ABSTRACT

Title of Dissertation: ROLE OF HEMAGGLUTININ-NEURAMINIDASE PROTEIN IN NEWCASTLE DISEASE VIRUS PATHOGENESIS

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The hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) is an important multifunctional envelope protein, which plays key roles in virus attachment, neuraminidase and fusion promotion activities. Using the established reverse genetics technique for the recovery of infectious NDV from cloned cDNA, the role of the HN protein in NDV pathogenesis was explored. By exchanging the HN gene between an avirulent NDV strain LaSota and a virulent NDV strain Beaudette C (BC), two chimeric viruses were generated. *In vitro* and *in vivo* studies done with the chimeras indicated an important role of the HN protein in modulating NDV virulence in hosts.

The HN gene of NDV has six glycosylation sites, two of which are not used for addition of carbohydrates. The exact role of the four functional glycosylation sites in NDV pathogenesis is unknown. To understand the importance of these sites in influencing NDV virulence, each site was eliminated individually and in combination on a cDNA clone of NDV strain BC. Four single mutant viruses with each of the sites (1-4) and one double mutant virus with sites 1 and 2 eliminated, were recovered. Results from this study established the key role of glycosylation site 4 and glycosylation sites 1 and 2 in combination, in influencing NDV pathogenesis.

A recent crystal structure of the HN protein of NDV gives valuable information about the location of amino acids involved in receptor-binding, neuraminidase and fusion promotion activities of the protein. To study the effect of mutagenesis of such key amino acids on NDV pathogenesis *in vivo*, five recombinant viruses with mutations in residues at the receptor-binding domains of the HN protein and in residues differing between the LaSota and BC strains of NDV, were generated. *In vitro* and *in vivo* studies with these viruses indicated the important role of certain residues in viral infectivity.

In summary, the importance of the HN protein in NDV pathogenesis was established. Further studies indicated the role of glycosylation sites in modulating NDV virulence. The relevance of certain key amino acid residues on the HN protein in NDV pathogenesis was also investigated.

ROLE OF HEMAGGLUTININ-NEURAMINIDASE PROTEIN IN NEWCASTLE DISEASE VIRUS PATHOGENESIS

by

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DEDICATION

I would like to dedicate this work to my father, Dr. Bhagabat Panda, and my mother, Mrs. Saraswati Panda, for their unconditional love and support and for being wonderful parents.

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LIST OF ABBREVIATIONS

ara C	cytosine arabinoside
BC	Beaudette C
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CPE	cytopathic effect
DF1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
ELISA	enzyme linked immunosorbent assay
EMEM	essential modified Eagle's medium
FBS	fetal bovine serum
GS	gene start
GE	gene end
HAd	hemadsorption
HA	hemagglutination assay
HDV	hepatitis delta virus
HE	hematoxylin eosin
HI	hemagglutination inhibition
HN	hemagglutinin-neuraminidase
hPIV3	human parainfluenza virus 3

ICPI	intracerebral pathogenicity index
IHC	immunohistochemistry
IVPI	intravenous pathogenicity index
kD	kilodaltons
L	large polymerase
M	matrix
mRNA	messenger RNA
MDT	mean death time
MOI	multiplicity of infection
MUN	2'-(4-methylumbelliferyl)- α-D-N-acetylneuraminic acid
NA	neuraminidase
NDV	Newcastle disease virus
nm	nanometer
NP	nucleocapsid protein
nt	nucleotide
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PI	post infection
RBC	red blood cell

RNA	ribonucleic acid
rNDV	recombinant Newcastle disease virus
RNP	ribonucleoprotein
RT-PCR	reverse transcription PCR
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPF	specific pathogen free
SV5	simian virus 5
U	unit/s
VSV	vesicular stomatitis virus

CHAPTER 1

1.1 Title

General Introduction

1.2 Introduction

Newcastle disease is a highly contagious and fatal viral disease that affects all species of birds. The clinical signs seen in birds affected by this disease vary widely and are dependent on factors like the virus strain, host species and age, immune status, environmental stress and presence of other organisms. In chickens, the disease may vary from sudden death with 100% mortality to subclinical disease (2). The disease has a worldwide distribution, and is a major threat to the poultry industries due to the huge economic loss associated with it.

Newcastle disease is caused by the Newcastle disease virus (NDV), which has been categorized into three main pathotypes depending on the severity of the disease in chickens: the lentogenic, mesogenic and velogenic strains. Lentogenic strains are avirulent and may cause mild respiratory infection. Mesogenic strains are of intermediate virulence and cause respiratory disorders with low mortality. Velogenic strains are highly virulent and cause high mortality. Velogenic strains are classified into neurotropic

velogenic strains, which cause neurological and respiratory disorders and viscerotropic velogenic strains, which cause hemorrhagic lesions in the digestive tract (2).

NDV is a member of the genus *Avulavirus* of the family *Paramyxoviridae,* in the order *Mononegavirales*. (78, 89). This family also includes other important pathogens such as the mumps virus, human parainfluenza virus, sendai virus, simian virus 5 and recently emerging nipah and hendra viruses. Recently, the nipah virus has been assigned as a Category C biological agent for biowarfare/bioterrorism by the Center for Disease Control (50). The velogenic strains of NDV have also been identified as potential animal pathogens which may be used as agents for bioterrorism, resulting in huge impacts on the a nation's agricultural economy (51). Thus, it is important to understand the molecular basis for pathogenesis of paramyxoviruses and to explore technologies for designing alternative safe vaccines for emergency preparedness.

NDV has a single stranded negative-sense RNA genome (89), which is 15,186 nucleotides long (29, 68, 108). The genomic RNA contains six genes encoding at least eight proteins (104, 137). Three proteins which constitute the nucleocapsid are the nucleoprotein (NP), the phospoprotein (P), and the large polymerase protein (L). The two external envelope proteins are the fusion protein (F) and the hemagglutinin-neuraminidase protein (HN). The inner layer of the virion is formed by the matrix protein (M). The two additional proteins formed by the editing process during P gene transcription, are the V and W proteins. NP binds to the genomic RNA forming the nucleoapsid core, whereas the P and L proteins are associated with the nucleocapsid core

and help in viral transcription and replication. The M protein helps in viral assembly. The F protein plays a direct role in the fusion process. It mediates fusion of the viral envelope with the host cell membrane, enabling viral entry into the cell membrane. The HN protein has functions in attachment, fusion promotion and removal of sialic-acid from progeny virions, thus, acting as a neuraminidase. The V protein of NDV functions as an alpha interferon antagonist (55). Function of the W protein is unknown (71, 137). In between each gene are short sequences called the intergenic sequences, the functions of which are unknown. At the beginning and end of each gene are conserved sequences known as the gene start (GS) and gene end (GE) sequences, respectively. These contain signals for transcription. The genome of NDV is flanked by a 55 nucleotide (nt) leader region at the 3' end and a 114 nt trailer region at the 5' end (68). These regions serve as cis-acting elements involved in replication and packaging of the viral RNA (71).

The HN protein of NDV plays an important role in virus life cycle. Besides playing a role in attachment of the virus to host cells, it cleaves sialic acid receptors from viral and cellular surfaces, preventing progeny virus from self-aggregating or adhering to cell membranes. HN also promotes cell fusion by interacting with the F protein of NDV. Thus, the HN has both hemagglutinating and neuraminidase activities (71). The HN protein is a type II glycoprotein possessing six glycosylation sites. Glycosylation in proteins helps in protein folding, maturation, stability and influences antigenicity and immunogenicity of the protein. While the role of glycosylation on the biological activities of the HN glycoprotein has been studied earlier (81), the effect of glycosylation of the

protein on virus infectivity has not been investigated extensively. It would be interesting to find out the role of the functional glycosylation sites of HN on the virulence of NDV.

Although the HN genes of virulent and avirulent NDV strains share a high percentage of homology, the viruses differ substantially in their virulence. Since HN is a multifunctional protein of NDV, which is actively involved in virus infectivity, studying the roles of the HN gene in virus pathogenesis would be important. By exchanging the HN gene between avirulent and virulent NDV strains, its role in pathogenesis could be determined.

The HN protein along with the F protein, is also the main target of immune response for NDV (82, 87). A recent report by Zeng *et al*., (152) indicated that the HN protein of NDV was responsible for induction of interferon-alpha and tumor necrosis factor. Since HN possesses immunogenic properties, studies on this protein may prove to be useful for the development of NDV vaccines.

Reverse genetics is a major breakthrough technique, which allows the production of infectious RNA viruses from cloned DNAs. It helps in the manipulation of the genomes of negative-sense RNA viruses. Lack of genetic recombination and inability of the naked RNA to initiate viral RNA synthesis has hindered genetic manipulation of single-stranded, negative sense RNA viruses. Reverse genetics makes it possible to introduce genetic changes directly into the negative-stranded RNA virus genomes. It has important implications in molecular biology studies and the development of attenuated

vaccines. The current vaccination protocol for NDV utilizes live attenuated lentogenic strains like the Hitchner B1 (47) and LaSota (42). As encountered in any live attenuated vaccine, NDV vaccine may still cause disease signs depending on the environmental conditions and stress. A highly stable and efficacious vaccine then becomes a desired objective. Using the reverse genetics system, we have investigated the role of the HN protein in NDV pathogenesis. We have further studied the importance of glycosylation sites in influencing viral infectivity. Additionally, the role of certain key amino acid residues of the HN protein involved in hemagglutination, neuraminidase and fusion promotion activities of NDV have also been investigated.

1.3 Research objectives

The specific objectives of the present study were:

1. To understand the role of the HN protein in NDV pathogenesis;
2. To study the importance of glycosylation sites on the HN protein of NDV and;
3. To investigate the role of individual key amino acids of HN involved in cell fusion, hemagglutination and neuraminidase activities.

CHAPTER 2

2.1 Title

Review of Literature

2.2 Classification

NDV is a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus* (78, 89). NDV is the only member of the genus *Avulavirus* (78). Other important members of the family *Paramyxoviridae* are the mumps virus, SV5 and parainfluenza virus type 2.

2.3 Virion

The NDV virus particles are pleomorphic in nature and range from 150-400 nm in size. The virions contain a long helical nucleocapsid structure which is 1,000 nm long and 17-18 nm in diameter. The envelope is covered with spike glycoproteins which are 8-12 nm in diameter. The genome of NDV is a single strand of RNA of negative sense, and has a molecular weight of 5.2 to 5.7 $\times$ 10^6 daltons (2). The genomic RNA consists of 15, 186 nucleotides (29, 68, 108). The helical nucleocapsid, rather than the free genome RNA, is the template for all RNA synthesis. The nucleocapsid protein (NP) and genome RNA together form a core structure, to which the phosphoprotein (P) and the large polymerase protein (L) are attached (71). This core forms the RNP or the transcriptive-

replicative complex and serves as the minimum infectious unit. The envelope of NDV contains two surface glycoproteins: the hemagglutinin-neuraminidase (HN) protein responsible for attachment of the virus to host cells and the fusion (F) protein required for fusion of the virus into the host cell membrane (49). The F and HN proteins are also the main targets of the immune response of NDV (82, 87). Internal to the envelope is the matrix (M) protein, which is thought to be important in viral assembly (105). Fig. 1 shows a schematic diagram of the NDV and Fig. 2 depicts an electron micrograph of the virus particle.

2.4 Genome organization

The NDV genome consists of six genes (3' NP-P-M-F-HN-L 5') encoding at least eight proteins (104, 137). The genomic RNA contains a 3' extracistronic region of 55 nucleotides, known as the leader, and a 5' extracistronic region of 144 nucleotides, known as the trailer (68). These regions are essential for replication of the genome, and they flank the six genes. At the beginning and end of each gene are conserved transcriptional control sequences, known as the gene start and gene end sequences, respectively. Between the gene boundaries are intergenic regions, which vary in length from 1- 47 nucleotides (12, 13, 68) (Fig. 3).

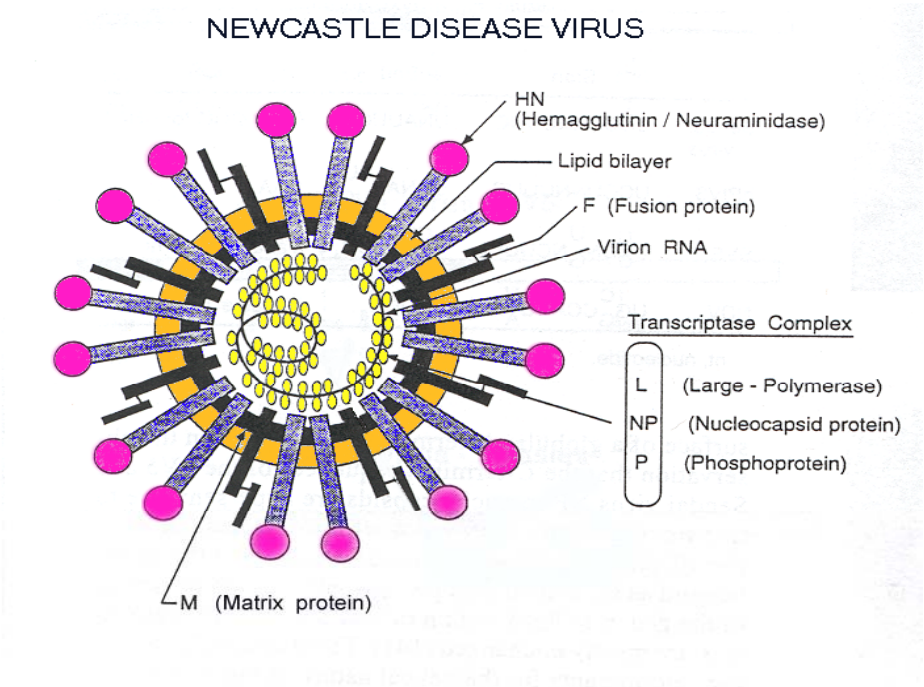


Figure 1. Schematic diagram of Newcastle disease virus particle (not drawn to scale)

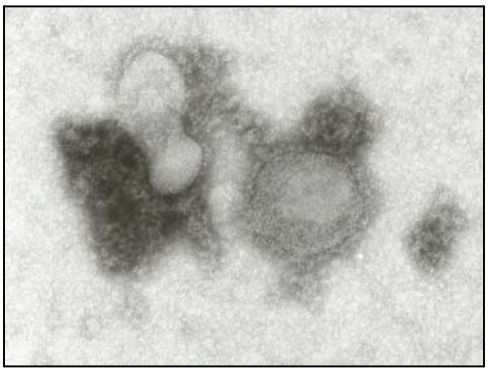


Figure 2. Electron micrograph of negatively stained pleomorphic Newcastle disease virus (strain Beaudette C) particles obtained from supernatant of infected chicken embryo fibroblast cells.

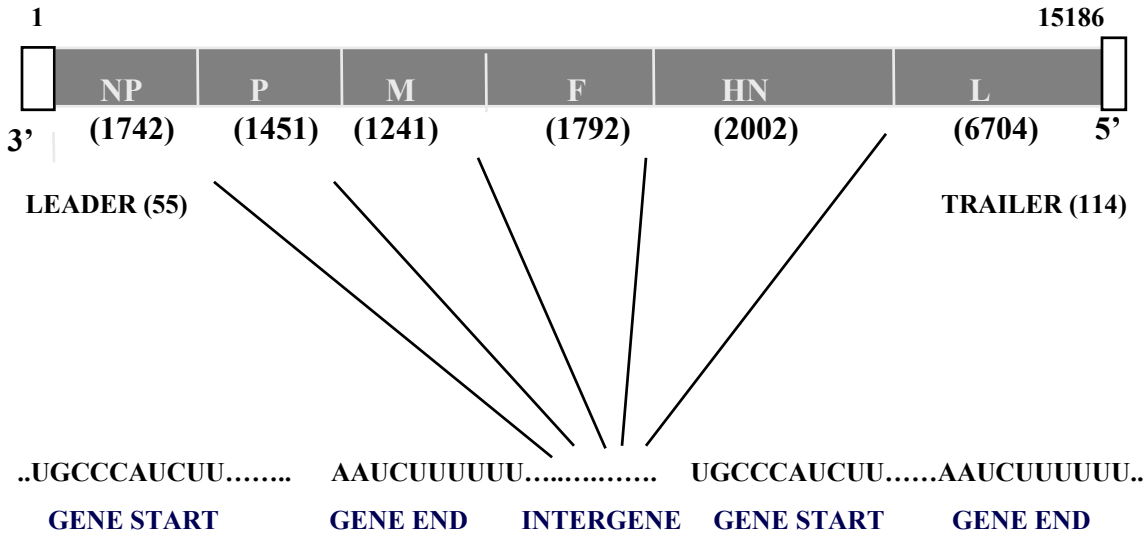


Figure 3. Genetic map of genomic RNA of NDV. NDV has a single-stranded, negative-sense RNA genome which is 15, 186 nucleotides in length. The length of leader, trailer and each gene is shown in parantheses. Each gene is flanked by conserved gene start and gene end sequences. The intergenic sequences present in between two genes range from 1-47 nucleotides in length.

2.5 Viral proteins

The six genes of NDV code for at least eight proteins: NP, P, M, F, HN, L, V and W. The V and W proteins are the two additional proteins formed by non-templated nucleotide addition through the RNA editing process during P gene transcription (137).

2.5.1 Nucleocapsid and its associated proteins

The nucleocapsid protein (NP) and genome RNA together form a core structure, to which the phosphoprotein (P) and the large polymerase protein (L) are attached (71). These three proteins form the transcriptive-replicative complex, which is the minimum infectious unit of NDV.

NP protein: The NP protein serves several functions in viral replication, including encapsidation of the genome RNA into a nucleocapsid (the template for RNA synthesis), association with the P-L polymerase during transcription and replication and most likely, interaction with the M protein during virus assembly. The intracellular concentration of unassembled NP is also considered to be a major factor controlling the relative rates of transcription and replication from genome templates (7, 8). The NP gene of NDV consists of 1747 nucleotides with a coding region of 489 residues. The molecular weight of the protein is predicted to be 54 kilodaltons (kD) (68).

