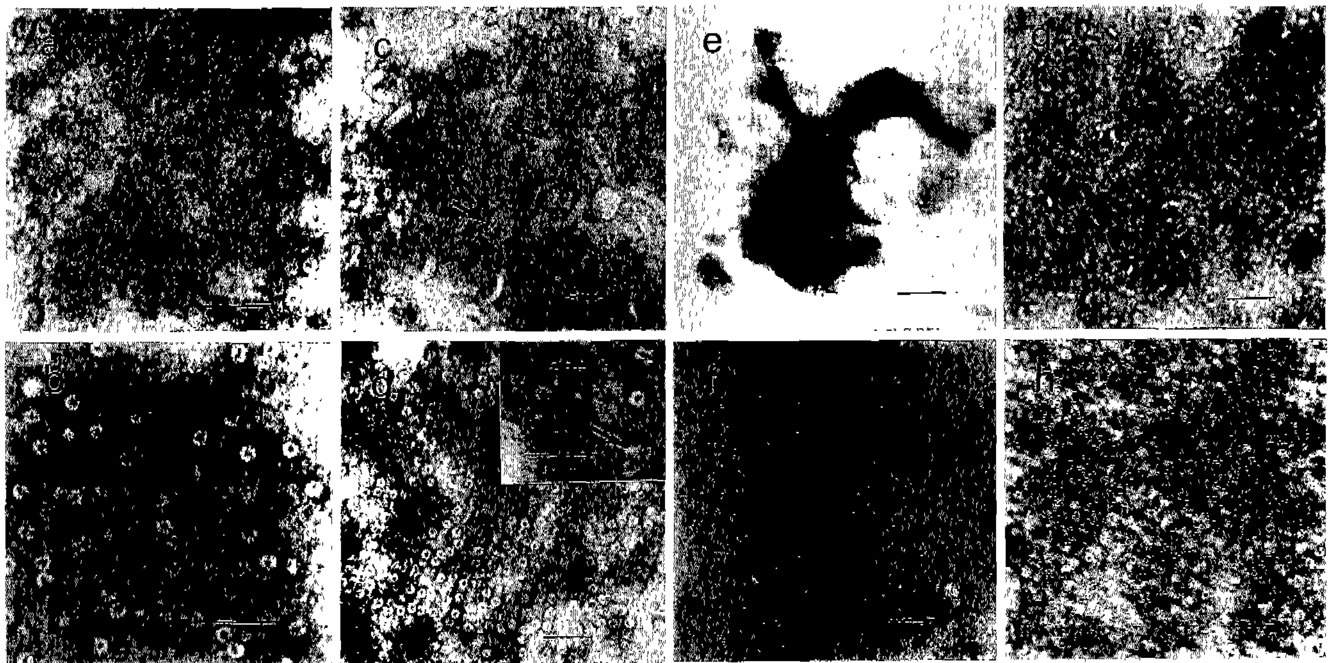

Fig. 3. Electron micrographs of the purified NDV recombinant NP and NP_{cfus} proteins that form ring- and nucleocapsid-like particles. (a) Samples from fractions 12 to 14 of the NP protein (Fig. 2a) which constitute the tip of the peak showed the presence of ring-like structures. (b) Similar structures were also observed in the NP_{cfus} protein of fractions 11 to 13 (Fig. 2b). (c) Examination of the NP protein from fractions 7 to 8 (Fig. 2a) revealed the nucleocapsid-like structure. (d) Comparatively short nucleocapsid-like structures (inset, indicated by arrows) were seen in a very small amount in the NP_{cfus} protein of fractions 2 to 6 (Fig. 2b). (e) The nucleocapsid structures which were released from the disrupted NDV particles. (f) and (g) NP and NP_{cfus} proteins from fractions 17 to 18 (Fig. 2a) and 14 to 15, (Fig. 2b) respectively. These fractions mainly consisted of a mixture of complete and incomplete ring-like particles. The NP_{cfus} proteins were also immunogold labeled with 5 nm gold particles (h) to locate the C-terminal portion of the NP_{cfus} protein. Gold particles which appeared as black granules seemed to be located on the surface of the ring-like structures suggesting that the C-terminal end is exposed on the surface of the ring-like particles. Each bar represents 100 nm.

