Cloning and expression of the HN gene from the velogenic viscerotropic Newcastle disease virus strain AF2240 in *Sf9* insect cells

H. K. A. Ong¹, A. M. Ali², A. R. Omar³ & K. Yusoff^{1*}

¹ Department of Biochemistry and Microbiology, ² Department of Biotechnology, and ³ Department of Veterinary Pathology and Microbiology, Universiti Putra Malaysia, 434400, Serdang, Selangor, Malaysia

(* Author for correspondence, E-mail: khatijah@fsas.upm.edu.my)

Received 29 December 1999; accepted 13 January 2000

Key words: baculovirus, cloning, haemagglutinin-neuraminidase, Newcastle disease virus, recombinant protein expression

Abstract

The haemagglutinin-neuraminidase (HN) gene of Newcastle disease virus (NDV) strain AF2240, amplified from the viral genomic RNA (~1.8 kb) was directionally cloned and inserted into a baculovirus expression vector system. The recombinant glycoprotein expressed in *Spodoptera frugiperda* (*Sf9*) cells showed haemagglutinin (HA), neuraminidase (NA) and hemadsorption activities. HA activity was detected in both extra- and intra-cellular recombinant HN (recHNAF2240) samples. In addition, both HA and hemadsorption activities were inhibited by polyclonal anti-NDV sera. Furthermore, significant expression of the recombinant protein was observed on the surface of infected cells. SDS-PAGE analysis revealed the presence of visually distinguishable bands between the 70 and 80 kDa in size that were absent in the wild-type samples. Western blot analysis showed that the distinct ~63 kDa band and a ~75 kDa band corresponded to the unglycosylated and glycosylated HN glycoprotein respectively as reported in another study. These observations indicated that the HN recombinant protein was not only expressed on the surface of the infected cells as well as with the viral coat protein, but also appears to be functional.

Introduction

Newcastle disease virus (NDV), an enveloped virus with a negative single-stranded RNA genome encoding for at least six genes, belongs to the *Rubulavirus* genus of the *Paramyxoviridae* family (Murphy, 1995). The Malaysian heat stable, viscerotropic-velogenic NDV strain AF2240 isolated in the 1960s, is a highly virulent strain causing 100% mortality to susceptible chickens and is commonly used as the challenge virus in vaccine trials (Ibrahim et al., 1980; Chulan et al., 1982; Ernawati and Ibrahim, 1984; Ideris et al., 1990). The haemagglutinin-neuraminidase (HN) surface glycoprotein, which has both haemagglutinating (HA) and neuraminidase (NA) activities, plays a major role in infection by mediating virus attachment (Scheid and Choppin, 1973) and also pose as a major target for immune response of the host (Schaper et al., 1988).

Tan et al. (1995) showed that the HN protein codes for 581 amino acids with a calculated *Mr* of 63.8 kDa in which there exists five potential asparagine-linked glycosylation sites. However, unlike the other 13 previously published NDV strains (Sakaguchi et al., 1989), a highly conserved sequence of acid amino residue at position 403 of the HN gene was missing in this strain (Tan et al., 1995) as well as another heat stable variant strain V4 (UPM) (Yusoff et al., 1996). In this report, the entire HN gene was cloned into the baculovirus expression vector system (BEVS) and expressed in insect cells, *Spodoptera frugiperda* (*Sf9*) which has the ability to grow in suspension to obtain high levels of recombinant glycoprotein (Smith et al., 1983). In addition, the HN glycoprotein of NDV is known to undergo glycosylation which is involved in proper folding and epitope recognition of the HN protein of NDV (Nishikawa et al., 1986; Yusoff et

al., 1988; Vidal et al., 1989). Since the BEVS is able to express recombinant proteins complete with post-transcriptional modification in insect cells, the highly glycosylated RecHNAF2240 protein could then be analysed in isolation from the rest of the NDV proteins and subsequently be used for the development of a potential subunit vaccine against NDV.