P protein: The P gene of NDV is 1451 nt long. The P gene ORF encodes an unedited version of mRNA, which results in formation of the P protein. RNA editing with the addition of one G nucleotide at the editing site (near the center of the ORF) produces an mRNA which encodes the V protein, whereas addition of two G nucleotides produces an mRNA that encodes the W protein. (71,137). The P protein is essential for viral RNA synthesis. This protein is highly phosphorylated in nature. It is an essential component of the viral RNA polymerase and the nascent chain assembly complex formed during viral RNA synthesis. The P protein associates with the L protein forming the viral polymerase (P-L) and thus, functions as a transcriptive and replicative factor. It also associates with the unassembled NP (NP^0) forming the P- NP^0 complex (44). This property of P protein has been suggested to prevent NP^0 from assembling RNA non-specifically (77). The predicted molecular mass of the polypeptide is 53 kD (27).

L protein: The L protein is the least abundant of the structural proteins (about 50 copies per virion). The L-gene is the most promoter-distal in the transcription map and thus the last to be transcribed. Its low abundance, its large size and its localization to transcriptionally active viral cores suggested that it might be the viral polymerase. The P and L proteins form a complex, and both proteins are required for polymerase activity with NP:RNA templates (26, 44, 76). The L protein is also responsible for capping and polyadenylation of the mRNAs. Polyadenylation is thought to result from polymerase stuttering on a short stretch of U residues. The L gene is 6704 nt long. The predicted molecular mass of the polypeptide is 242 kD (151).

2.5.2 Matrix Protein

M is the most abundant protein in the virion. The M gene of NDV is 1241 nt long. Its predicted molecular mass is 40 kD (14). The M protein interacts with the nucleocapsid and the envelope proteins of the virion. This protein is considered to be the central organizer of viral morphogenesis, interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucleocapsids. The self-association of M and its contact with the nucleocapsid may be the driving force in forming a budding virus particle (105).

2.5.3 Envelope glycoproteins

NDV possesses two integral membrane glycoproteins namely, the hemagglutinin-neuraminidase (HN) glycoprotein which is involved in cell attachment and the fusion (F) glycoprotein which mediates pH-independent fusion of the viral envelope with the plasma membrane of the host cell.

HN protein: The HN glycoprotein of NDV is a multifunctional protein and a major antigenic determinant of the virus. It is responsible for the adsorption of the virus to sialic acid-containing receptors. In addition, HN mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of virions and the surface of infected cells. In addition to the hemagglutinating and neuraminidase activities, HN also has a fusion promoting activity, through interacting with the fusion glycoprotein of NDV (71). Previous research has indicated that for fusion to occur, a type-specific interaction

between the F and HN proteins is required. It is proposed that HN undergoes a conformational change on attachment to its ligand and thereby triggers a conformational change in the F protein to release the fusion peptide (70). Studies have shown that a point mutation in the globular domain of HN that abolishes its receptor recognition, neuraminidase and fusion activities, also interferes with its ability to interact with F (32). Research on the role of the HN protein in the mechanism of cell membrane fusion indicate that the binding of the HN protein to the receptor induces conformational changes of residues near the hydrophobic surface of the protein and this change in turn, triggers the activation of the F protein, which initiates membrane fusion (140). The HN gene is 1998 nt long with a coding region having 577 amino acid residues. The HN of some strains of NDV is synthesized as a biologically inactive precursor (HN_0), and 90 residues from the C-terminal are removed to activate the molecule (83, 84, 94). The HN proteins are type II integral membrane proteins that span the membrane once. The N-terminus of the HN protein consists of the cytoplasmic domain, followed by the transmembrane region and the stalk region. The protein contains a single hydrophobic domain, located near the N-terminus, that acts as a combined signal and anchorage domain targeting the nascent chain as it emerges from the ribosome to the membrane of the endoplasmic reticulum and ensuring the translocation of the polypeptide chain across the membrane, bringing about the stable anchoring of the protein in the lipid bilayer (71). The C-terminus end of the protein is composed of the globular head or the ectodomain. This region of the HN protein is the main site of attachment of the virus to host cells. The HN glycoprotein of NDV contains six *N*-linked carbohydrate chains (81). These carbohydrate chains are added to the protein in the rough endoplasmic reticulum as it

undergoes modifications in various cell compartments during its formation (67). All but one of the six glycosylation sites on the HN protein, are located in the C-terminal external domain. The secondary structure prediction shows that the C-terminal external domain is mostly arranged in beta-sheets, while alpha-helices are predominantly located in the N-terminal domain of the protein (118). The predicted molecular weight of the HN protein is 74 kD (12). The HN protein forms an oligomer consisting of di-sulfide linked homodimers that form a non-covalently linked tetramer (75). There has been debate for many years as to whether the HN molecules contain combined or separate active sites for hemagglutinating and neuraminidase activities. Recent studies and analysis of the crystal structure of HN of NDV have predicted that there resides a single site in the HN protein that binds sialic acid tightly (hemagglutination activity), but hydrolyses the molecule slowly (neuraminidase activity) (24).

F protein: The F protein of NDV mediates viral penetration by fusion between the virion envelope and the host cell plasma membrane, in a pH-independent manner. After fusion, the nucleocapsid is delivered to the cytoplasm. Later in infection, the F protein expressed at the plasma membrane of infected cells can mediate fusion with neighboring cells to form syncytia (giant cells). Syncytia formation is a hallmark of NDV infection in host cells. It is a typical cytopathic effect caused by the virus and can lead to tissue necrosis and might also be a mechanism of virus spread. The F protein is a type I integral membrane protein and is synthesized as an inactive precursor (F_0) that is cleaved by a host-cell protease. This cleavage releases a new N-terminus of F_1, thus forming the biologically active protein, consisting of disufide-linked chains F_1 and F_2 (122). The

cleavage of F_0 is a key determinant for pathogenicity of paramyxoviruses. Viruses that have multiple basic residues in the cleavage site of the F protein have proteolytic cleavage of the F_0 molecule intracellularly by subtilisin-like proteases such as, furin, during transport of the protein through the trans Golgi network. Paramyxoviruses that have single basic proteins in the F cleavage site cannot be cleaved intracellularly and require exogenous proteases for cleavage activation (102, 122). The F gene is 1792 nt long. F_0 has a predicted molecular mass of 66 kD. F_1 is 55 kD and F_2 is 12.5 kD (13). The fusion peptides are thought to intercalate into target membranes, starting the fusion process.

2.6 Stages of replication of NDV

All aspects of replication of NDV occur in the cytoplasm. In cell-culture, single-cycle growth curves for virulent NDV strains can be as short as 10 hours. During the late stages of infection, the effect of viral replication can result in complete shut off of host macromolecular synthesis. Initiation of infection starts with the adsorption of the virus to the cell receptors and subsequent fusion into host cellular membrane. Finally, progeny viruses mature by budding through the plasma membrane. Cleavage of the F_0 precursor glycoprotein to F_1 and F_2 by host cell proteases is required for progeny virus to become infective (41, 94).

2.6.1 Virus adsorption and entry

Molecules containing sialic acid serve as cell surface receptors for NDV. Sialic acid is found both on glycoproteins and on lipids making NDV accessible to a wide range of host cell receptors. Upon adsorption of the virus to the cellular receptors, the viral membrane fuses with the host cellular plasma membrane at neutral pH. This results the release of the viral nucleocapsids into the cytoplasm of the host cell. The M protein is considered to make several contacts with the nucleocapsid. After the release of the nucleocapsid into the cytoplasm, disruption of the M-nucleocapsid complex is necessary. The driving force for this process is unknown in paramyxoviruses.

2.6.2 Transcription

All viral mRNA synthesis begins at the 3′ end of the genome of NDV. The cis-acting promoter sequences function as initiators of leader RNAs and the antigenome. The viral RNA polymerase has to first transcribe the leader RNA before beginning mRNA synthesis at the NP gene start signal. RNA replication of Sendai virus requires the genome length to be a multiple of six ("Rule of Six" theory), for efficient replication (10). NDV also follows the rule of six principle for efficient replication. This hexamer rule is most likely related to the finding that each NP subunit of the nucleocapsid is associated with exactly six nucleotides (37). The efficiency of the promoter at the 3′ end depends on its position relative to the NP subunits. Apart from sendai virus, the rule is important for RNA replication in PIV3, SV5 and measles (35, 90, 133); but not in RSV (120). Once the nucleocapsid is released in to host cell cytoplasm the leader mRNA is synthesized first, on entry of the NP and P/L polymerase at the 3′ end of the genome. The

leader sequence contains the regulatory elements needed for gene expression. After synthesis of the leader RNA, reinitiation begins at the gene start of the first gene (NP) and terminates at the gene end. This results in release of the first capped and polyadenylated mRNA (147). Transcription continues in this start-stop manner until the L mRNA is synthesized. The intergenic regions located in between the genes are not transcribed. Initiation of a downstream mRNA depends on termination of the upstream mRNA consistent with a single viral RNA polymerase entry site at the 3′ end of the genome. The frequency with which the viral RNA polymerase reinitiates the next mRNA at gene junctions is not perfect. This leads to a gradient of mRNA abundance that reduces according to the distance of the location of a particular gene from the 3′ end of the genome (11).

2.6.3 Replication

The (-) genome replicates via a full-length complementary copy known as the antigenome (+). After translation of the primary transcripts and accumulation of the viral proteins, antigenome synthesis begins. The (-) genome is then synthesized by making a complementary copy of the antigenome. During this process, the viral RNA polymerase which was engaged in mRNA synthesis till now, copies the same genomic template, but this time, ignores all the junctional signals, and synthesizes an exact complementary copy (7, 93). Both the genome and antigenome are assembled into encapsidated nucleocapsid. The leader and trailer regions of the genome contain specific sequences for initiating encapsidation (7). The processes of transcription and replication are tightly regulated. When unassembled NP is limiting, the viral RNA polymerase is preferentially engaged in

mRNA synthesis, raising the intracellular levels of unassembled NP and all other viral proteins. When unassembled NP levels are sufficient, some viral RNA polymerase activity switches from transcription to replication, thereby lowering the levels of unassembled NP, as each initiation of encapsidation utilizes many NP monomers to finish the assembled genome chain (66). The RNA synthesis of NDV is shown in Fig. 4A.

2.6.4 Virus assembly and release

The intracellular site of nucleocapsid assembly is in the cytoplasm. The first step in viral assembly is the encapsidation of genomic RNA into nucleocapsid. The nucleocapsids are thought to be assembled in two steps: first, there is an association of free NP subunits with the genome or template RNA to form the helical ribonucleoprotein (RNP) structure, followed by the association of the P-L protein complex (64). In contrast to the antigenomes, Paramyxovirus mRNAs are not encapsidated. The assembly of the viral envelope takes place at the cell surface. The viral integral membrane glycoproteins (F and HN) are synthesized in the endoplasmic reticulum and undergo step-wise conformational maturation before transport through the secretory pathway. Folding and maturation of the glycoproteins of NDV occur in the cell with the help of many folding enzymes and molecular chaperones. Only correctly folded and assembled proteins are transported out of the endoplasmic reticulum. In the Golgi apparatus, the carbohydrate chains on the HN glycoproteins are modified extensively. Cleavage of the F proteins with multiple basic cleavage sites, occurs in the trans Golgi apparatus. Finally, the glycoproteins are transported to the plasma membrane (33). In NDV, the assembly of the

envelope occurs at the cell surface and release of the virus takes place by budding (38). Fig 4B shows the life cycle of NDV.

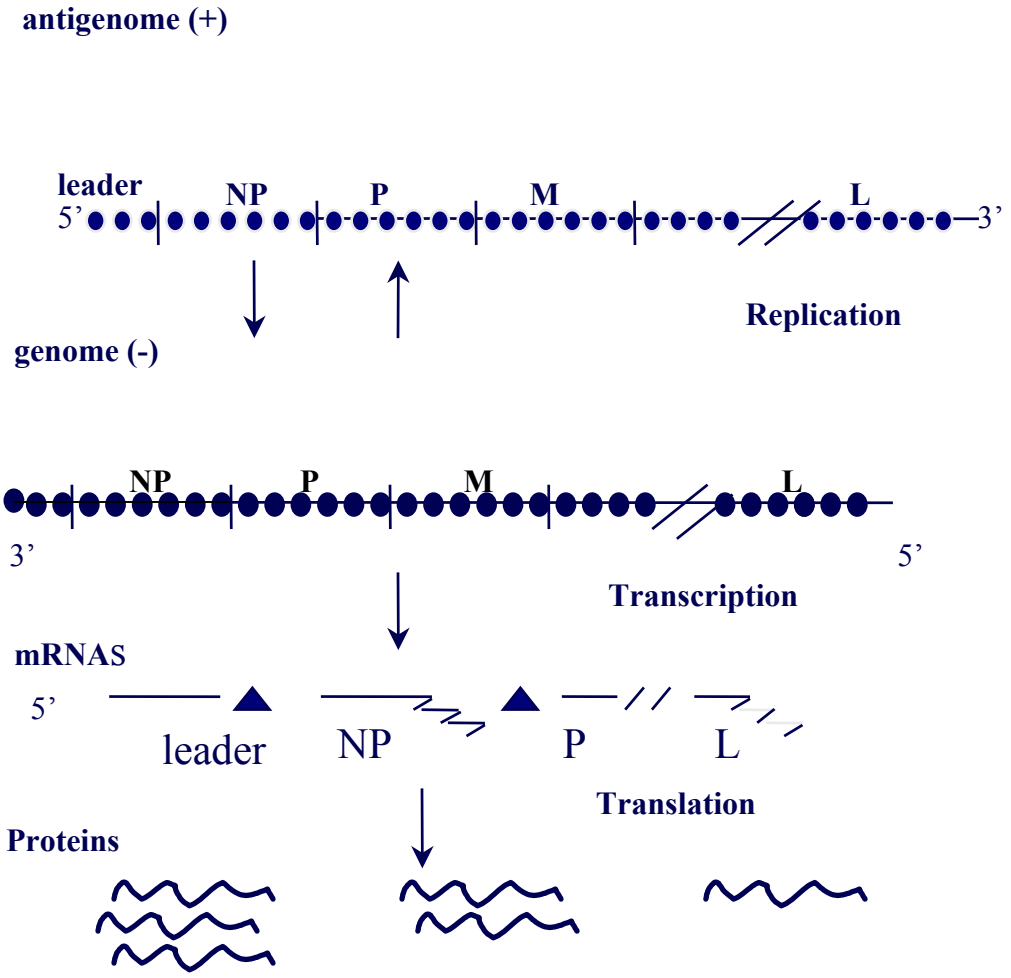


Figure 4A. Paramyxovirus RNA synthesis: schematic representation. Genome and antigenome are shown as nucleocapsids with ovals representing the NP subunits and vertical lines representing the gene junctions. Capped (triangles) and polyadenylated (zig zag lines) are formed by the viral polymerase protein during transcription. These in turn are translated into the respective proteins (squiggly lines). (Figure modified from Collins, et al., 1996. Respiratory Syncytial virus, In Fields, B.N. Knipe, D. M. and Howley, P. M. (ed) . Virology 3rd ed. Raven Press, New York).

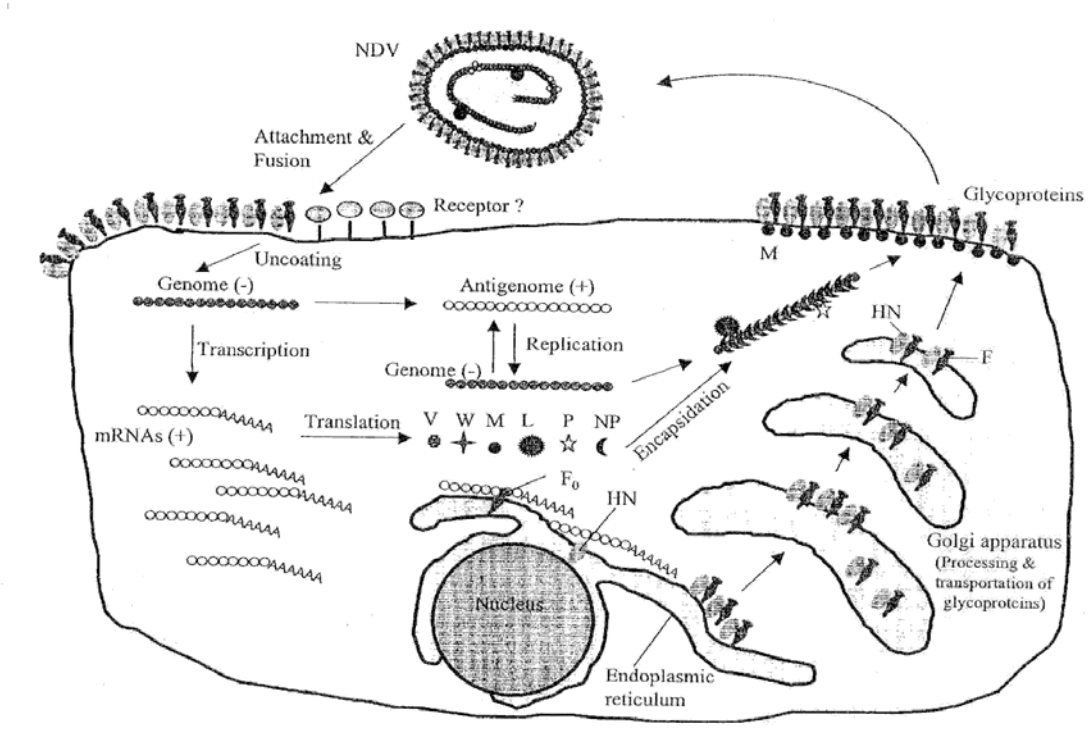


Figure 4B. The life cycle of NDV. (Figure from review article by Yusoff, K., and W. S. Tan. 2001. Newcastle disease virus: macromolecules and opportunities. Av. Pathol. 30:439-455).