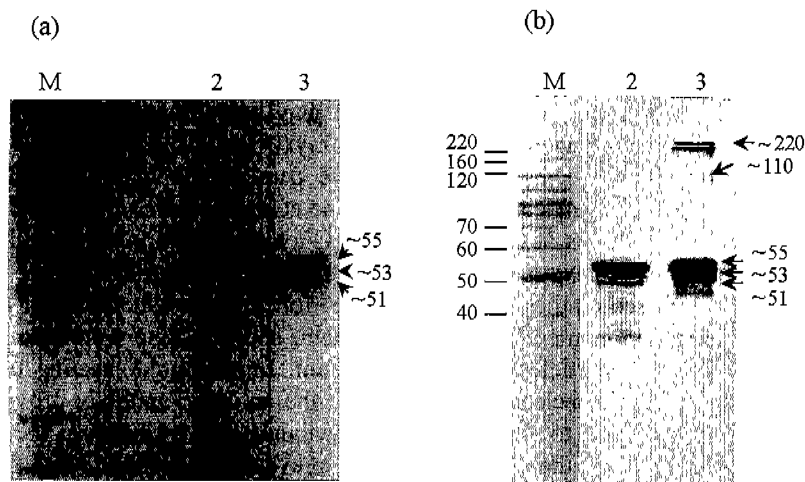

Fig. 4. Analysis of NP protein under reducing and non-reducing conditions. NP protein was analyzed by SDS-12% PAGE under both reducing and non-reducing conditions (a), then Western blotted and probed with the anti-NDV chicken serum (b). (a) Under reducing conditions, NP protein migrated as a major band of about 53 kDa (lane 2). The protein under non-reducing conditions migrated as three bands with molecular masses of approximately 51, 53 and 55 kDa (lane 3). (b) A very small portion of the protein under non-reducing conditions migrated as a dimer (about 110 kDa) and a tetramer (about 220 kDa), which could only be seen after prolonged incubation with the anti-NDV chicken serum on a Western blot (lane 3, indicated by arrows). GelBase Software (UVP Inc, USA) was used to estimate the relative amount of each band as percent of total. Lane 1, molecular mass markers are indicated in kDa.

nm gold particles. The gold particles which appeared as black granules were located on the surface of the ring-like particles (Fig. 3h), suggesting that the C-terminus of the NP protein is exposed on the surface of the ring-like particles.

Reducing and non-reducing SDS-PAGE of NP particle
The ring- and nucleocapsid-like particles migrated as a major band of monomer, about 53 kDa, under reducing conditions (Fig. 4a and b, lane 2). Under non-reducing conditions, more than 95% of the protein migrated as monomers which appeared as three distinct bands with apparent molecular masses of about 55 (about 24% of total protein), 53 (about 48%) and 51 (about 24%) kDa (Fig. 4a and b, lane 3), and only a very small amount ($<$ 5%) of the protein was detected as dimer (about 110 kDa) or tetramer (about 220 kDa) on a Western blot after prolonged incubation with the anti-NDV chicken serum (Fig. 4b, lane 3). In the presence of 1 mM of $CuSO_4$ as the oxidizing agent and analyzed under reducing and non-reducing conditions, the protein profiles were similar to that of untreated samples (data not shown).

Discussion

The capsid of viruses comprises many copies of either one or a few polypeptide chains that package(s) the nucleic acid genome and protects it from nuclease activities. A substantial body of evidence showed that capsid proteins of different viruses synthesized either in the eukaryotic or prokaryotic system can self-assemble and encapsulate their nucleic acids. In the case of NDV, a member of the *paramyxovirus*, neither the mechanism(s) of NP assembly nor genome encapsulate is clearly understood. To date, the three dimensional structures of the paramyxoviral nucleocapsids or their subunits at atomic resolution have not been resolved. Perhaps the heterogeneous length, and inherent fragility and flexibility of the isolated nucleocapsids, as shown by electron microscopy (10), do not allow the nucleocapsids to crystallize easily. In contrast, the capsids of other viruses such as hepatitis B virus (26), tobacco mosaic virus (15), foot-and-mouth disease virus (1), common cold virus (3), tomato bushy stunt virus (13), and polio virus (14) which form either a rod-shaped or spherical shell have been studied in detail at atomic resolution.

In order to gain an insight into the architecture of the NDV nucleocapsid, we therefore employed *E. coli* to express high levels of its subunit because the synthesis of this protein does not require post-translational processing such as membrane translocation, acylation or glycosylation. The proteins expressed in this system self-assembled predominantly into ring-like particles, a phenomenon which was not inhibited by fusing a peptide of 29 amino acids containing two antigenic sequences to its C-terminal end. The diameter (24 ± 2 nm) of the ring-like particles is significantly larger than the width of the viral nucleocapsids that has been reported to be around 18 nm (10). Besides, the diameter of the central hole (7 ± 1 nm) is around 2 nm larger than that of the hollow of intact nucleocapsid (5 nm) (10). The ring-like particles which form a nucleocapsid-like herringbone structure appeared to be loosely held compared to that of the virion derived nucle-

ocapsid. Moreover, the heterogenous lengths of the nucleocapsid-like structures were significantly shorter than those of intact virions. These observations imply that the vRNA may serve as a template for the NP proteins to associate into a relatively compact and longer nucleocapsid. In most orientations, the ring-like particles contain spikes radiating from the outer edge of the rings, suggesting that these spikes constitute the projections of the viral nucleocapsid that show a similar herringbone structure to that of other paramyxoviruses (10). Based on these observations we propose that several NP monomers form a ring-like particle and that several of these particles assemble to form a nucleocapsid. It is, however, unclear at this stage whether the NP subunits assembled to form the ring- and nucleocapsid-like particles inside the cells or/ and during preparation of the cell lysates. Theoretically, sectioning of the bacterial cells expressing the protein subunits with an ultramicrotome followed by electron microscopy should be able to provide a clearer picture of this process.