Materials and methods

Viruses, cell lines, vectors

NDV strain AF2240 was cultivated in 9 to 10-day-old embryonated chicken eggs, purified by 30–60% (w/v) sucrose gradient centrifugation at $275\,000 \times g$ for 4 h at 4 °C, and stored in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, pH 8.0). *Spodoptera frugiperda* (Sf9) cells obtained from RIKEN Cell Bank, Japan were grown in SF900 medium (Gibco, USA). The cells were infected with the wild-type baculovirus, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) obtained from Invitrogen (USA). Extracellular viruses (ECV) from the wild type (wt) and recombinant baculovirus-infected cells were used as inoculum in spinner flask culture of Sf9 for propagation of AcMNPV and recombinant AcMNPV respectively. The baculovirus transfer vector pCR Bac and the AcMNPV DNA were obtained from Invitrogen (USA).

Cloning strategy of the HN gene

Viral RNA of NDV was extracted by a standard proteinase K method (Yusoff et al., 1988). Specific primers flanking the HN region were synthesised based on the published sequence of the HN gene (Tan et al., 1995) with the addition of a *Bam*HI site and an *Eco*RI site at the forward 5'- ATG GAT CCA ACG TCC GTT CTACCG CAT CAC CA-3' and reverse 5'- ATG AAT TCC GAT CCT TGG CAT TGC AGA AG-3' primers, respectively. The forward primer corresponding to the 5' end of the sensed RNA strand of the HN gene contains the initiation codon for the HN protein (underlined). One step RT-PCR was performed using the high fidelity Titan RT-PCR (Boehringer, Mannheim, Germany). The resulting PCR products, digested with *Bam*HI and *Eco*RI were ligated into a similarly digested pCR Bac transfer vector and transformed into competent TOP10F' *E. coli* cells (Invitrogen). Positive transformants were selected on LB plates supplemented with ampicillin (100 µg/ml) and

plasmids were extracted using Wizard Plus Miniprep kit (Promega). Analysis of the recombinant plasmid (pAF2240-1) was done with a double digest using *Bam*HI and *Eco*RI. The plasmid pAF2240-1 was also verified by PCR using the baculovirus polyhedrin forward and the baculovirus (+15) reverse primers (Invitrogen), and partial sequencing at the 5' and 3' terminal ends of the insert (Sanger et al., 1977).

Transfection of recombinant transfer vectors into Sf9 insect cells

Sf9 insect cells were seeded in 3 ml of SF900 serum free medium at 2×10^6 cells/ml. The pAF2240-1 plasmid containing the 5' *lacZ* region was co-transfected with a triple-cut, linearized AcMNPV DNA contributing the 3' *lacZ* region into prepared cells using InsectinePlus liposomes (Invitrogen, USA). The transfection mixtures were incubated at 27 °C for 72 h after which 2 ml of the medium from the mixture were harvested and used for the recombinant viral plaque assay to identify and isolate pure blue recombinants in the presence of Blu-gal (Gibco). Blue plaques formed at 10 days post infection (p.i.) were isolated and transferred into 6 well tissue culture plates with 2 ml of SF-900 medium containing 5×10^5 log-phased Sf9 cells per well. Isolation of the viral DNA from the infected cell lysates was done using Easy-DNA kit (Invitrogen). A standard PCR analysis using the recombinant baculovirus forward and reverse PCR primers (Invitrogen) flanking the insert region was performed to confirm the presence of the insert in the viral genome.

Large-scale virus propagation

Approximately 8 ml of the recombinant-virus infected cell lysates from the second generation (P2) were used to infect 500 ml of Sf9 cells separately at 2×10^6 cells/ml in 1 l spinner flask (Technomara). The culture was incubated at 27 °C with constant stirring (60 rpm) for 6 days after which it was harvested and stored at 4 °C. A plaque assay using a large-scale, high titer stock showed that the recombinant virus had a titer of 6.5×10^7 pfu/ml.