The mechanism of viral assembly at the cell membrane is not known. The viral M proteins are thought to play a major role in bringing the assembled ribonucleoprotein core to the appropriate place on the plasma membrane to form a budding virion (105). It is believed that the F and HN glycoprotein cytoplasmic tails make important contacts with the M protein, which, in turn, associates with the nucleocapsid. The assembled patch at the plasma membrane is then release by budding.

2.7 Reverse genetics

Reverse genetics is a technique that allows the generation of viruses possessing a genome derived from cloned cDNAs. When compared to the positive-sense RNA viruses, genome manipulation of negative-sense RNA viruses is more cumbersome. This is because the negative-sense RNA viruses require the virion RNA to be assembled into an active transcriptase-replicase complex for the genome to initiate viral infection. After many years of research, the techniques to manipulate the genomes of nonsegmented negative-strand RNA viruses have now been developed (116). This was first done with the successful recovery of the rabies virus in 1994 (126). Rabies virus was rescued when plasmids encoding the N, P and L proteins, as well as a plasmid coding the entire antigenome, all under the control of the bacteriophage T7 RNA polymerase promoter, were transfected into cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase protein.

Rescue of the rabies virus using reverse genetics procedures was followed by recovery of several other viruses such as the vesicular stomatitis virus (73, 149), SV5 (45), human respiratory syncitial virus (21), Sendai virus (40, 62), rinderpest virus (6), parainfluenza virus (36, 48) and measles virus (113). Reverse genetics systems to recover infectious NDV from cloned cDNAs were first reported in 1999 (98, 106). Currently reverse genetics systems are available for lentogenic strains LaSota (54, 98, 106), B1 (95) and for mesogenic strain Beaudette C (69). The availability of reverse genetics technology for NDV and other RNA viruses have provided means to genetically define and study these viruses. The reverse genetics technique for the rescue of NDV is depicted schematically in Fig. 5.

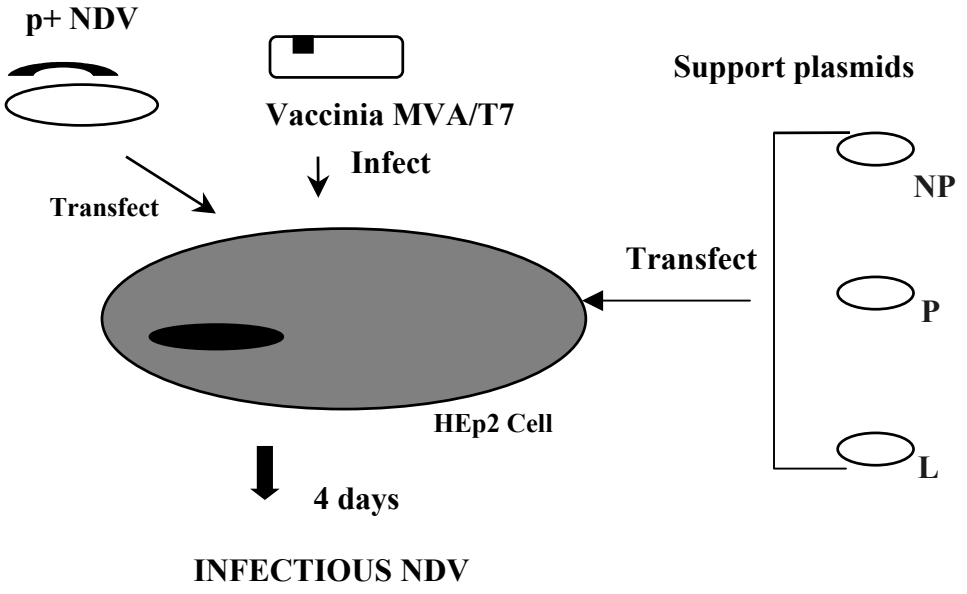


Figure 5. Schematic procedure for the recovery of infectious NDV (Beaudette C) from cDNA. Intracellular expression of antigenomic full-length cDNA (p+NDV) and polymerase complex NP, P and L mRNAs encoded in plasmids were co-transfected into HEp-2 cells. All the plasmids under the control of the T7 RNA polymerase promoter sequence were transcribed by the T7 RNA polymerase, supplied by the recombinant vaccinia MVA/T7 strain. Infectious NDV was generated entirely from cloned cDNA with procedures explained by Krishnamurthy *et al*. (69).

CHAPTER 3

3.1 Title

The Role of the Hemagglutinin-Neuraminidase protein in NDV pathogenesis

3.2 Abstract

The hemagglutinin-neuraminidase (HN) protein of NDV plays a crucial role in the process of infection. However, the exact contribution of the HN gene in NDV pathogenesis is not known. In this study, the role of the HN gene in NDV virulence was examined. Using reverse genetics procedures, the HN gene was exchanged between a virulent recombinant NDV strain, rBeaudette C (rBC), and an avirulent recombinant NDV strain, rLaSota. The hemadsorption and neuraminidase activities of the chimeric viruses showed significant differences from their parental strains, but heterotypic F and HN pairs were equally effective in fusion promotion. The tissue tropism of the viruses was shown to be dependent on the origin of the HN protein. Chimeric virus with the HN protein derived from the virulent virus exhibited tissue predilection to that of virulent virus and vice versa. The chimeric viruses with reciprocal HN proteins either gained or lost virulence, determined by standard intracerebral pathogenicity index test in chickens and mean death time in chicken embryos devised to classify these viruses, indicating that virulence is a function of the amino acid differences in the HN protein. These results are consistent with the hypothesis that virulence of NDV is multigenic, and cleavability of F protein alone does not determine the virulence of a strain.

3.3 Introduction

Newcastle disease virus (NDV), the only member of the genus *Avulavirus*, belongs to the family *Paramyxoviridae* (78). NDV is an important pathogen of many species of birds, which invokes trade barriers and causes significant economic losses in the commercial poultry industry worldwide. NDV isolates display a spectrum of virulence in chickens which vary from a fatal to an inapparent infection (2). Strains of NDV are classified into three major pathotypes, depending upon the severity of disease produced in chickens. Avirulent strains are termed lentogenic; intermediately virulent strains are termed mesogenic, and highly virulent strains are termed velogenic.

The surface of NDV particles contains two important functional glycoproteins; fusion (F) and hemagglutinin-neuraminidase (HN) proteins. In general, membrane glycoproteins drive the assembly and budding of enveloped RNA viruses (134), and are the key players in determining the host range and tissue tropism. The F protein mediates both virus-cell and cell-cell fusion (46). The F protein is synthesized as a non-fusogenic precursor, F0, and only becomes fusogenic after cleavage by host cell proteases into disulfide-linked F1 and F2 polypeptides (117). The cleavability of F protein is directly related to the virulence of viruses *in vivo*. A high content of basic amino acid residues at the F0 cleavage site is correlated to virulence (17, 148). Recent studies with recombinant NDV generated by reverse genetics techniques showed that modification of a lentogenic F cleavage site into a velogenic cleavage site increased the virulence of the strain (103, 106), but did not reach the virulence level of velogenic strains. This result indicated that

the efficiency of cleavage of the F0 protein is not the sole determinant responsible for the virulence of NDV.

The HN protein of NDV is a multifunctional protein. It possesses both the receptor recognition and neuraminidase activities associated with the virus. It recognizes sialic acid-containing receptors on cell surfaces; it promotes the fusion activity of the F protein, thereby allowing the virus to penetrate the cell surface; and it acts as a neuraminidase by removing the sialic acid from progeny virus particles to prevent progeny viral self-agglutination (71). Thus, HN protein plays an important role in viral infection. Although the functions of the HN protein in NDV infection are well studied, its role in NDV pathogenesis is presently not known. We have recently shown that the cleavability of F protein alone does not convert an otherwise non-pathogenic strain into a highly virulent pathotype (103). Other gene products of NDV may also be involved in imparting virulence to a strain.

In this study, we exchanged the HN gene between a virulent recombinant NDV strain rBeaudette C (rBC) and an avirulent recombinant NDV strain rLaSota, thus, generating chimeric recombinant NDV viruses, rBC LaSoHN and rLaSo BCHN. Here, we show that the HN gene-swapped viruses were viable, and their tropism and virulence were altered depending on the nature of the HN gene sequence. The rBC LaSoHN virus showed reduced virulence, while the rLaSo BCHN virus showed an increase in virulence. The rBC and and rLaSo BCHN viruses were distributed systemically in chicken embryos, while the rLaSota and rBC LaSoHN viruses remained in the respiratory tract and never

reached systemic sites. These altered pathogenic properties evidenced by the exchange of the HN gene indicate its essential role in the tropism and virulence of NDV.

3.4 Materials and Methods

3.4.1 Cells and Viruses

DF1 cells (a chicken embryo fibroblast cell line) were maintained in DMEM, and Vero and HEp2 cells were maintained in EMEM media, with 5% fetal bovine serum (FBS). NDV strains, LaSota and BC were received from the National Veterinary Services Laboratory, Ames, IA. They were propagated in the allantoic cavity of embryonated chicken eggs. After two days, the allantoic fluid was harvested and the virus was purified (103). Briefly, the allantoic fluid was harvested and clarified by low speed centrifugation at 1800 × g for 30 min. Virus was pelleted by ultracentrifugation at 35, 000 × g for 18 h and the pelleted virus was resuspended in 4 ml sterile PBS. The virus was then layered on top of a discontinuous sucrose gradient made with 3 ml of 55% and 5 ml of 20% sucrose in PBS. The gradient was then ultracentrifuged at 43, 000 × g for 1 h. The virus band at the interface of the 20% and 55% sucrose gradients was collected and pelleted at 35, 000 × g for 18 h. The virus pellet was resuspended in 500 µl of PBS and stored at 4^{o}C.

3.4.2 Construction of plasmids and recovery of chimeric viruses

Full-length antigenomic cDNAs of BC and LaSota strains of NDV, designated as pBC and pLaSota respectively, were cloned into a low-copy number plasmid vector pBR322. These cDNA clones were used to rescue the retcombinant viruses, rBC and

rLaSota respectively, as described elsewhere (54, 69). A chimeric rBC virus containing LaSota HN gene in place of its own, and the reciprocal recombinant rLaSota virus containing BC HN gene, were generated for this study (Fig.6). The unique *Mlu*I and *Age*I sites in pBC and *Mlu*I and *SnaB*I sites in pLaSota at the F-HN and HN-L intergenic regions were exploited for exchanging the HN genes between the full-length plasmids. The LaSota HN gene was amplified by PCR using *Mlu*I (+) primer: 5′ AACT***ACGCGT***TGTAGATGACCAAAGGACGATATACGGGTAG 3′ and *Age*I (-) primer: 5′ GATC***ACCGGT***ACGTATTTTGCCTTGTATCTCATTGCCACTTAC 3′. The BC HN gene was amplified using the *Mlu*I (+) primer described above and *Sna B*I (-) primer: 5′ GATC***TACGTA***TTTTGCCTTGTATCTCATTGCCACTTAC 3′. The resulting full-length clones were designated as pBC LaSoHN and pLaSo BCHN, respectively. These plasmids were used to recover the recombinant chimeric viruses, rBC LaSo HN and rLaSo BCHN, using reverse genetics procedures (69).

Full-length antigenomic cDNAs of LaSota and BC viruses were cloned into a low-copy number plasmid vector, pBR322 (54, 69). They were designated as pBC and pLaSota, respectively. Reverse genetics procedures were used to rescue the recombinant viruses (rLaSota and rBC), as described elsewhere (54, 69). A chimeric rBC virus infectious clone containing LaSota HN gene in place of its own, and the reciprocal recombinant consisting of rLaSota infectious clone bearing the Beaudette C HN gene, were generated (Fig.6). In pBC, the unique *Mlu*I site in the intergenic region of F and HN genes and the unique *Age*I site in the intergenic region of HN and L genes were used to

exchange the HN genes between rBC and rLaSota virus infectious clones. PCR was done using LaSo *Mlu*I (+) primer:

5′ AACT***ACGCGT***TGTAGATGACCAAAGGACGATATACGGGTAG 3′ and

LaSo *Age*I (-) primer:

5′ GATC***ACCGGT***ACGTATTTTGCCTTGTATCTCATTGCCACTTAC 3′

which created overhangs of *Mlu*I and *Age*I sequences flanking the pLaSota HN gene. The HN gene from the full-length pBC plasmid was removed by digesting the full-length pBC plasmid with *Mlu*I and *Age*I enzymes. The resulting full-length plasmid of pBCΔ HN was then ligated with the pLaSota HN gene PCR product, after digestion with *Mlu*I and *Age*I enzymes, to obtain the full-length pBC LaSo HN plasmid. In pLaSota, the unique *Mlu*I site in the intergenic region of F and HN genes and the unique *SnaB*I site in the intergenic region of HN and L genes were used to exchange the HN genes between rBC and rLaSota infectious clones. For the insertion of the HN gene of pBC into pLaSota, PCR was done with the full-length pBC plasmid using primers flanking its HN gene. Primers used were: BC *Mlu*I (+) primer:

5′ AACT***ACGCGT***TGTAGATGACCAAAGGACGATATACGGGTAG 3′

and BC *Sna B*I (-) primer:

5′ GATCACCGG***TACGTA***TTTTGCCTTGTATCTCATTGCCACTTAC 3′

These primers introduced overhangs of *Mlu*I and *SnaB*I sites at the beginning and end of the pBC HN gene, respectively. The PCR product was then digested with *Mlu*I and *Sna B*I enzymes and ligated to the full-length pLaSota to obtain pLaSo BC HN plasmid. These plasmids were used in the recovery of the recombinant chimeric viruses, rBC LaSo HN and rLaSo BCHN respectively, using reverse genetics procedures (69).

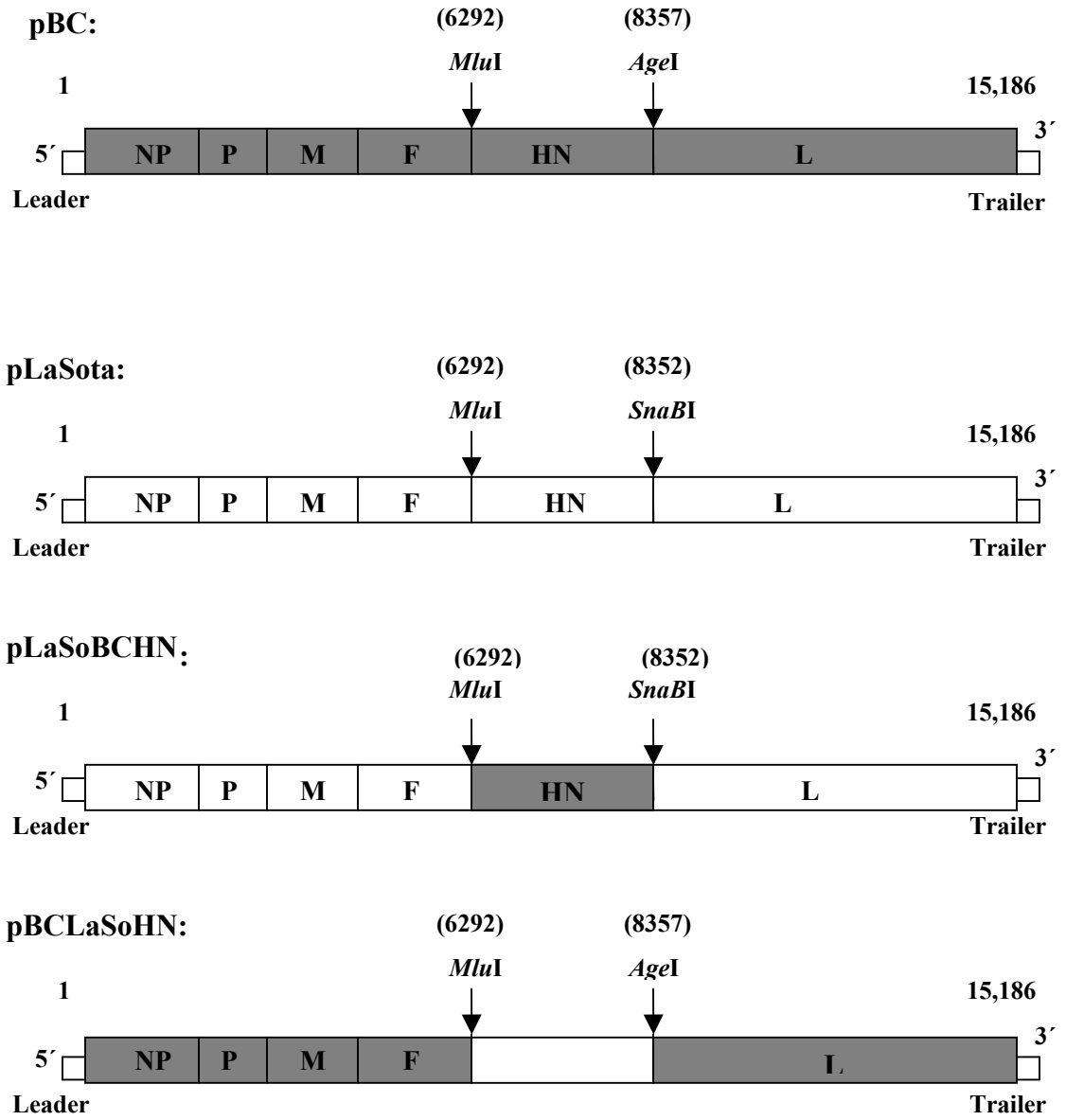


Figure 6. Schematic representation of the strategy for the exchange of the HN gene between rBC and rLaSota viruses. The drawing is not to scale. Two unique restriction sites (*Mlu* I and *Age* I in rBC virus and *Mlu* I and *Sna B* I in rLaSota virus) introduced in the HN intergenic region while constructing the full-length genome of NDV (54, 69), were exploited for the HN gene swap. The HN gene was exchanged as a single restriction

fragment between the rBC and rLaSota viruses, using *Mlu* I and *Age* I and *Mlu* I and *Sna B* I sites, respectively. The first and last nucleotide of the entire full-length genome of the parental rBC virus are shown. The approximate location of the restriction sites used are indicated (arrows).