Both the ring- and nucleocapsid-like particles gave identical results when analyzed on SDS-PAGE, under reducing or non-reducing conditions, suggesting that the particles are made up from the same subunits. It is likely that these subunits possess at least three different conformations due to intra-molecular disulfide bonds, because under non-reducing conditions, two extra monomer bands, each constitute about a quarter of the total protein were present; one migrated slightly faster (51 kDa) and another slower (55 kDa) than the reduced sample (53 kDa). This finding accords with that of nucleocapsids isolated from intact virions (24), suggesting that the NP monomer produced in *E. coli* folds to its native conformations. Furthermore, the amino acid sequence of the NP monomer contains 3 cysteine residues (18) which would allow in different combinations, the formation of three intra-molecular disulfide bonding patterns. The significance of the multiple forms of NP monomers is still unknown, but their existence may reflect requirements for conformationally distinct NP monomers in the assembly of the ring- and nucleocapsid-like structures.

Only a very small amount of inter-molecular disulfide bonds was detected, suggesting that the conformations of the ring- and nucleocapsid-like particles are mainly held by non-covalent forces, which provide a possible explanation to the observation that the nucleocapsids tend to fracture randomly and easily during isolation. Furthermore, the contacts between the monomers and the ring-like particles have to be maintained in a manner that they remain flexible enough for the nucleocapsid to be tightly coiled within the irregular shape of the viral particles. Sequence analysis of the NP proteins of two newly emergent deadly paramyxoviruses, Hendra virus (GenBank accession number: AF017147) and Nipah virus (GenBank accession number: AF212302), revealed that they do not contain any cysteine residues, but they have been reported to possess similar herringbone-like structures (18, 7). This further suggests that inter-molecular disulfide bonds do not play any important role in the architecture of paramyxoviral nucleocapsids.

We demonstrate in this report that fusion of the 29 amino acids inclusive of the *myc* and His-tag sequences to the C-terminal portion of the full-length NP protein did not impair ring assembly, but the nucleocapsid-like structures formed were shorter than those of non-fusion NP protein, and that they were also present in a very small amount. Immunogold labeling of the ring-like particles with the anti-*myc* antibody proved that the C-termini of the subunits are exposed on the surface of the particles and prevent the adjacent rings from stacking onto each other to form a longer nucleocapsid-like structure. Prevention of the polymerization of the symmetrical ring-like particles into flexible and heterogeneous nucleocapsids will ease the study of the three dimensional structures of the nucleocapsid, in particular, its subunits by crystallography and cryo-electron microscopy.

Expression of the NP protein in *E. coli* and its assembly into ring- and nucleocapsid-like particles, and the fractionation of these two forms of particles on sucrose gradient centrifugation, can be easily employed to identify the minimum contiguous sequences that are required for NP-NP interactions. This can, in principle, be achieved by deletion mutagenesis of the NP gene, expression of the mutants in *E. coli*, and followed by electron microscopy. The generated mutants can also be used to study the mechanism of genome encapsidation by allowing the purified mutants to assemble *in vitro*, in the presence and absence of the viral RNA and other nucleic acids. The system can also be extended to investigate the domains involved in NP-P, NP-L and NP-M interactions to understand the regulatory events in the life cycle of NDV and other paramyxoviruses.

Acknowledgements

This study was supported by the IRPA Grant No. 01-02-04-0107 from the Ministry of Science, Technology and Environment of Malaysia. C.L. Kho is supported by the National Science Fellowship of Malaysia.

References

1. Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands, and F. Brown. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9Å resolution. *Nature* 337, 709-716.
2. Alexander, D.J. 1988. Newcastle disease virus-An avian paramyxoxirus, p. 11-22. *In* D.J. Alexander (ed.), Newcastle disease. Kluwer Academic Publishers, London.
3. Arnold, R. and M.G. Rossmann. 1990. Analysis of the structure of a common cold virus, human rhinovirus 14, refined at a resolution of 3.0Å. *J. Mol. Biol.* 211, 763-801.