Expression of the HN protein

A standard haemagglutination (HA) assay was carried out on infected cell lysates, virus infected cells (intracellular) and infected cell supernatants (extracellular) by a standard end-point titration using 50 µl of two-fold dilutions and 50 µl of 1% (v/v) chicken

red blood cells (RBC). Neuraminidase (NA) activities of the infected cells were determined by a standard NA test (Aminoff, 1961). For hemadsorption analysis, *Sf9* cells were plated onto 60 mm tissue culture dishes containing 3 ml of medium at 2×10^6 cells/ml and infected at an m.o.i. of 10 with different viruses. At 48 h.p.i., the cells were rinsed three times with phosphate-buffered saline (PBS), pH 7.4 and overlaid with 1% RBC. The latter was allowed to adsorb for 30 min and then washed three times in PBS. Cell monolayers were then examined by phase contrast microscopy for the presence of adsorbed RBC. Haemagglutination inhibition (HI) assays were done by using four haemagglutinating units of either the recHNAF2240 virus or the NDV strain AF2240 virus with a serial dilution of NDV polyclonal anti-sera. Similarly, the hemadsorption inhibition assays were performed by incubating at room temperature the monolayers of the infected *Sf9* cells with NDV polyclonal anti-sera, prior to the addition of 1% RBC.

Indirect Immunofluorescence (IIF)

Indirect immunofluorescence was performed as described by Rossier et al. (1989) with slight modifications. Briefly, *Sf9* cells were infected with wild-type baculovirus (AcMNPV) and recombinant baculovirus (recHNAF2240) at an m.o.i. of 10 and incubated for 72 h. Infected cells were washed with phosphate-buffered saline (PBS, pH 7.4), resuspended thoroughly and 10 μ l of the suspension was added to each slide well to be tested. The slides containing the evenly spread cells were incubated at 37 °C for 30 min to allow attachment of the cells and then fixed with acetone. These cells were then incubated with NDV polyclonal anti-sera (1:200 dilution) at 37 °C for 1 h. After 3 washes with PBS, the cells were incubated as above with a goat anti-chicken FITC-conjugated secondary antibody (1:200) (KPL). A similar washing step was repeated after which the slides were then air-dried, mounted in buffered glycerol (10% PBS, pH 8.0 and 90% glycerol) with a cover-slip and examined under UV microscopy.

SDS-PAGE and Western Blotting

Cell lysates infected with recombinant (recHNAF2240) and wild-type (wt) viral samples were individually pelleted and boiled for 5 min in sample buffer prior to electrophoresis in 12% (w/v) SDS-PAGE (Laemmli, 1970). Gels were then either stained with

Figure 1. Agarose gel electrophoresis of plasmids, digested plasmids, RT-PCR product and PCR product. Supercoiled DNA marker and 1 kb marker are in lanes 1 and 8 respectively. Ethidium bromide stained gels indicated a non-transformed plasmid (lane 2) and a putative recombinant plasmid (lane 3). Upon digestion with *Eco*RI and *Bam*HI, the presence of the insert was detected (lane 5) but not from the non-transformed plasmid (lane 4). A single RT-PCR product of the HN gene (lane 6) and a PCR product using specific primers flanking the HN insert of the recombinant plasmid was also detected (lane 7).

Coomassie blue or electroblotted onto PVDF membranes for Western blot analysis (Wisdom, 1994). The membranes were blocked with milk blocking solution (KPL) and reacted with NDV polyclonal anti-sera (1:200). Blots were developed using alkaline phosphatase-conjugated goat anti-chicken and BCIP/NBT colour reagents according to manufacturer's (KPL) instructions.