3.4.3 RNA extraction and RT-PCR of recovered chimeric viruses

The recovered recombinant chimeric viruses rBC LaSoHN and rLaSo BCHN were grown in the allantoic cavity of 9-day-old embryonated chicken eggs. After 2 days, the allantoic fluid was harvested, clarified and the virus was purified as described above. Viral RNA was extracted from the recovered viruses using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done with the extracted RNA, using the Thermoscript RT kit (Invitrogen) to synthesize the first strand cDNA. The genomes of recovered chimeric viruses were entirely sequenced after RT-PCR to confirm the presence of the substituted gene. The sequence of the forward and reverse primers used for the RT-PCR of the substituted LaSota HN gene in the BC genome were: 5′AACTACGCGTTGTAGATGACCAAAGGACGATATACGGGTAG 3′ and 5′GATCACCGGTACGTATTTTGCCTTGTATCTCATTGCCACTTAC 3′ respectively. Forward and reverse primers used for the RT-PCR of the HN gene of pBC in the LaSota genome were: 5′AACTACGCGTTGTAGATGACCAAAGGACGATATACGGGTAG 3′ and 5′ GATCACCGGTACGTATTTTGCCTTGTATCTCATTGCCACTTAC 3′ respectively.

3.4.4 Growth characteristics of viruses

The growth kinetics of rBC LaSoHN and rLaSo BCHN were performed by multiple-cycle growth conditions in DF1 cells. Virus was inoculated at an MOI of 0.01 to DF1 cells grown in DMEM with 5% FBS at 37^{o}C. The medium of cells infected with rLaSo BCHN contained 1 μg/mL of acetyl trypsin. Supernatant was collected at 8 h intervals until 56 h post-infection (P.I). The virus content in the samples was quantitated

by plaque assays in DF 1 cells. Briefly, supernatant, collected from virus-inoculated samples earlier, was serially diluted, and 100µl of each serial dilution was added per well of confluent DF1 cells. After 60 min adsorption, cells were overlaid with DMEM (containing 2% FBS and 0.9% methyl cellulose), and incubated at 37^{o}C for 3-4 days. The cells were then fixed with ethanol and stained with crystal violet for enumeration of plaques.

3.4.5 Neuraminidase assay

A fluorescence-based neuraminidase (NA) assay was done as described by Potier *et al.* (110). Briefly, serial two-fold dilutions of virus samples were prepared in 50 µl volumes of enzyme buffer (32.5 ml of 0.1 2-N-Morpholinoethanesulfonic acid [MES], pH 6.5 and 4.0 ml of 0.1M calcium chloride made up to a final volume of 100 ml with Milli-Q water) in a 96-well plate. Ten µl of 12.5% v/v dimethyl sulfoxide (DMSO) was added to all wells of an assay plate (black 96-well Microfluor plates, Franklin, MA). Ten µl of each virus dilution was transferred in duplicate (2 rows) to the assay plate, starting with the most dilute in column 11. Ten µl of enzyme buffer alone was added to each well of column 12 (blank wells). The reaction was initiated by the addition of 30 µl per well of substrate mix (1 volume of 325 mM MES, pH 6.4, 3 volumes of 10 mM calcium chloride and 2 volumes of 0.5mM 2'-(4-methylumbelliferyl)- α-D-N-acetylneuraminic acid [MUN] (Sigma) to give a final concentration in the assay of 100 µM MUN. The reaction was incubated at 37^{o}C for 15 min with shaking, and terminated by the addition of 150 µl per well of 0.014 M sodium hydroxide in 83% (v/v) ethanol. NA activity of the recovered viruses was measured with the 4-methylumbelliferone released from the fluoregenic

substrate, MUN. Released 4-methylumbelliferone was quantified by fluorometric determination with an excitation wavelength of 360 nm and emission wavelength of 450 nm. Readings from the substrate blanks were subtracted from the virus sample readings, and the mean values of duplicate readings calculated.

3.4.6 Hemadsorption (HAd) assay

Virus was inoculated to confluent monolayers of Vero cells in 6-well plates at an MOI of 10. After 18 to 24h, the media was decanted and the cells were overlaid with guinea pig RBCs in PBS at a concentration of 1×10^8 cells/ml. The plates were kept at 4°C for 15 min. The unbound RBCs were removed by washing twice with PBS. The RBCs bound to the virus-infected cells were lysed with 0.05 M ammonium chloride and the released hemoglobin was measured at 549 nm in a spectrophotometer.

3.4.7 Fusion Index assay

The fusogenic abilities of the recombinant viruses were examined as described by Kohn (65). Virus was inoculated to confluent ($1X10^6$cells/ml) Vero cells in 6-well plates at an MOI of 0.1. Cells were maintained in 5% DMEM medium at 37^0C and 5% CO_2. After observing CPE within 48-72 h, media was removed and cells were washed once with 0.02% EDTA and then incubated with 1 ml of EDTA for 2 min at room temperature. The cells were then washed with PBS and fixed with methanol for 20 min at room temperature. Staining of the cells was done with hematoxylin-eosin (HE) stain (Hema 3). Quantitation of fusion was done by expressing the fusion index as the ratio of total number of nuclei to the number of cells in which these nuclei were observed (i.e., the

mean number of nuclei per cell). The NA, HAd and fusion index values of all viruses were expressed as a percentage of these values compared to the wild-type virus, rBC, in which these values were considered to be 100 per cent.

3.4.8 Immunohistochemistry

The tissue tropism of recombinant NDV was examined in 9-11-day-old embryonated chicken eggs. Briefly, 10^3 PFU of each of the recombinant NDV was inoculated into developing chicken embryos by the chorio-allantoic route. The infected embryos were chilled at 48 h PI, and tissues such as spleen, kidney, small intestine, large intestine, lung, heart, and brain were collected and fixed for cryosectioning and processing as described by Sheela *et al.*, (132). The tissues were cryosectioned and 3-5-umeter sections were cut for immunohistochemistry. All sampled tissues were examined by immunohistochemistry using the following protocol to detect the viral HN protein (IHC/HN). The sections were rehydrated in three changes of PBS, 10 min each, followed by treatment with proteinase K (10 ug/mL) for 30 min at 37^{o}C. The sections were then washed three times with PBS, fixed with ice-cold acetone and washed again in PBS. To quench endogenous peroxides, the sections were flooded with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After washing with three changes of PBS at 10-min intervals, the tissues were blocked with normal horse serum (VectaStain kit, Vector Laboratories, Burlingame, CA) for 30 min. The sections were then incubated with a 1: 500 dilution of the primary NDV monoclonal antibody (72) cocktail against HN (10D11, AVS, 15C4 and B79) for 1h at room temperature. After washing, sections were incubated with VectaStain secondary antibody (Vector Laboratories, Burlingame, CA)

for 30 min, as recommended by the manufacturer. After a further washing cycle, the sections were incubated with VectaStain ABC reagent for 30 min. Substrate was NovaRed (Vector Laboratories, Burlingame, CA). Sections were counterstained lightly with hematoxylin and coverslipped with Vectamount (Vector Laboratories, Burlingame, CA) for a permanent record.

3.4.9 Mean Death Time (MDT) in chicken embryos

The virulence of the recovered viruses was determined by the mean death time in embryonated SPF chicken eggs (1). A series of 10-fold dilutions of infected allantoic fluid was made in sterile PBS, and 0.1 ml of each dilution was inoculated into the allantoic cavity of each of the five 9-day old embryonated eggs. The eggs were incubated at 37^{o}C and examined four times daily for 7 days. The time that each embryo was first observed dead was recorded. The highest dilution at which all embryos died was considered the minimum lethal dose. The MDT was recorded as the mean death time in hours for the minimum lethal dose to kill the embryos. The MDT has been used to classify NDV strains into velogenic (taking under 60 h to kill); mesogenic (taking between 60 h to 90 h to kill) and lentogenic (taking more than 90 h to kill).

3.4.10 Pathogenicity studies in chickens

To test the pathogenicity of the recovered viruses *in vivo*, intra cerebral pathogenicity index (ICPI) test was performed according to standard procedures (1). For ICPI, 10^3 PFU/ chicken of each virus was inoculated intracerebrally into groups of ten 1-day-old specific-pathogen-free (SPF) chicks. The inoculation was done using a 27-guage

needle attached to a 1 ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical symptoms and mortality once every 12 h for a period of 8 days. Equal numbers of chickens were used in each experiment. Each experiment had mock-inoculated controls that received a similar volume of sterile PBS by the respective routes. The ICPI values were calculated as described by Alexander (1). Briefly, the birds were scored daily: 0 if normal, 1 if sick, and 2 if dead. The ICPI value was the mean score per bird per observation. Highly virulent (velogenic) viruses give values approaching 2; avirulent (lentogenic) viruses give values close to 0. To assess the survivability rates of birds inoculated with the recombinant viruses, one-day-old chicks were inoculated intracerebrally with 10^3pfu/chick. Infected chicks were observed daily for 8 days for signs of paralysis and death. Percent survival of inoculated chicks was plotted over time.

3.5 Results

3.5.1 Recovery of recombinant parental and chimeric NDV

The recovery of recombinant NDV from infectious cDNA clones, derived from a mesogenic strain of NDV, BC, and a lentogenic NDV strain, LaSota, have been reported earlier from our laboratory (54, 69). We used the established reverse genetics system to examine the role of the HN gene in the virulence of NDV, in this study. The HN genes of an avirulent recombinant NDV virus rLaSota and a virulent NDV virus were exchanged

in an otherwise identical genomic background. To ensure the presence of the intended HN gene swaps, the entire cDNA clone of each chimeric virus was sequenced using Big dye terminator (Applied Biosystems) method. The supernatants from transfected HEp2 cells were passaged two times in DF1 cells to recover infectious NDV. Virus-positive supernatants were used to inoculate the allantoic cavities of 9-11-day-old embryonated SPF eggs. The allantoic fluid harvested after embryo deaths were analyzed in a hemagglutination (HA) assay. Allantoic fluid with a positive HA titer was used for the isolation of viral RNA, followed by a sequence analysis of an RT-PCR fragment that covered the HN gene swap region. Nucleotide sequencing confirmed that the introduced HN gene exchanges between rBC LaSoHN and rLaSo BCHN were retained in the recovered viruses. The recovered rBC virus bearing the LaSota HN gene in place of its own HN gene was called rBC LaSoHN virus, and rLaSota virus with its HN gene replaced by the HN gene of BC virus was designated as rLaSo BCHN. These exchanges were stable and were seen even after 5 sequential passages of these viruses at high MOI in DF1 cells and in 9-day-old embryonated chicken eggs (data not shown).

3.5.2 Growth of the recombinant viruses *in vitro*

The growth characteristics of the parental and chimeric viruses were assessed by multi-step growth curves (Fig.7). The differences in the growth kinetics of HN gene swap viruses were marked until 16 h PI. When compared to its parental rLaSota virus, the growth rate of rLaSo BCHN virus was more than two-fold higher at 16 h PI, while the rBC LaSoHN virus was 1.4-fold lower than its parental rBC virus. The parental and gene swap viruses grew to similar titers after 32 h PI. These studies showed the relevance of

HN in virus growth *in vitro*. No differences were observed in the plaque sizes of recombinant viruses with reciprocal HN swap viruses when compared to their parental viruses. These results indicated that heterotypic F and HN proteins were compatible in the chimeric viruses.

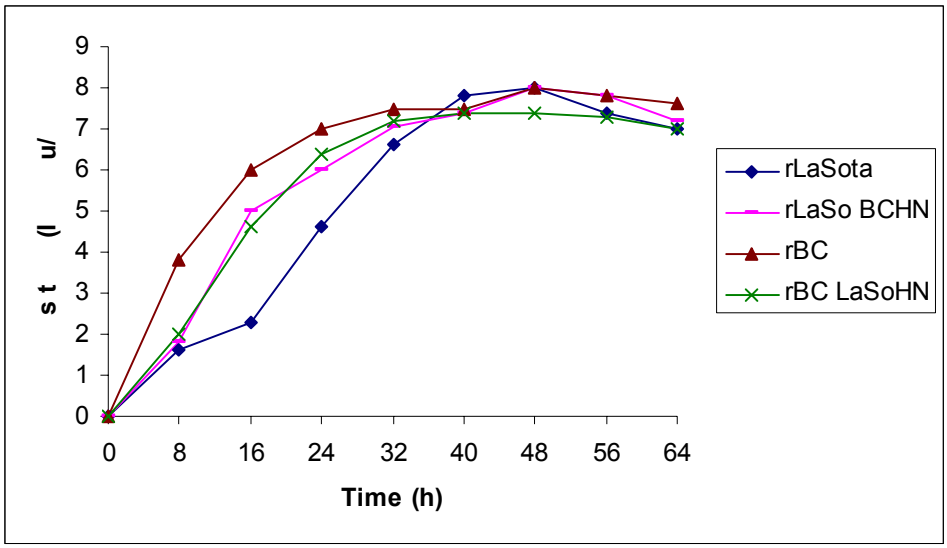


Figure 7. The growth kinetics of parental and chimeric viruses in chicken embryo fibroblast (DF1) cells. Multiple cycle growth conditions were employed to assess the differences in the growth of these viruses. DF 1 cells were infected with each of these viruses at an MOI of 0.01. Supernatants collected at 8h intervals were used for determining the virus titers by a plaque assay. The values represented are the means from three independent experiments.

3.5.3 Biological activities of mutant viruses

We analyzed whether the origin of HN protein determines the biological activities of NDV. The NA, HAd and fusion activities of the parental and chimeric viruses are shown (Fig.8). The percent biological activity of each chimeric or parental virus is shown relative to the rBC virus, whose biological activities were considered to be 100%. The NA and HAd values of rBC LaSoHN was only 50% of the parental rBC virus, while there was an 80 % increase in the HAd and a 20 % increase in the NA values of rLaSo BCHN virus over the rLaSota virus. The fusogenic abilities of the parental and chimeric viruses did not vary significantly. These studies reiterate the importance of HN protein in the attachment and neuraminidase functions of NDV in the context of a viral infection. These differences in the attachment and elution may translate into differences in viral growth kinetics *in vivo*. Furthermore, these results demonstrate that a combination of F and HN proteins derived from heterologous viruses are fully functional in inducing fusion.

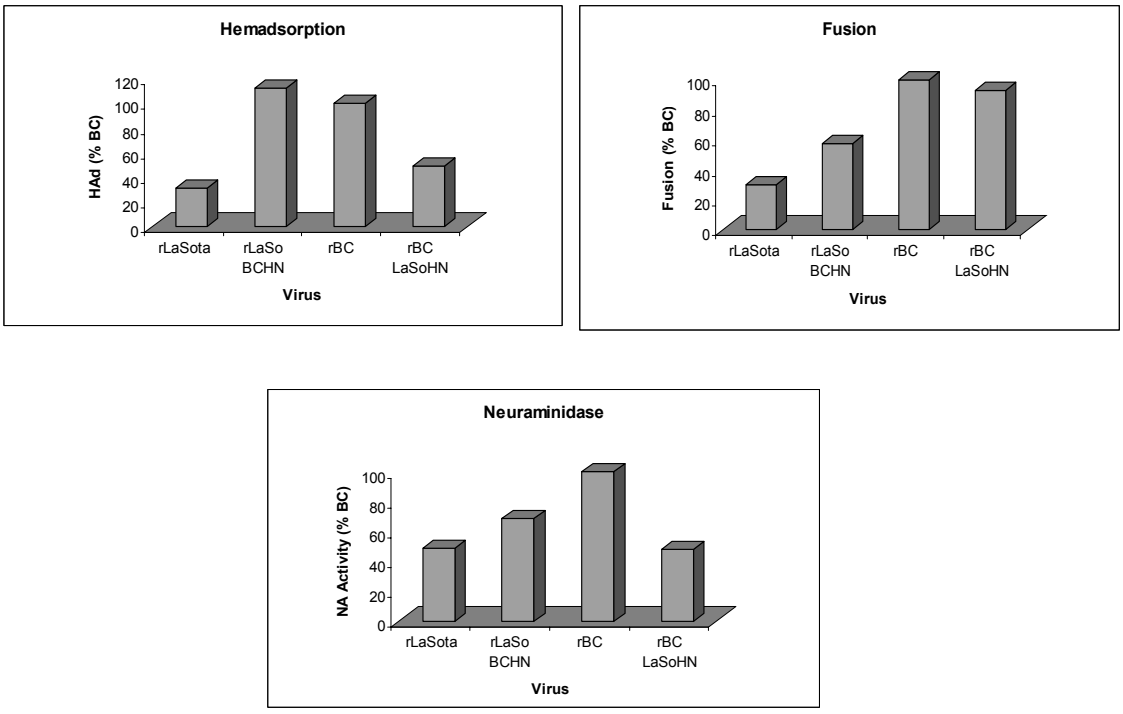


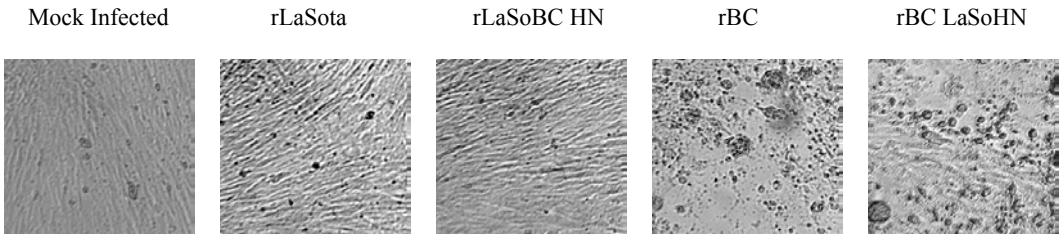
Figure 8. The *in vitro* biological activities of the parental and HN chimeric viruses. The hemadsorption (HAd), neuraminidase (NA) and fusogenicity of the parental and HN chimeric viruses were examined. The percent biological activities of these viruses are compared to the rBC virus, whose activities were considered to be 100%. The HAd was measured as the percentage of the hemoglobin released from guinea pig RBCs attached to infected cells. The NA activity of purified parental and chimeric viruses was measured by a fluorometric assay. The fusion index (FI) was estimated in Vero cells infected at an MOI of 0.1. The cells were stained with H&E stain and the FI was calculated. The FI is the ratio of the total number of nuclei to the number of cells in which the nuclei were observed, i.e., the mean number of nuclei per cell. The mean of three independent experiments is shown.