4. Blaskovic, D. and B. Styk. 1967. Laboratory methods of virus transmission in multicellular organisms, p. 163-233. *In* K. Maramorasch and H. Koprocvski. (eds.), Virology, Vol. 1. Academic Press, New York.
5. Buchholz, C.J., D. Spehner, R. Drillien, W.J. Neubert, and H.E. Homann. 1993. The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. *J. Virol.* 67, 5803-5812.
6. Choppin, P.W. and R.W. Compans. 1975. Reproduction of paramyxoviruses, p. 95-178. *In* H. Fraenkel-Conrat and R.R. Wagner (eds.), Comprehensive Virology. Plenum, New York.
7. Chua, K.B., W.J. Bellini, P.A. Rota, B.H. Harcourt, A. Tamin, S.K. Lam, T.G. Ksizek, P.E. Rollin, S.R. Zaki, W.J. Shieh, C.S. Goldsmith, D.J. Gubler, J.T. Roehrig, B. Eaton, A.R. Gould, J. Olson, H. Field, P. Daniels, A.E. Ling, C.J. Peters, L.J. Anderson, and B.W.J. Mahy. 2000. Nipah virus: a recently emergent deadly paramyxovirus. *Science* 288, 1432-1435.
8. Compans, R.W. and P.W. Choppin. 1967. The length of the helical nucleocapsid of Newcastle disease virus. *Virology* 33, 344-346.
9. Compans, R. W. and P.W. Choppin. 1967. Isolation and properties of the helical nucleocapsid of the parainfluenza virus SV5. *Proc. Natl. Acad. Sci. USA.* 57, 949-956.
10. Finch, J. T. and A.J. Gibbs. 1970. Observations on the structure of the nucleocapsids of some paramyxoviruses. *J. Gen. Virol.* 6, 141-150
11. Fooks, A.R., J.R. Stephenson, A. Warnes, A.B. Dowsett, B.K. Rima, and G.W.G. Wilkinson. 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. *J. Gen. Virol.* 74, 1439-1444.
12. Hamaguchi, M., T. Yoshida, K. Nishikawa, H. Naruse, and Y. Nagai. 1983. Transcriptive complex of Newcastle disease virus 1. Both L and P proteins are required to constitute an active complex. *Virology* 128, 105-117.
13. Harrison, S.C. and A. Jack. 1975. Structure of tomato bushy stunt virus. Three- dimensional x-ray diffraction analysis at 16 Å resolution. *J. Mol. Biol.* 97, 173-191.
14. Hogle, J.M., M. Chow, and D.J. Filman. 1985. Three dimensional structure of polio virus at 2.9Å resolution. *Science* 229, 1358-1365.
15. Holmes, K.C., G.J. Stubbs, E. Mandelkow, and U. Gallwitz. 1975. Structure of tobacco mosaic virus at 6.7Å resolution. *Nature* 254, 192-196.
16. Hosaka, Y., H. Khano, and S. Ikeguchi. 1966. Studies of the pleomorphism of HVJ virion. *Virology* 29, 205-221.
17. Hosaka, Y. and K. Shimizu. 1968. Lengths of the nucleocapsids of Newcastle disease and mumps viruses. *J. Mol. Biol.* 35, 369-373.
18. Kho, C.L., W.S. Tan , and K. Yusoff. 2001. Sequence analysis of the nucleoprotein of a Newcastle disease virus heat resistant strain: Comparison with other members of the *Paramyxoviridae. J. Biochem. Mol. Biol. & Biophys.* (in press).
19. Krishnamurthy, S. and S.K. Samal. 1998. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *J. Gen. Virol.* 79, 2419-2424.
20. Markwell, M.A. and C.F. Fox. 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. *J. Virol.* 33, 152-166.
21. Peeples, M.E. 1988. Newcastle disease virus replication, p.45-78. *In* D.J. Alexander (ed.), Newcastle disease. Kluwer Academic Publishers, London.
22. Phillips, R.J., A.C.R. Samson, and P.T. Emmerson. 1998. Nucleotide sequence of the 5-terminus of Newcastle disease virus and assembly of the complete genomic sequence: agreement with the "rule of six". *Arch. Virol.* 143, 1993-2002.
23. Samson, A.C.R. 1988. Virus structure, p. 23-44. *In* D.J. Alexander (ed.), Newcastle disease. Kluwer Academic Publishers, London.
24. Smith, G.G. and L.E. Hightower. 1981. Identification of the P proteins and other disulfide-linked and phosphorylated proteins of Newcastle disease virus. *J. Virol.* 37, 256-267.
25. Stone-Hulslander, J. and T.G. Morrison. 1997. Detection of an interaction between the HN and F proteins in Newcastle disease virus-infected cells. *J. Virol.* 71, 6287-6295.
26. Wynne, S.A., R.A. Crowther, and A.G. Leslie. 1999. The crystal structure of the human hepatitis B virus capsid. *Mol. Cell.* 3, 771-780.