Results

Cloning and molecular identification of the recombinant virus

Amplification of the total RNA extracted from pure NDV strain AF2240 produced a single band of approximately 1.8 kb (Figure 1, lane 6) and it appeared to be correlated with the size of the entire HN open reading frame (ORF) as reported earlier (Tan et al., 1995). The putative recombinant plasmid was detected from the plasmid minipreps (Figure 1, lanes 2 and 3). Further analysis using restriction enzymes, *Bam*HI and *Eco*RI which cut the insert from the pAF2240-1 plasmid, showed two bands, one at ~1.8 kb and the other

Figure 2. Agarose gel showing the PCR detection of the HN insert in the DNA isolated from pure recombinant (recHNAF2240) virus (lane 3) and the polyhedrin region of the wt virus infected cells (lane 4). The presence the HN insert and the polyherin region can be observed from a recombinant and wt virus mixture (lane 5). A PCR product; identical in size with the HN insert from the recombinant virus infected cells was detected from the recombinant plasmid using the same primers (lane 2) when compared with the 1 kb ladder (lane 6). Negative control (lane 1).

at ~4.8 kb (Figure 1, lane 5). PCR verification on the recombinant plasmid with the appropriate primers flanking the insert region, produced a PCR product slightly higher than 1.8 kb (Figure 1, lane 7). The presence of the HN gene, in frame with the start codon and its correct orientation was confirmed by sequencing (data not shown). The transfection on *Sf9* insect cells showed the typical morphology of viral infection at all stages of the transfected cells under the influence of the polyhedrin promoter, namely; the increase in cell diameter and nuclei, followed by cessation of cell growth and detachment (O'Reilly et al., 1992). Plaque assay on the harvested medium generated clearing zones with blue haze in the presence of Blu-gal in the monolayer, indicating the presence of the recombinant virus. A PCR analysis on the viral DNA extracted from recombinant and wt-infected cells showed a ~2 kb band and a 830 bp, band respectively (Figure 2, lanes 3, 4 and 5). The expected 2 kb PCR product resembled the size of the insert and the size of the DNA contributed by the transfer vector where as the 830 bp product

Figure 3. Hemadsorption assay on virus infected and uninfected cells with 1% chicken RBC. (A) recHNAF2240 infected cells; (B) wild-type infected cells; (C) uninfected cells.

appears to be the typical polyhedrin region flanked by the primers on the wt strains.

Expression of the recombinant HN protein in Sf9 cells

Virus-infected cell lysates (intracellular and extracellular RecHNAF2240) were able to agglutinate chicken

Figure 4. Indirect immunofluorescence (IIF) on recombinant virus infected Sf9 cells showing high fluorescence staining with NDV polyclonal anti-sera.

red blood cells (1%) as can be seen from a standard spot-test. The HA titer was at 16 HAU from the recombinant virus infected cell lysates which were tested after 6 days post-infection (d.p.i.). However, no agglutination activity could be detected from the wt-infected cell lysates. Sf9 cells that were infected with the recombinant virus were also able to hemadsorb 1% chicken RBC (Figure 3A). In addition, the recombinant virus infected cell lysate exhibited haemagglutination and hemadsorption inhibition in the presence of NDV polyclonal anti-sera. The wt-infected Sf9 cells (Figure 3B) and uninfected cells (Figure 3C) were also negative for any adsorption ability. Moreover, a RecHNAF2240 sample (total protein concentration of 1.6 mg/ml) had NA activity at absorbance 549 nm showing a value of 0.265 whereas the wt-infected cells and the uninfected cells at a similar concentration were negative.

Indirect immunofluorescence (IIF)

Further analysis using IIF showed that the NDV polyclonal anti-sera produced a strong fluorescence signal on the surface of recombinant virus infected Sf9 cells but no staining pattern was seen on the control (wt-infected cells) and the uninfected cells (Figure 4).