3.5.4 Cytopathogenicity and Tissue tropism

The cytopathic effects (CPE) of the HN swap viruses differed significantly. At 48 h PI, the rLaSo BCHN virus showed more extensive CPE in primary CEF cells than the rLaSota virus (Fig. 9). Similarly, rBC LaSoHN virus showed markedly less CPE than the rBC virus. The rapid destruction of cell monolayers by the rLaSo BCHN virus was consistent with the increase in the receptor recognition and neuraminidase activities of the virus. In the rBC LaSoHN virus, the reduction in the receptor binding and NA activities may have resulted in the differential CPE observed in CEF cells.

On the other hand, the tissue distribution of the chimeric viruses after chorio-allantoic inoculation of 10-day-old chicken embryos showed that the HN protein probably determines tissue tropism. The chimeric rBC LaSoHN and the lentogenic rLaSota viruses did not reach the brain of inoculated embryos, while with the rBC and rLaSo BCHN viruses, NDV-specific antigen could be demonstrated in different parts of the brain. (Fig.10). NDV antigens were localized in the alveolar epithelium and peribronchiolar space with rBC and rLaSo BCHN viruses, while chicken embryos inoculated with rLaSota and rBC LaSoHN viruses showed discrete staining of the bronchial and bronchiolar epithelium. The rLaSota and rBC LaSoHN viruses were also distributed in the villous epithelial cells of the small and large intestines, in addition to the lungs. There was a wide dissemination of the rBC and rLaSo BCHN viruses in chicken embryonic tissues such as lung, brain, kidney, intestines and spleen. The extent and distribution of antigen-positive areas in these tissues were marked and extensive.

24h post infection:



48h post infection:

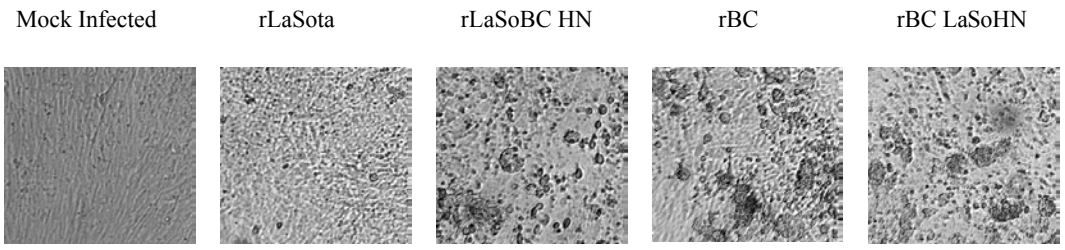


Figure 9. The cytopathogenicity of the parental and chimeric viruses in DF1 cells. The cells were infected at an MOI of 0.1with each of these viruses. After 24 and 48h, the cyotopathic effects (CPE) of each virus infected monolayer was examined under an inverted microscope. The differences in the CPE of each virus infected monolayer at 24 and 48h are shown. The figures in top panel represent CPE observed at 24h post-infection. The bottom panel shows the CPE at 48h post-infection for different viruses.

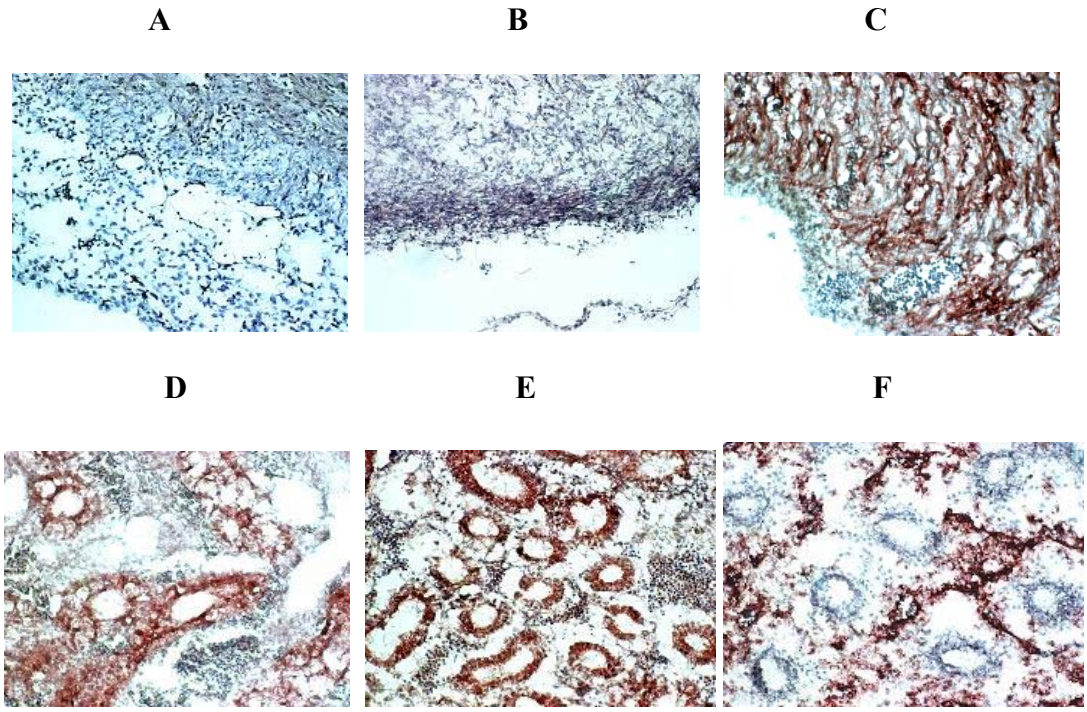


Figure 10. The tissue tropism of recombinant NDV in developing chicken embryos. Groups of 9-11-day-old chicken embryos were inoculated with 10^3 PFU of each of the parental and HN chimeric NDV by the chorio-allantoic route. Frozen sections from infected embryos were processed for immunohistochemistry after 48 h. Tissue sections were stained with primary NDV monoclonal antibody cocktail followed by VectaStain (Vector Laboratories) and VectaStain ABC reagent. The substrate was NovaRed (Vector Laboratories). Representative sections from each virus infected embryo are shown. While rLaSota and rBC-LaSoHN viruses showed no staining in the cerebellum (A and B), the cerebellum showed extensive antigen-specific staining in the molecular layer in rLaSo-BCHN virus (C), Viral antigens were localized in the bronchial and bronchiolar epithelium with rLaSota (D) or rBC-LaSoHN (E) viruses, but diffuse interstitial staining was noticed with rLaSo-BCHN virus (F). Magnification X 20.

3.5.5 Pathogenicity studies of mutant viruses

We wished to determine whether the differences in *in vitro* biological characteristics of the chimeric viruses would translate into increased or decreased virulence in chickens or chicken embryos. The MDT of rLaSo BCHN was decreased to 84 h when compared to the parental rLaSota virus, which had a MDT of 96 h, indicating an enhancement in the virulence of the chimeric virus. The rBC virus had a MDT of 62h while rBC LaSoHN took 72h to kill embryonated SPF eggs, indicating the degree of attenuation imparted by the LaSoHN to the rBC genetic background. The ICPI values indicated altered virulence properties in chimeric viruses when compared to the parental viruses (Table 1). The recombinant rLaSo BCHN had an ICPI of 0.75 when compared to its lentogenic parental strain rLaSota with an ICPI of 0.00, despite both of them being isogenic, except for the swapped HN gene. The rBC LaSoHN virus, on the other hand, showed a reduced ICPI value of 1.02 when compared to its mesogenic parental virus rBC with an ICPI of 1.58. The survivability of 1-day-old chicks inoculated intracerebrally with the recombinant viruses is shown (Fig.11). The rLaSota virus induced 0% mortality after 8 days of inoculation. The rLaSo BCHN virus showed a dramatic increase in virulence compared to its parent rLaSota, with only 20% of the birds surviving on day 8. After inoculation with rBC virus, 20% of the birds survived on day 3 and by day 4, all the inoculated birds were dead. On the contrary, the rBC LaSoHN virus showed decreased virulence compared to its parent rBC, with 20% of the birds surviving on day 5 and 100% mortality on day 6. The gain or loss of virulence in these viruses, therefore, appears to be solely mediated by the HN gene.

Table 1. HN chimeras of NDV and virus pathogenicity *in vivo*.

Virus	ICPI [a]	MDT [b]
rLaSota	0.00	96
rLaSo BC HN	0.75	84
rBC	1.58	62
rBC LaSo HN	1.02	72

The virulence of the mutant and parental viruses was evaluated by intracerebral pathogenicity index (ICPI) in day-old-chicks, and minimum death time (MDT) in embryonated chicken eggs. [a] ICPI was determined by inoculating 10^3 PFU of each virus/ bird, into groups of 10 one-day-old SPF chicks, *via* the intracerebral route. The birds were observed daily for 8 days and at each observation, scored 0 if normal, 1 if sick, and 2 if dead. The ICPI value is the mean score per bird per observation. Highly virulent viruses give values approaching 2 and avirulent viruses give values approaching 0. [b] MDT in hours for the minimum lethal dose to kill 9-day-old embryonated chicken eggs by allantoic route of inoculation. Highly virulent viruses take under 60h to kill the embryos whereas avirulent viruses take more than 90h to kill the embryos.

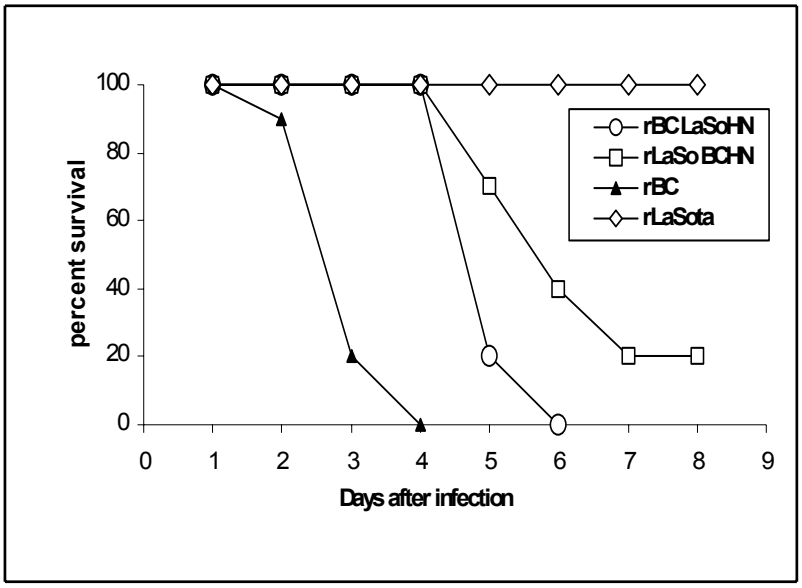


Figure 11. The survivability of 1-day-old chicks inoculated with the parental and HN chimeric viruses. Specific-pathogen-free 1-day-old chicks were inoculated intracerebrally with 10^3 PFU/chick. Infected chicks were observed daily for 8 days for signs of paralysis and death. Percent survivors for each virus are plotted over time.

3.6 Discussion

The HN gene plays an important role in the pathogenesis of paramyxoviruses (34). The HN gene of NDV is a multifunctional protein with receptor recognition, neuraminidase activity on sialic acid-containing receptors, and fusion promotion (71). Transfection studies with HN mutants of NDV have highlighted the importance of the different regions of the HN protein in the biological activities of the protein (31, 80, 81). However, the contribution of the HN gene in NDV pathogenesis in the context of virus infection is not known. In this study, using reverse genetics techniques, a chimeric rBC virus (a recombinant moderately virulent, mesogenic NDV strain) containing the LaSota (a recombinant avirulent, lentogenic NDV strain) HN gene in place of its own and the reciprocal recombinant, consisting of rLaSota bearing the BC HN gene, were generated to assess the effect of HN gene substitution on viral pathogenesis. The hypothesis tested in the present study was that differences in biological functions of HN protein displayed by different strains of NDV *in vitro* are important determinants of pathogenesis *in vivo*. The pathogenicity of the chimeric NDV was examined in the natural host, chicken. The results of this study indicated that the *in vitro* biological characteristics of the HN are good indicators of *in vivo* pathogenicity and the HN gene makes an important contribution to the overall virulence of NDV isolates.

The sequence analysis of the chimeric viruses confirmed the presence of the substituted HN gene. These chimeric viruses reached similar titers with similar kinetics, compared to those viruses with homologous proteins. This suggested that heterologous

pairs of HN and F proteins were fully functional despite being derived from different viruses. This probably reflects the high level of amino acid sequence identity between these two different NDV strains (98.4% in F and 97.1% in HN). The BC and LaSota strains used in this study are phylogenetically closely related (15). Therefore, it would be interesting to examine whether the HN and F protein pairs derived from distantly-related NDV strains, are also functional *in vitro* and *in vivo*.

In vitro studies with these viruses yielded results that supported the hypothesis of the importance of the HN gene in NDV virulence. The parental rBC virus showed higher HAd and NA activities, compared to that of the parental rLaSota virus. Interestingly, the rLaSo BCHN virus also had increased HAd and NA activities when compared to its parent rLaSota. These activities were decreased in the rBC LaSoHN virus when compared to the parental rBC virus. These results indicated that the magnitude of the HAd and NA activities of NDV are solely determined by the amino acid differences in the HN protein. The fusion promotion activity of HN appeared to be independent of HAd and NA activities. Despite the fact that the HAd and NA activities of BC and LaSota viruses were at different levels, the heterotypic HN proteins in the chimeric viruses were fully functional in fusion promotion. Our results are in agreement with those of Sergel *et. al*., (129) who have shown that the fusion promotion activity of HN did not correlate with the level of NA activity.

Several reports have suggested that a type-specific functional interaction between F and HN proteins of paramyxoviruses is required for cell fusion (32, 53, 138). Studies of

hybrid HN proteins by three different laboratories have all implicated the membrane-proximal ectodomain in virus-specific fusion promotion activity (31, 141, 144). With this approach, the specificity of the NDV F protein has been mapped to amino acids 55-141 of the NDV HN protein, a sequence adjacent to the HN protein transmembrane domain (31). However, we have demonstrated that chimeric NDV containing heterotypic F and HN proteins were compatible, since they were derived from phylogenetically closely-related virus strains (15). It was suggested that a specific interaction occurs between the F protein HR2 domain and the HN protein domain from amino acids 124 to 152 for fusion promotion (43). There was only one amino acid difference from phenylalanine to tyrosine (F132I) between BC and LaSota viruses in the proposed F and HN interacting domain, which may be the reason for the heterotypic interaction of the F and HN proteins from distantly-related strains of NDV.

It has been proposed that for influenza virus, the neuraminidase activity may be important for removing respiratory tract mucin sialic acids, allowing the virus to reach target cells (22). There is a precedent for decreased virulence as a result of decreased neuraminidase activity for influenza viruses in animals (91, 145). To the contrary, the C-28 variant of human parainfluenza virus with deficient neuraminidase caused more intense disease in cotton rats (111). Examination of a temperature-sensitive NDV variant and two sequential revertant viruses revealed that alterations in neuraminidase can compensate for alterations in binding (131, 136). The original NDV variant, with an amino acid substitution at position 129, was deficient in binding erythrocytes; a second mutation, at position 175, reduced neuraminidase activity but restored binding; the third

sequential mutation, at position 193, partially restored neuraminidase activity. Thus, the balance between the receptor binding and neuraminidase activities appear to be critical to the virulence of NDV. The early induction of CPE in CEF cells by rLaSo BCHN and diminished CPE of rBC LaSoHN, when compared to their parental types with homologous HN in our study, might have resulted from these changes in receptor recognition and NA activities. Increased attachment and NA activities are consistent with the early growth and CPE of these viruses with virulent HN as against those viruses containing avirulent HN showing a delay in reaching the peak titers and inducing CPE.

A small amino acid motif in the fusion protein precursor [F0], termed the cleavage site, has been identified as a pathotype determinant. Thus, viruses with multibasic amino acids at the cleavage site are designated virulent, and those characterized by monobasic amino acids at the cleavage site are avirulent (17, 18, 19, 20). Cleavage of F0 polypeptides into F2 and F1 is essential for a virus particle to become infectious (92). In chickens, the F protein of pathogenic NDV is cleaved by ubiquitous intracellular furin-like proteases, and the F protein of non-pathogenic viruses is only cleaved by trypsin-like proteases secreted from a limited number of tissues (39). Thus, virulent viruses can spread rapidly throughout the host and cause disease. In comparison, the avirulent motif can be cleaved only in the respiratory tract and gut, where trypsin-like proteases are available. The replication of these viruses is, therefore, restricted to these organs (92). However, in our studies with chicken embryos, where F proteins with either mono or multibasic amino acids at the cleavage site are cleaved with equal efficiency due to the availability of trypsin-like proteases, the HN gene sequence appears to determine

the tropism of the virus to different tissues and organs. Especially, the discrete replication of viruses with avirulent HN in bronchial and bronchiolar epithelium, widespread distribution of the viruses with virulent HN in the lung parenchyma, and the systemic distribution of the viruses with virulent type HN in several organs including the brain, indicates that the HN protein, independent of F protein cleavability, determines the viral tropism in chicken embryos. This may be the result of differences in the receptor recognition and NA activities between the HN proteins of BC and LaSota viruses. It would be interesting to study the tropism of these viruses in chickens, where F cleavability may play a major role in virulence via natural routes of infection. A set of carefully-defined mutations in the HN protein may indicate the amino acid residues involved in the tropism and virulence of NDV.