Protein analysis with SDS-PAGE and Western Blotting

SDS-PAGE analysis revealed the presence of a distinct band of approximately 63 kDa of the recombinant infected cell lysate samples which was not detected on the wt samples. In addition, three other faint bands were detected from the recombinant virus samples between 70 to 80 kDa range (Figure 5, lane 3) that appeared to show a different protein banding pattern as compared to the wt-infected (Figure 5, lane 4) and uninfected cell lysates samples (Figure 5, lane 5). Using the NDV polyclonal anti-sera, western blot analysis indicated the appearance of two distinct protein bands from the recombinant samples which bound strongly to the anti-sera (Figure 6, lane 3). When the size of these protein bands were compared with the original purified NDV cultured from embryonated eggs, probably the protein is glycosylated HN protein (~75 kDa) (Figure 6, lane 2) and that 63 kDa protein probably corresponds to unglycosylated form (Tan et al., 1995).

Discussion

The focus of this study was to investigate the possibility of cloning the HN structural gene of NDV strain AF2240 obtained from an RT-PCR process in a baculovirus expression vector system to enable an expression of recombinant glycosylated HN proteins that are biologically active. This approach would complement further analysis on the functionality of HN structural gene in isolation from the rest of the NDV genes and its potential as a subunit vaccine against NDV. The insertion of two different restriction enzyme sites at the primer ends allowed the amplification of an RT-PCR product having complementary ends with the transfer vector. This strategy ensured a directional cloning of the insert into the transfer vector in the correct orientation. The verification of the recombinant transfer plasmid using restriction enzyme analysis, PCR and sequencing the terminal ends have shown that the entire HN structural gene is intact.

The subsequent transfection of the linearized baculovirus AcMNPV DNA and the recombinant transfer vector into low passaged Sf9 cells, produced well-separated recombinant virus (45 plaques per plate) which can be easily detected and isolated in the presence of Blu-gal since only the recombinant virus has the full length *lacZ* region. The ~2 kb PCR band generated by virus isolates from the blue plaques indicated

Figure 5. 12% SDS-PAGE gel stained with Coomassie blue, comparing banding patterns of infected cell lysates. Mid-range molecular weight marker (Promega) (lane 1); Benchmark protein ladders (Gibco) (lane 6); purified NDV (lane 2); recHNAF2240 infected cell lysates (lane 3); wt (AcNMPV) infected cell lysates (lane 4) and uninfected cell lysates (lane 5).

Figure 6. Western Blot analysis of various virus-infected cell lysates using NDV polyclonal anti-sera. Purified NDV (lane 2) showing the presence of the glycosylated HN indicated by a small arrow head. The recHNAF2240 infected cell lysates in lane 3 indicating the putative unglycosylated RecHNAF2240 and glycosylated RecHNAF2240 at ~63 kDa and ~75 kDa, respectively (shown with a small arrow head). The wt (AcNMPV) in lane 4 and uninfected cell lysates in lane 5. The apparent MW are detected in lanes 1 and 7 (Mid-range protein marker, Promega) as well as lane 6 (Benchmark protein ladders, Gibco).

Figure 7. The homologous recombination of the recombinant plasmid (pAF2240-1) with the linearized baculovirus DNA occurring at the *lacZ* and ORF 1629 region. The gene of interest with the start codon was inserted downstream of the polyhedrin promoter.

that the HN gene had been inserted into the polyhedrin region, downstream of the polyhedrin promoter. When compared with the non-blue plaques from the same mixture, a distinct difference in the size of the PCR product (~830 bp that corresponded to the polyherin region only) was obtain. These observations appear to show that the gene of interest has recombined in a homologous manner with the linearized baculovirus DNA in *Sf9* cells which could replicate and eventually form recombinant viruses (Figure 7). Based on the results obtained, the recombinant HN expressed

in *Sf9* insect cells that have been infected with the recombinant virus appears to show similar biological activity as the original HN in NDV, especially with regard to HA and NA activities as well as hemadsorption of chicken RBC. A significant HA activity of the extracellular fraction strongly suggests that the recombinant HN glycoprotein might have incorporated into the envelope of the baculovirus. HA activity was also detected from the intracellular fraction though the level of expression appears to be lower.

Similar results were reported by Nagy et al. (1990,

1991) showing expression of the HN gene of Hitchner B1, a lentogenic strain of NDV. However, in our case, NA activity was only detected after the recombinant HN from the extracellular fraction were concentrated by ultracentrifugation. In addition, a significant hemadsorption activity of the cells infected by the recombinant virus acknowledges the fact that the recombinant HN appears to have translocated to the cell surface. This finding was further strengthened when high levels of fluorescence signals were detected with IIF using NDV anti-sera polyclonal antibodies. Moreover, both HA and hemadsorption activities were inhibited by NDV anti-sera polyclonal antibodies indicating the biological functionality of the expressed protein which was also observed in the Baculovirus HN expression of parainfluenza type 3 virus (Coelingh et al., 1987) and NDV strain Hitchner B1 (Nagy et al., 1991). In another study, serum samples from chickens immunised with the killed reCHNAF2240 virus via the intra-muscular (i.m.) route were able to show substantial immune response with a range of HI titer between 8 to 32.

The study done using SDS-PAGE and western blot analysis to investigate the expression of the recombinant protein revealed that the baculovirus expression system was able to produce both the glycosylated (~75 kDa) and unglycosylated (~63 kDa) recombinant protein products as was reported elsewhere (Ong et al., 1999). Although the unglycosylated protein appears to be more distinct when viewed after Coomassie blue staining, both proteins were detected under a western blot analysis. There appears to be a high possibility that the unglycosylated HN product could have been synthesised earlier followed by the glycosylated form (Ong et al., 1999) resulting in the expression of both forms of glycoprotein with the earlier being more intense. However, it was apparent that the sizes of the glycosylated and unglycosylated HN fragments appear to correspond with the authentic glycosylated HN protein based on the western blot (~75 kDa) and its predicted size of 63.8 kDa from previous report (Tan et al., 1995) as well as another study involving tunicamycin treatment confirming its *N*-linked glycosylation (Ong et al., 1999). A time course study using [³⁵S] methionine-labelled samples of recombinant infected cell lysates indicated that the expression of the HN-recombinant proteins was most obvious at 48 h post-infection (h.p.i.) and the synthesis of the glycosylated form was inhibited in the presence of tunicamycin (Ong et al., 1999).

In addition to the expression of both the recombin-

ant glycosylated and unglycosylated viral glycoproteins in insect cells which were noted to be similar in size to the authentic viral protein by various researchers (Mackow et al., 1989; Nagy et al., 1990; Ghiasi et al., 1991, 1992, 1994), the presence of partially glycosylated (Ghiasi et al., 1992, 1994) as well as the absence of the unglycosylated form (Coelingh et al., 1987) were observed. The various level of glycosylated viral proteins were attributed to the formation of precursor or cleaved proteins which appears to co-express with the glycosylated form (Nagy et al., 1990; Ghiasi et al., 1992, 1994). Despite the presence of these additional unglycosylated or partially glycosylated forms, the recombinant baculovirus-expressed protein products were able to induce significant protective immune response in their respective hosts (Nagy et al., 1990; Ghiasi et al., 1992, 1994).

Although significant differences have been noted between the expressed glycosylated protein products of insect and mammalian cells (Marz et al., 1995), this study provides substantial evidence to conclude that the recombinant HN produced by the reCHNAF2240 virus in insect cells are biologically functional and able to translocate as well as express on the cell surface in the form of authentic NDV HN surface glycoprotein. Since the HN of the pure NDV virus is highly glycosylated (Tan et al., 1990), the formation of a functional glycosylated recombinant HN product would provide the basis for a suitable model to further explore the immunological properties of the HN glycoprotein and may support the role as a potential subunit vaccine against NDV.

Acknowledgements

This study was supported by the Malaysian Ministry of Science, Technology and Environment IRPA Grant No. 01-02-04-0107.

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