The pathogenicity of the chimeric viruses was assessed by ICPI and MDT tests. Interestingly, the results showed that the chimeric rBC virus bearing the HN gene of LaSota (the rBC LaSoHN virus) was markedly less virulent than the parental rBC virus, and the reciprocal chimeric rLaSo BCHN virus was also mildly virulent, compared to its parental rLaSota virus. Chicks inoculated with rLaSo BCHN had lower survival rates, compared to chicks inoculated with rLaSota, and chicks inoculated with rBC LaSoHN had higher survivability than the ones inoculated with rBC. MDT results also showed a gain of virulence of rLaSo BCHN and a loss of virulence of rBC LaSoHN, compared to rLaSota and rBC, respectively. Thus, when the HN gene of avirulent LaSota strain replaces the HN gene of virulent BC strain in the BC virus, there was a reduction in virulence of the chimeric virus (rBC LaSoHN). Exactly, the opposite effect was observed

in the chimeric virus, rLaSo BCHN, with enhanced virulence. This "gain or loss of function" between the chimeric and parental virus strains was a further indication that the observed differences were associated with the HN protein.

In summary, using reciprocal HN gene swaps between avirulent and virulent NDV in an otherwise identical genetic background, we have demonstrated that the reduced receptor recognition and neuraminidase activities, inherent to avirulent HN, translate into differences in the tropism and virulence of the virus. Avirulent HN bearing chimeric virus remain restricted in growth to limited tissues and organs, besides being attenuated in virulence relative to the parental type. On the other hand, virulent HN imparted ability to spread systemically, and enhanced the virulence of an otherwise avirulent NDV.

It will be interesting to study the molecular mechanism by which the HN gene determines tropism and virulence. The terminal globular head of the HN protein contains the active sites involved in virus attachment and neuraminidase activities (24, 57, 58, 86, 150). Recent results suggest that a specific interaction between the F protein HR2 domain and the HN protein membrane-proximal ectodomain spanning amino acids 124 to 152 occurs for fusion promotion (43). There are only 17 amino acid differences between the HN proteins of BC and LaSota viruses. With the availability of an established reverse genetics system for avirulent and virulent NDV strains, it would be of special interest to mutate these residues on the HN protein involved in its biological functions and observe the effects of these mutations in virus infectivity *in vitro* and *in vivo*.

CHAPTER 4

4.1 Title

Loss of carbohydrate from the Hemagglutinin-Neuraminidase protein of Newcastle Disease Virus affects virulence

4.2 Abstract

The hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) is an important determinant of its virulence. Using reverse genetics procedures, we investigated the role of each of the four functional *N*-linked glycosylation sites (G1-G4) of the HN glycoprotein of NDV on its pathogenicity. The *N*-linked glycosylation sites G1-G4 at residues 119, 341, 433 and 481, respectively, of a virulent NDV strain Beaudette C (BC) were eliminated individually by site-directed mutagenesis on a full-length cDNA clone of BC. A double mutant was also created by eliminating the first and second glycosylation sites at residues119 and 341(G12), respectively. Infectious virus was recovered from each of the cDNA clones of the HN glycoprotein mutants, employing a reverse genetics technique. The G4 and G12 mutant viruses replicated to lower titers than the parental virus. The differences in the biological activities of the mutant viruses, such as neuraminidase and hemadsorption activities, were negligible in comparison to the parental virus. However, the fusogenicity of glycosylation mutant G4 was significantly

lower and that of the double mutant virus (G12) was significantly higher than that of the parental virus. Cell surface expression of the G4 virus was significantly lower than the parental virus. The antigenic reactivities of the mutants to a panel of monoclonal antibodies against the HN protein indicated that removal of glycosylation from the HN protein increased (G1, G3 and G12) or decreased (G2 and G4) the formation of antigenic sites, depending on their location. In standard tests devised to assess the virulence of these strains, such as the intracerebral and intravenous pathogenicity indices, mutants G4 and G12 had significantly lower indices than the parental BC virus, indicating loss of virulence. These results indicate that glycosylation of the HN glycoprotein of NDV may play a role in the virulence of NDV, and that elimination of the fourth glycosylation site (residue 481) at the globular head, or more than one glycosylation site (residues 119 and 341) in the stalk and globular head, may attenuate the virus significantly. This approach can be used for the generation of recombinant NDV vaccines.

4.3 Introduction

Newcastle disease virus (NDV) is a member of the family *Paramyxoviridae*, and has been assigned to the genus *Avulavirus* in the subfamily *Paramyxovirinae* (78, 89). It causes a serious respiratory and neurological disease in all species of birds and is an economically-important infectious agent, causing significant losses to the poultry industry. Newcastle disease varies in the degree of severity, ranging from an inapparent infection to severe disease causing 100% mortality. NDV strains are categorized into three main pathotypes, depending on the severity of disease produced in chickens.

Avirulent strains that do not cause disease are known as lentogenic, viruses of intermediate virulence that cause respiratory disease are called mesogenic, while virulent viruses that cause high mortality are termed velogenic. Velogenic strains are further classified as viscerotropic or neurotropic. Viscerotropic velogenic strains produce lethal infections in which hemorrhagic lesions in the intestine are prominent; whereas, neurotropic velogenic strains produce high mortality preceded by neurological signs, with the absence of hemorrhagic intestinal lesions (2).

NDV contains a single-stranded, negative sense, non-segmented RNA genome. The genomic RNA is 15,186 nucleotides in length (68, 108). The genomic RNA contains six genes that encode at least seven proteins (104, 137). The envelope of NDV contains two glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins. The F glycoprotein mediates fusion of the viral envelope with cellular membranes (16). The HN glycoprotein of NDV is a multifunctional protein. It recognizes sialic acid- containing receptors on cell surfaces; it promotes the fusion activity of F protein of NDV, thereby allowing the virus to penetrate the cell surface, and it acts as a neuraminidase by removing the sialic acid from progeny virus particles to prevent viral self-aggregation (71).

The HN glycoprotein is a type 2 homotetrameric integral membrane protein which undergoes *N*-linked glycosylation (81, 115). This process occurs in the rough endoplasmic reticulum of host cells when an *N*-linked carbohydrate attaches covalently as a core oligosaccharide side chain to asparagines on the nascent polypeptide chain in

response to the consensus sequence motif NXT (Asn-X-Thr) or NXS (Asn-X-Ser), where X is any amino acid except aspartic acid and proline. The structure of the oligosaccharide side chain is then extensively modified as the protein moves through the membrane systems of the cell (67). *N*-linked glycosylation influences many properties of glycoproteins, including initiation and maintenance of folding of the proteins into their biologically-active conformation, maintenance of protein stability and solubility, intracellular transport of the proteins to various subcellular compartments and the cell surface, and influencing the antigenicity and immunogenicity of the protein (101,112).

The HN glycoprotein sequence of NDV strain Beaudette C (BC) contains six predicted sites for addition of *N*-linked carbohydrates (residues 119, 341, 433, 481, 508 and 538) (81). A previous study has shown that four of these addition sites (G1, G2, G3 and G4 at residues 119, 341, 433 and 481, respectively) are used, while two addition sites (G5 and G6 at residues 508 and 538, respectively) are not used (81). The same study has shown that G1 and G2 play little role in maturation, but modulate the biological activities of the protein; whereas, G3 and G4 influence both folding and activity of the protein (81). In that study, the role of individual oligosaccharide chains in the activities of the HN glycoproteins was examined using a plasmid transfection system. Thus, the role of each oligosaccharide chain in viral replication and pathogenesis could not be determined. In our current study, a reverse genetics system was used to generate recombinant viruses with mutations in the glycosylation sites of the HN protein. These mutations eliminated each of the four functional glycosylation

sites individually (G1, G2, G3 and G4), and in G1 and G2 in combination. This allowed the determination of the role played by the individual glycans in the context of viral replication and pathogenesis. Our results showed that elimination of the residue at G4, and combined elimination of residues at G1 and G2, significantly attenuated the pathogenicity of the viruses. It also indicated that G4 played an important role in the transport of the HN protein. Interestingly, elimination of G1 and G2 significantly increased fusion promotion activities of the viruses. This study provides a useful tool for studying the impact of glycosylation of the HN protein of NDV on virus pathogenicity, and may be able to provide insights for designing better recombinant attenuated NDV vaccines.

4.4 Materials and Methods

4.4.1 Cells and virus

Chicken embryo fibroblast cell line (DF1) was obtained from Dr. Douglas Foster (University of Minnesota) and grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum. HEp 2 cells, which were used for transfection, were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum. NDV strain BC and other recombinant viruses were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The modified vaccinia Ankara (MVA) recombinant that expresses the T7 RNA polymerase (a generous gift of Dr. Bernard Moss, National Institutes of Health) was grown in primary chicken fibroblast (CEF) cells.

4.4.2 Introduction of HN mutations into full-length NDV cDNA

Construction of plasmid pNDVfl carrying the full-length cDNA of the NDV strain BC has been described previously (69). To introduce mutations into the HN gene of pNDVfl, a *Age* I- *Mlu*-I fragment containing the HN gene was excised from pNDVfl and subcloned into plasmid pGEM-7Z (+) (Promega, Wisconsin, MA). Using appropriate oligomers, site-directed mutagenesis (9) was performed on the cDNA clone of the HN gene to generate a panel of HN mutants, as shown in Fig. 12. Mutants were named with a G (for glycosylation site) and the number of the functional glycosylation site mutated (1-4). For generating cDNA clone G1, the forward primer used was 5′ -^{6766}***C***A***G***AACAGCGGGTGGGGGGCACCT ^{6789}-3′ and reverse primer 5′- ^{6765} CGCAGCTCCATTAATCTGATAAGAGAG ^{6739}- 3′; for cDNA clone G2, forward primer, 5′- ^{7432} ***C***A***G***GACACATGCCCAGATGAG ^{7452}-3′ and reverse primer 5′- ^{7431}GTATCGCTTGTATATTACATATTTCCC ^{7405}-3′; for cDNA clone G3, forward primer 5′- ^{7708}***C***A***G***AAAACAGCCACTCTTCATAGTCCC ^{7734}-3′ and reverse primer 5′- ^{7707}GCTGACTGTCATAGGATATAATAACGC ^{7681}-3′; and for cDNA clone G4, forward primer 5′ -^{7852}***C***A***G***CACACCTTGCGAGGGGTA^{7872}-3′ and reverse primer 5′- ^{7851}CCTATAGAAGATTAGGGGATATGG ^{7828}-3′. Bold, italicized alphabets represent the mutated nucleotides, that changed the corresponding amino acid. To eliminate each glycosylation site, the asparagine residue in the NXT/NXS sequence motif was changed to glutamine. A double HN mutant, G12, was also created by eliminating glycosylation sites 1 and 2. This was done by mutating the second glycosylation site on the cDNA clone, G1, using the primer specific for making the G2 cDNA clone. All

mutant HN cDNAs were sequenced to their entirety to confirm the presence of the intentional mutations. The mutagenized *Age* I- *Mlu* I fragments were then excised from pGEM 7Z(+) and inserted into the *Age* I- *Mlu* I site of pNDVfl.

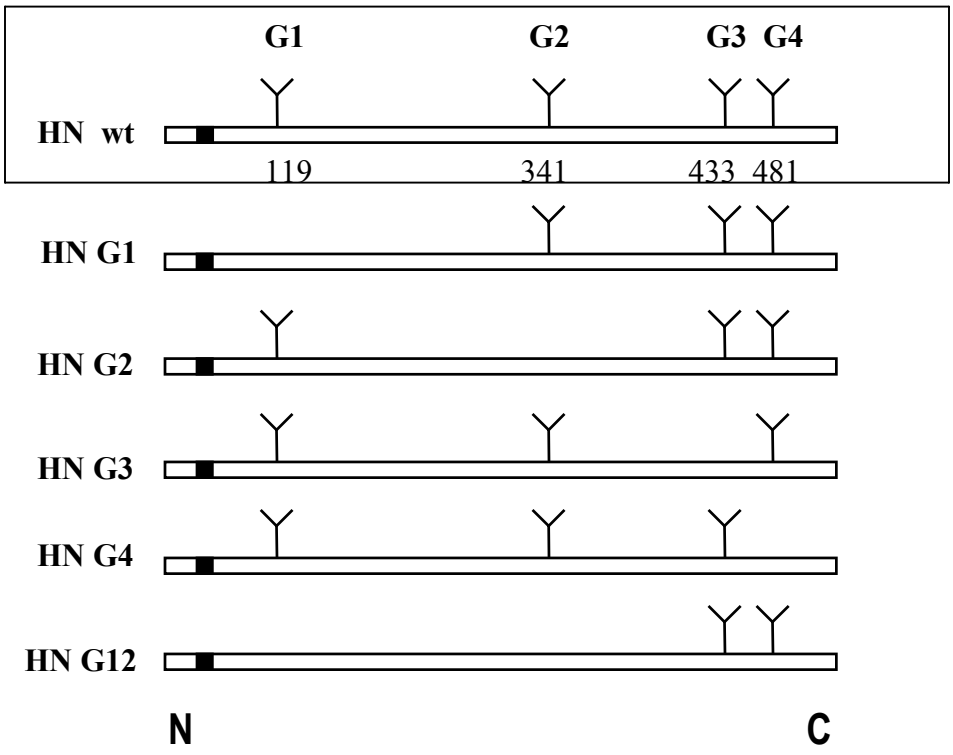


Figure 12. Schematic representation of the HN glycosylation mutants generated by site-directed mutagenesis. The HN wild-type (HN wt) protein is shown within the box at the top of the figure. Symbols: ▭ , 571 amino acid polypeptide chain ▬, N-terminal transmembrane (TM) anchor; Y, functional *N*-linked glycosylation sites; the residue number indicates the Asn residue in the HN amino acid sequence. The designations of the carbohydrate chains (G1, G2, G3 and G4) are indicated above each site. HN glycosylation mutants are represented in a similar fashion, with the designated name shown at the left of each diagram. HN wt has four functional Asn-linked glycosylation sites, represented by G1, G2, G3 and G4, present at amino acid residues 119, 341, 433

and 481, respectively. HN G1 mutant has its glycosylation site G1 at residue 119 eliminated, mutant HN G2 has its G2 glycosylation site at residue 341eliminated, mutant HN G3 has its G3 glycosylation site eliminated, mutant HN G4 has its G4 glycosylation site at residue 481 eliminated, and the double mutant HN G12 has two of its glycosylation sites at residues 119 and 341eliminated. All glycosylation sites were eliminated by mutating the asparagine (Asn) residue at each site to glutamine (Glu), using site-directed mutagenesis.

4.4.3 Generation of recombinant viruses

Transfection and recovery of recombinant NDV were performed as previously described (69). Briefly, HEp2 cell monolayers were infected at a multiplicity of infection (MOI) of 3 with recombinant vaccinia virus strain MVA/T7 expressing the T7 polymerase. HEp2 cells were used for transfection since they are permissive for NDV growth and resistant to cytopathic effects (CPE) caused by vaccinia virus MVA strain. Cells were grown to 80% confluency in 6-well plates, and washed twice with optiMEM (Invitrogen) just before transfection. The cells were transfected with 5μg of each of the five glycosylation mutant full-length cDNA clones (i.e., G1, G2, G3, G4 and G12) together with 2.5 μg NP, 1.5 μg P and 1.0 μg L support plasmids. These were added in a volume of 0.2 ml of optiMEM/well. Transfection was done with Lipofectamine^{TM} Reagent (Invitrogen), according to the manufacturer's instructions. Cells were incubated at 37^{o}C for 8h. The supernatant mixture was then removed and replaced with 3 ml of DMEM. Cells were maintained at 37^{o}C for 3-4 days and passaged again in HEp2 cells to remove the residual vaccinia virus. The supernatants from the second HEp2 passage were used to infect DF1 cells and, after 2-3 days, the cells were examined for typical CPE produced by NDV in the form of syncytia. Infectious virus was thus obtained entirely from cloned cDNA. Each recombinant virus was plaque-purified on DF1 cells and was used to generate virus stocks by growth in 9-11-day-old chicken embryonated eggs. Recovered viruses were designated rBC for the virus obtained from transfection with wild-type plasmid construct pNDVfl, and rG1, rG2, rG3, rG4 and rG12 for the respective mutant viruses obtained from plasmid constructs G1, G2, G3, G4 and G12, respectively.

4.4.4 RT-PCR and sequence analysis of the HN gene

RNA was isolated from the recovered HN mutant viruses by using TRIzol reagent (Invitrogen). RT-PCR was performed using the Thermoscript RT-PCR kit (Invitrogen) with primers

P1 (5′- ^{6202}GTGAACACAGATGAGGAACG ^{6221}-3′ positive sense) and P1R (5′- ^{9369}ATATCATTGGGGAGGAGGCCG ^{9349}-3′ negative sense) to amplify the HN gene. The amplified cDNA fragments were then sequenced using the Themosequenase kit (Amersham) to confirm the presence of the introduced mutations in the recovered viruses. Primer used to confirm the mutation at G1 (amino acid residue at position119 on the HN gene) was: 5′- ^{6623}CATCTGCACTTGGTTCCAATC ^{6643}-3’ for mutation at G2 (residue 341): 5’- ^{7294} TGGGTGGCCAACTACCCAGG ^{7313}- 3’ for mutation at G3 (residue 433): 5’- ^{7660}GGGTCATCATACTTCTCTCCCGCG ^{7683}-3’ for mutation at G4 (residue 481): 5’- ^{7660}GGGTCATCATACTTCTCTCCCGCG ^{7683}-3′ for mutation at G12 (residues119 and 341): 5′- ^{6623}CATCTGCACTTGGTTCCAATC ^{6643}-3′ and 5′ -^{7294}TGGGTGGCCAACTACCCAGG ^{7313}-3′. The HN cDNA amplified by RT-PCR of the recovered viruses were also sequenced entirely to ensure that the rest of the sequence of the protein remained unchanged.

4.4.5 Characterization of the mutant viruses

The growth rate of each recombinant virus was examined by multiple-cycle growth conditions. The DF1 cells were infected with each virus at an MOI of 0.01. A sample of the supernatant was harvested at 8h intervals for a period of 64h. The amount

of virus in the supernatant was determined by plaque assay on DF1 cells. Size and morphology of the observed plaques were also examined.

The receptor recognition properties of the recombinant viruses were evaluated by their ability to adsorb guinea pig red blood cells (RBCs) (65). Confluent monolayers of DF1 cells were infected with each virus at an MOI of 10. After 24h at 37^{o}C, the medium was removed and the infected cell monolayer was overlaid with guinea pig RBCs in PBS at a concentration of 1×10^{8} cells/ml. The cells were incubated for 15 min at 4^{o}C. The unbound RBCs were washed two times with PBS. The RBCs bound to the virus-infected cells were lysed with 0.05 M ammonium chloride. The released hemoglobin (hemadsorption activity of the virus) was measured at 549 nm in a spectrophotometer.

The neuraminidase (NA) activity of the recombinant viruses was determined by a fluorescence-based NA assay according to the procedures of Potier *et al.* (110). Briefly, serial two-fold dilutions of virus samples were prepared in 50 µl volumes of enzyme buffer (32.5 ml of 0.1 2-N-Morpholinoethanesulfonic acid [MES], pH 6.5 and 4.0 ml of 0.1M calcium chloride made up to a final volume of 100 ml with Milli-Q water) in a 96-well plate. Ten µl of 12.5% v/v dimethyl sulfoxide (DMSO) was added to all wells of an assay plate (black 96-well Microfluor plates, Franklin, MA). Ten µl of each virus dilution was transferred in duplicate (2 rows) to the assay plate, starting with the most dilute in column 11. Ten µl of enzyme buffer alone was added to each well of column 12 (blank wells). The reaction was initiated by the addition of 30 µl per well of substrate mix (1 volume of 325 mM MES, pH 6.4, 3 volumes of 10 mM calcium chloride and 2 volumes

of 0.5mM 2'-(4-methylumbelliferyl)- α-D-N-acetylneuraminic acid [MUN] (Sigma) to give a final concentration in the assay of 100 μM MUN. The reaction was incubated at 37°C for 15 min with shaking, and terminated by the addition of 150 μl per well of 0.014 M sodium hydroxide in 83% (v/v) ethanol. NA activity of the recovered viruses was measured with the 4-methylumbelliferone released from the fluoregenic substrate, MUN. Released 4-methylumbelliferone was quantified by fluorometric determination with an excitation wavelength of 360 nm and emission wavelength of 450 nm. Readings from the substrate blanks were subtracted from the virus sample readings, and the mean values of duplicate readings calculated.

The ability of each recombinant virus to form syncytium was determined according to the procedures described by Kohn (65). Briefly, confluent monolayers of Vero cells were infected at an MOI of 0.1. After 48h at 37°C, the medium was removed, and cells were washed once with 0.02% EDTA and incubated with 1 ml of 0.02% EDTA for 2 min at room temperature. The cells were then washed with PBS and fixed with methanol for 20 min at room temperature. Staining of the cells was done with 1% hematoxylin-eosin stain, using the Hema 3 Stain Set (Biochemical Sciences, Inc., NJ). The syncytia were quantitated by determining the fusion index for each virus. Fusion index is the ratio of the total number of nuclei to the number of cells in which these nuclei are present, (i.e., the mean number of nuclei per cell).

4.4.6 Radioimmunoprecipitation and analysis of stability of HN protein

DF1 cells were infected with recombinant viruses at an MOI of 10. After 4h incubation at 37^{o}C, the medium was removed, and the cells were washed with PBS and starved in 5 ml of methionine-cysteine-free medium (Invitrogen) for 30min. The cells were then pulse-labeled for 2h, with medium containing 100 μCi of ^{35} [S] methionine-cysteine (1000 Ci/mmol; Amersham, Arlington Heights, IL) per mL. After labeling, cells were washed in PBS and lysed in cold radioimmunoprecipitation (RIPA) buffer {20mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100}. The cell lysates were centrifuged to remove the nuclei. Immunoprecipitations were performed by adding anti-NDV-specific polyclonal antibody to the cell lysates and incubating for 3h at 4^{o}C. Immune complexes were precipitated by incubating with *Staphylococcus aureus* protein A for 30min. Immune complexes were then solubilized by boiling in buffer H (1% SDS, 0.15 M Tris HCl, pH 7.5). Solubilized proteins were analyzed on 12% SDS polyacrylamide gels (SDS-PAGE). A portion of each solubilized protein was digested with endoglycosidase H (Endo H) (Boehringer-Mannhiem Corp). Briefly, Endo H (1.5 mU) and sodium citrate buffer (0.05 M, final concentration), pH 5.5, were added to the immunoprecipitated proteins and incubated overnight at 37^{o}C. The digestion was stopped by boiling and the samples were analyzed on 10% SDS-PAGE.

To assess the stability of the HN proteins of the recombinant viruses, equal amounts of each purified virus sample were analyzed on 10% SDS-PAGE gel. The concentration of the viral proteins of each recombinant virus was standardized using the

Micro BSA™ protein assay reagent kit (Pierce, IL). A premixed protein molecular weight marker (Low range 14.4-97.4 kD) (Boehringer Mannheim Corp) was also run on the gel, to distinguish the viral proteins based on their molecular weights. The HN protein of NDV has an approximate molecular weight of 74 kD.The gel was stained with Commassie blue to visualize the viral proteins. Stability of the HN protein of the recombinant viruses was also assessed by radioimmunoprecipitation studies. Immunoprecipitated complexes described earlier were stored at 4°C and run in SDS-PAGE gels on days 1, 3, 5 and 7 post-immunoprecipitation. The HN protein in the radiographs was captured using the Kodak ID ™ Camera and the amount of protein was determined with the Fuji Image Gauge 3.46 software.

4.4.7 Cell surface expression of HN proteins

DF1 cells were grown to 70% confluence in 6-well plates. Virus was inoculated at an MOI of 0.1 and the cells were maintained in DMEM medium with 5% fetal bovine serum at 37°C. After 48h, the cells were washed with PBS and fixed onto the plate with 3% paraformaldehyde in PBS for 30 min. After washing 3 times with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 30min. Following three further washes with PBS, the cells were treated with 1:500 dilution of primary antibody (HN-specific monoclonal antibody) in PBS at room temperature for 30min. The cells were then rinsed with PBS and incubated with affinity-purified, fluorescein-labeled goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories), for 30 min. The cells were washed once again with PBS and visualized under a Nikon Eclipse TE 300 (Nikon, Japan) fluorescent microscope.

4.4.8 Flow cytometry

DF1 cells were infected with each virus at an MOI of 1. After 16h, the cells were washed once with calcium-and magnesium-free PBS containing 0.02% sodium azide (PBSA). The cells were lifted off the dish with PBS containing 50 mM EDTA. The cell suspensions were washed once with PBSA and then bound with a mixture of a cocktail of NDV HN-specific monoclonal antibodies (72) at a dilution of 1:200, and incubated on ice for 30min. The cells were washed three times with PBSA and bound with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories), for 30min on ice. Cells were then washed three times with PBSA. The fluorescence of 10,000 cells was measured with a FACS Vantage flow cytometer (Becton Dickinson).

4.4.9 Enzyme Linked Immunosorbent Assay (ELISA)

The reactivity of the HN-specific monoclonal antibodies (MAbs) of NDV was tested in a standard ELISA test. The MAbs used were 15C4, AVS, B79, and 10D11 (72). The MAb 15C4 neutralizes and inhibits hemagglutination (HA) of all lentogenic, mesogenic, and velogenic NDV strains, but not the pigeon paramyxovirus-1 (PPMV-1) strain. Antibody 10D11 also inhibits HA activity, but inhibition is more selective and is limited to the mesogenic and domestic, or indigenous, velogenic strains of NDV. MAb B79 reacts in all serologic assays with an antigenic site common to all serotype 1 avian paramyxoviruses. AVS is paramyxovirus-1 (PMV-1) lentogenic strain specific MAb, which reacts with avirulent strains only. The MAb 8, which does not react with any of the NDV strains but only with infectious bursal disease virus, was used as a negative control.

Equal amounts of purified parental and mutant recombinant viruses were used as antigens. The concentration of the viral proteins of each recombinant virus was standardized using the Micro BSA^{TM} protein assay reagent kit (Pierce). A 1:2000 dilution of MAbs was used in the ELISA test. Affinity-purified peroxidase-labeled goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories) was used as the secondary antibody. The results of the ELISA were graded as + to ++++, depending on the reactivity of MAbs to the virus. Uninfected cell antigen was used as a negative control.

4.4.10 Pathogenicity studies

The virulence of the recombinant viruses was determined by three *in vivo* tests: the mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) tests.

MDT was determined to examine the pathogenicity of the recombinant viruses in embryonated chicken eggs as described previously (1). Briefly, a series of 10-fold dilutions of fresh, infective allantoic fluid were made in sterile PBS, and 0.1 ml of each dilution was inoculated into the allantoic cavity of each of five 9-day-old embryonated chicken eggs. The eggs were incubated at 37^{o}C and examined four times daily for 7 days. The time that each embryo was first observed dead was recorded. The highest dilution that killed all embryos was considered the minimum lethal dose. The MDT was recorded as the mean death time in hours for the minimum lethal dose to kill the embryos. The MDT has been used to classify NDV strains into velogenic (taking under 60h to kill);

mesogenic (taking between 60h to 90h to kill) and lentogenic (taking more than 90h to kill).

To examine the pathogenicity of recombinant viruses *in vivo* in chickens, the ICPI and IVPI tests were performed as described elsewhere, with modifications (1). For ICPI, 10^3 PFU of each virus/bird was inoculated into groups of 10 one-day-old SPF chicks, via the intracerebral route. The inoculation was done using a 27-guage needle attached to a 1ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. For IVPI, 10^3 PFU of each virus/bird was inoculated intravenously into groups of 10 six-week-old chickens. The dispenser was set to dispense 0.1 ml of inoculum per inoculation. In both studies, the birds were observed for clinical symptoms and mortality every 12 h for a period of 8 days for ICPI, and 10 days for IVPI studies, respectively. Each experiment had mock-inoculated controls that received a similar volume of sterile PBS by the respective routes. The ICPI and IVPI values were calculated as described by Alexander (1).

4.5 Results

4.5.1 Generation of recombinant NDV containing the HN *N*-linked glycosylation site mutations

Previously, recovery of recombinant NDV from an infectious cDNA clone (pNDVfl) derived from a mesogenic strain of NDV, BC, has been reported from our laboratory (69). In this study, the established reverse genetics system was used to determine the role of *N*-linked carbohydrates of the HN protein on the biological activities of NDV. To achieve this goal, the *Age*I-*Mlu*I subclone containing the HN gene derived from the full-length clone of BC (pNDVfl) was mutated. Each of the four functional *N*-linked glycosylation sites (81) were altered by site-directed mutagenesis changing asparagine, the first amino acid residue of the acceptor site, to glutamine (Fig. 12). Substitutions were made by altering the first and third positions (bold and underlined) of each asparagine codon to create a codon for glutamine (**A**A**T** or **A**A**C** to **C**A**G**). Glutamine was chosen because it is structurally similar to asparagine, differing by only a single methylene group. Moreover, a minimum of two nucleotide changes are necessary for the altered sequence to revert to any codon specifying asparagine, thereby reducing the likelihood of direct reversion of the targeted codon during virus replication *in vivo*. To examine the effect of the combined loss of two *N*-linked glycosylation sites, a double mutant was also created by eliminating the *N*-linked glycosylation sites 1 and 2 (G1 and G2, respectively). The mutant HN cDNA subclones were then inserted into the full-length cDNA of BC (pNDVfl), thus generating four single mutant (G1, G2, G3 and G4) and one double mutant (G12) cDNA clones. To ensure the presence of the

introduced mutations, the entire HN cDNA clone was sequenced using dideoxy chain termination method.

Recombinant viruses expressing wild-type HN, rBC; expressing mutant protein G1, rG1; expressing mutant protein G2, rG2; expressing mutant protein G3, rG3; expressing mutant protein G4, rG4; and expressing mutant protein G12, rG12 were recovered by transfection of HEp2 cells with full-length mutant HN cDNA clones and support plasmids, and amplification of viruses in DF1 cells. Recovered viruses were subjected to RT-PCR and the HN genes were sequenced to their entirety to confirm the presence of the introduced mutations. To determine the stability of each HN mutation, the recovered viruses were passaged five times in 9-11-day-old embryonated chicken eggs and the sequence of the HN gene was determined in viruses recovered at each passage level. These sequence analyses showed that the introduced HN mutations were unaltered, even after five egg passages (data not shown).

4.5.2 Growth and plaque morphology of mutant viruses

The efficiency of replication of the parental (rBC) and mutant (rG1, rG2, rG3, rG4 and rG12) viruses were compared in a multi-step growth cycle. DF1 cells were infected in triplicate with each virus at an MOI of 0.01, and samples were collected at 8h intervals for quantitation by plaque assay on DF1 cells (Fig. 13). The replication kinetics of the mutant viruses were comparable to that of the parental virus. Interestingly, rG4 and rG12 viruses showed delayed growth kinetics (1.5 to 2.0 log units lower) compared to the parental and other mutant viruses. However, after 40h, the virus titer of rG4 reached a

level comparable to that of the parental virus, but rG12 was 1.0 log unit lower than that of the parental virus. From 48h onwards, the differences in viral growth were insignificant between the parental and mutant viruses. The onset of cell fusion was also delayed for rG4 and rG12 viruses. The rG4 virus took 48h while the rG12 virus took 24h longer than the parental virus to produce syncytium in DF1 cells. Most mutant viruses had similar plaque sizes as the parental virus, measuring 3-5 mm in diameter, except the mutants rG4 and rG12, which had considerably smaller-sized plaques (1-2 mm in diameter), as shown in Fig. 14.

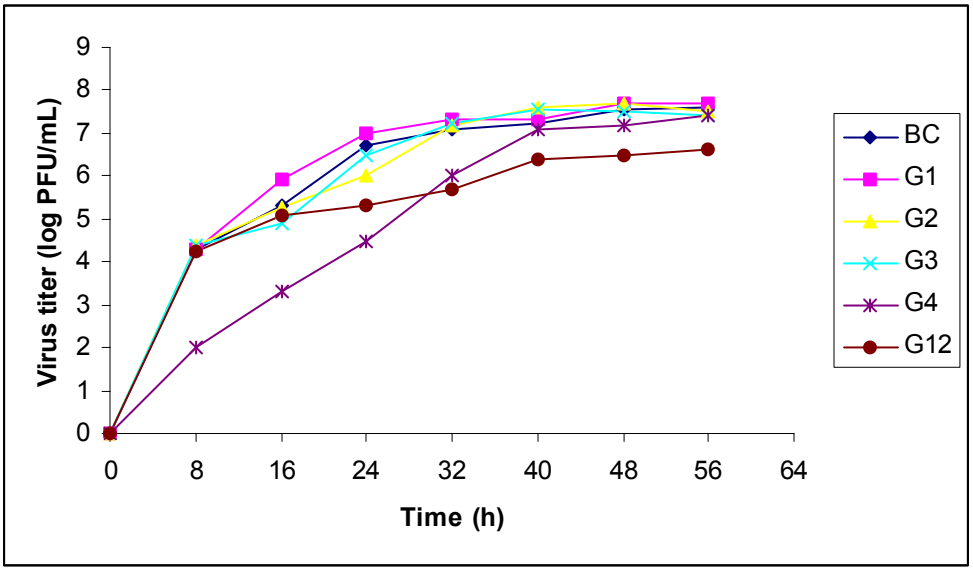


Figure 13. Growth kinetics of viruses in tissue culture. Multicycle growth of parental and HN glycosylation mutant viruses in chicken embryo fibroblast (DF1) cells. Cells were infected at an MOI of 0.01 with the indicated parental or chimeric virus. Samples were taken at 8h intervals and virus titers were determined by plaque assay. Values are averages from three independent experiments.

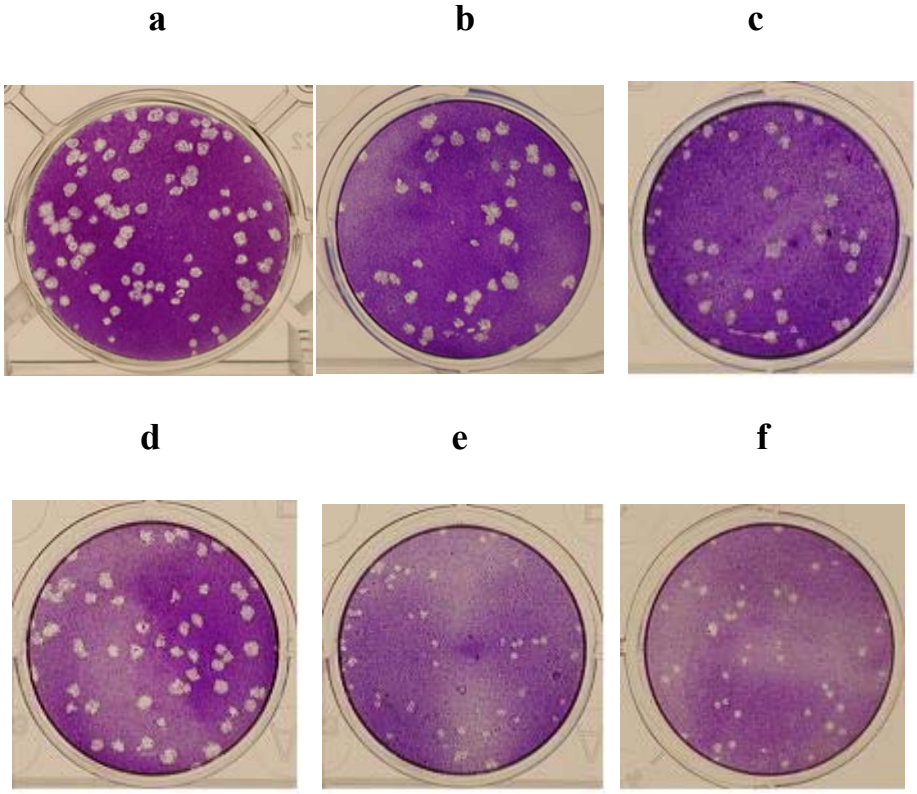


Figure 14. Plaque morphology in DF1 cells by wild-type and HN glycosylation mutant viruses 4 days post-infection. Recovered viruses were titrated in duplicates in 12-well plates. Supernatant collected from virus-inoculated samples was serially diluted and 100µl of each serial dilution was added per well to confluent DF1 cells. After 60min of adsorption, cells were overlaid with DMEM (containing 2% fetal bovine serum and 0.9% methyl cellulose) and incubated at 37^{o}C for 3-4 days. The cells were then fixed with ethanol and stained with crystal violet for observation of plaques. Plaque size and morphology of a) rBC; b) rG1; c) rG2; d) rG3; e) rG4 and f) rG12 viruses.

4.5.3 Analysis of the HN protein of glycosylation mutants by radioimmunoprecipitation assay

To confirm that the mutated glycosylation sites actually lead to the loss of carbohydrates in the HN protein, DF1 cells infected with recombinant NDVs expressing the mutant HN proteins were labeled with ^{35}[S] methionine-cysteine 4-6h after infection. The labeled proteins were immunoprecipitated from cell lysates with NDV-specific polyclonal antibodies and analyzed by SDS-PAGE (Fig. 15 A). One half of the cell lysates were treated with endoglycosidase H (Endo +) and the other half was undigested (Endo H -). Both the samples were subjected to SDS-PAGE analysis (Fig. 15B). Our results showed that Endo H-digested mutant HN proteins (Endo H +) comigrated with the parental viruses on polyacrylamide gels. In the undigested samples, the HN proteins of single mutants (G1, G2, G3 and G4) migrated slightly faster on polyacrylamide gels than the parental HN protein, while the HN protein with mutations in two sites (G12) migrated faster than the single-mutant HN proteins. Protein missing site 1 migrated slower than protein missing sites 2, 3, or 4. These results corroborated with earlier findings with transfected mutant plasmids (81) that glycosylation sites at residues 119, 341, 433 and 481 are actually used in the HN protein of NDV. Further, the slightly slower migration of the G1 mutant than the other mutants, reported earlier and also in our study, may be due to a smaller carbohydrate linked to site 1 than those linked to other sites (81). Variation in size of carbohydrate side chains has been observed in other glycoproteins (97, 130). This study confirmed the usage of glycosylation sites 1-4 on the HN protein of the viruses. None of the precipitated proteins, Endo H-digested or undigested, including the parental HN protein, showed any partially-resistant side-chains reported earlier (81) in

polyacrylamide gels. There were no partially Endo H-resistant species after Endo-H digestion of metabolically-labeled NDV HN protein. However, the amount of precipitated protein with rG4 mutant virus was extremely small (Figs.15A, B and C). Commassie blue staining of purified virus run in 10% SDS-polyacrylamide gels also showed extremely small amounts of HN protein of rG4 virus (Fig. 16). This suggests that mutation of the glycosylation site 4 (Asn-481) inhibited the maturation of HN glycoprotein of NDV. This was further seen in immunoprecipitation experiments (Fig. 15C) in which the immunoprecipitated cell lysates were stored at 4^{o}C and analyzed on polyacrylamide gels on days 1, 3, 5 and 7 after immunoprecipitation. It was found from HN protein quantitation results (data not shown) that there was gradual degradation of the HN proteins of all mutants with increasing time points. Thus, the involvement of some other mechanism other than instability of the protein may be the reason for production of relatively lesser amounts of HN protein in cells infected with glycosylation mutant rG4. Pulse-chase experiments also indicated that the small amounts of rG4 HN protein precipitated were not due to the instability of the protein, since all proteins were immunoprecipitated to the same levels after a 2-12h chase (data not shown).

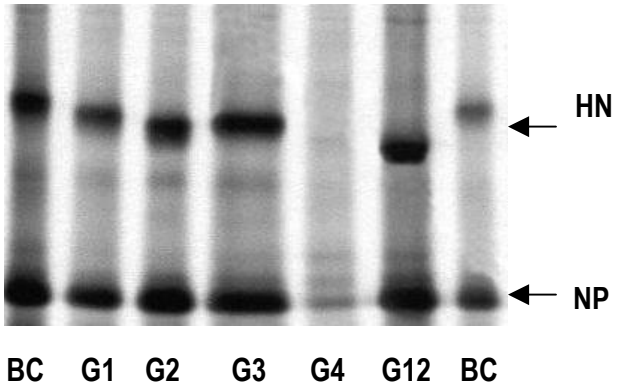


Figure 15 A. Expression of HN protein of glycosylation mutants and parental virus with radioimmunoprecipitation assay. DF1 cells infected with each of the viruses for 4-6h and proteins were labeled with [^{35}S] methionine-cysteine for 2h. Equal amounts of cell lysates were immunoprecipitated with a cocktail of NDV-specific polyclonal antibodies and *Staphylococcus aureus* protein A, and analyzed with SDS-PAGE.

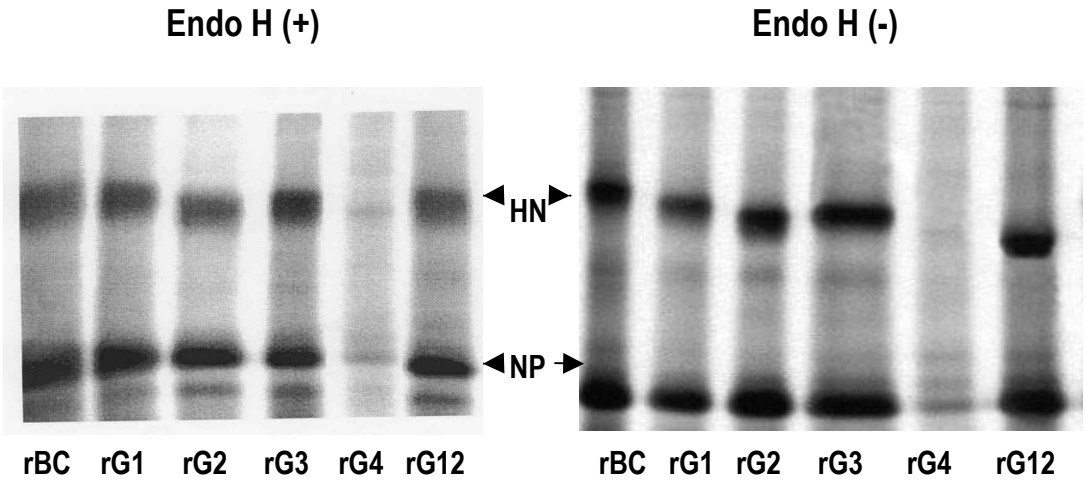


Figure 15 B. Immunoprecipitation of parental rBC and HN glycosylation mutants from infected cells and treatment with Endo H. DF1 cells infected with each of the viruses for 4-6h and proteins were labeled with [^{35}S] methionine-cysteine for 2h. Equal amounts of

cell lysates were immunoprecipitated with a cocktail of NDV-specific polyclonal antibodies and *Staphylococcus aureus* protein A, and analyzed with SDS-PAGE. The immunoprecipitates were digested (+) or mock-digested (-) with endoglycosidase H (Endo H) and then analyzed by SDS-PAGE.

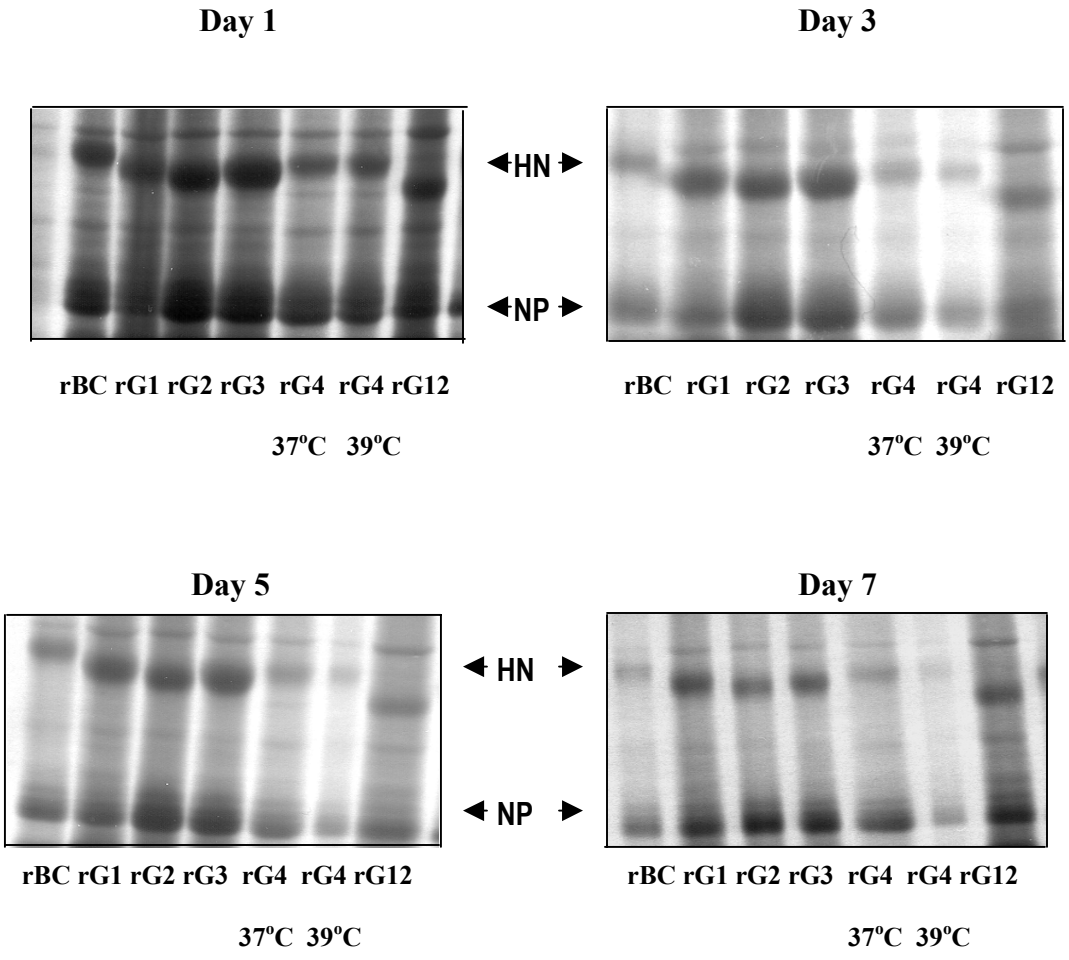


Figure 15C. Analysis of stability of the HN protein of glycosylation mutants and parental virus with radioimmunoprecipitation assay. DF1 cells infected with each of the viruses for 4-6h and proteins were labeled with [^{35}S] methionine-cysteine for 2h. Equal amounts of cell lysates were immunoprecipitated with a cocktail of NDV-specific polyclonal

antibodies and *Staphylococcus aureus* protein A. Immunoprecipitates were stored at 4^{o}C and stability of the NDV HN protein was assessed by running each sample in SDS-PAGE gels on days 1, 3, 5 and 7 after immunoprecipitation. Cells infected with the virus rG4 were maintained at 37^{o}C and 39^{o}C to check whether this mutant was temperature-sensitive. The HN proteins of all mutants showed gradual degradation with time. The amount of rG4 HN protein immunoprecipitated was lesser when compared to the HN protein precipitated from other mutants.

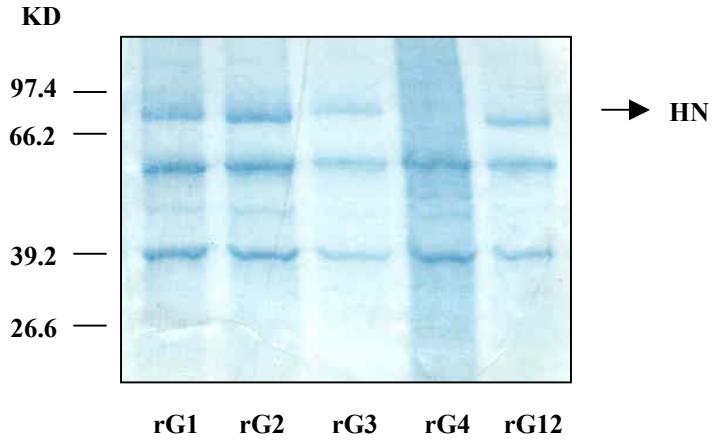


Figure 16. Expression of HN protein glycosylation mutants. Equal amounts of proteins from parental and mutant purified viruses were analyzed by SDS-PAGE. The migration and protein stability of the HN protein from each virus was examined after Commassie blue staining. The HN protein of mutant rG4 virus was found to be degraded. A protein molecular weight marker was also run along with the purified viral samples to assess the viral proteins based on their molecular weights. The molecular weight of the HN protein of NDV is approximately 74kD. Three independent experiments with purified viral proteins confirmed the degradation of the HN glycoproteins of the rG4 mutant virus.

4.5.4 Reactivity of glycosylation mutant HN proteins with monoclonal antibodies

It is suggested that formation of antigenic sites present only on mature HN molecule is a measure of maturation of the protein (81). In order to examine the effect of loss of carbohydrate on the formation of antigenic sites, the parental and mutant viruses were tested for reactivities to a panel of HN-specific monoclonal antibodies (15C4, 10D11, B79 and AVS) by ELISA. Purified parental and mutant viruses were used as antigens. ELISA results showed that all mutant proteins reacted with most of the antibodies tested except the mutants rG2 and rG4. None of the mutants and the parental virus reacted to MAb AVS, which recognizes only avirulent strains, and MAb 8, which recognizes a totally unrelated virus, as expected. The mutant rG1, rG3 and rG12 viruses showed higher rates of reactivity than the other mutants with MAb B79, and rG1 and rG3 viruses showed higher reactivity to MAb 15C4, compared to the parental rBC and other mutant viruses. The rG4 and rG2 viruses were poorly reactive to the 15C4 and B79 Mabs, but were totally unreactive to the MAb 10D11. Table 2 shows the reactivity of HN-specific MAbs to parental and mutant viruses. Thus, loss of different carbohydrate side chains differentially influences the formation of antigenic epitopes. The presence of the G2 and G4 mutations eliminated the detection of a particular epitope recognized by the 10D11 MAb. The varying efficiency of binding to 15C4 and B79 antibodies by rG4 and rG2 viruses suggests that a significant population of these mutant proteins remain at least partially misfolded.

Table 2. Monoclonal antibody (MAb) reactivity, Endo H sensitivity and cell surface expression of HN glycosylation mutant viruses.

	Monoclonal antibody reactivity					Endo H sensitivity[†]	Cell surface expression[‡]
Virus	15C4	10D11	B79	AVS	8		
rBC	+++	+	+	-	-	S	Yes
rG1	++++	+	+++	-	-	S	Yes
rG2	++	-	-	-	-	S	Yes
rG3	++++	+	+++	-	-	S	Yes
rG4	++	-	+	-	-	S	Yes
rG12	+++	+	+++	-	-	S	Yes

The reactivity of the HN specific MAbs to the HN protein of NDV was tested by an ELISA. Equal amounts of proteins from parental and mutant purified viruses were used as coating antigens. The MAbs were diluted 1:2000 of MAbs in dilution buffer. Affinity-purified, peroxidase-labeled goat anti-mouse immunoglobulin (KPL, Gaithersburg, MD) was used as the secondary antibody. The data are presented as the relative amount of HN captured by each of the MAbs: ++++, absorbance $_{450}$ > 2.0; +++, absorbance $_{450}$ > 1.1; ++, absorbance $_{450}$ 0.9-1.0; + absorbance $_{450}$ 0.5-0.8; and -, absorbance $_{450}$ < 0.4. Data are from three independent experiments. [†] Abbreviations: S, Carbohydrate side chains were sensitive to Endo H digestion. Data are from three independent experiments. [‡] Cell

surface expression was analyzed by indirect immunofluorescence with anti-HN MAb cocktail. Data are from three independent experiments.

4.5.5 Cell surface expression of glycosylation mutant HN proteins

To study the transport of the mutant HN proteins synthesized in virus-infected cells from the cytoplasm to the cell surface, indirect immunofluorescence studies were carried out. The infected cells were fixed with 3% paraformaldehyde, washed and then permeabilized with 0.1% Triton X-100. The cells were viewed under a fluorescent microscope after being treated with NDV HN-specific monoclonal antibody followed by incubation with affinity-purified fluorescein-labeled goat anti-mouse immunoglobulin. As shown in Fig.17, all mutant and the parental viruses showed the presence of surface immunofluorescence in fixed cells, and internal cytoplasmic immunofluorescence in fixed and permeabilized cells. Flow cytometry was used to quantitate these results. DF1 cells infected with each of these viruses were incubated with HN-specific MAb cocktail, and surface expression was determined by flow cytometry. The percentage of cells expressing the different mutated HN proteins was very similar except for rG4 virus, which had a slightly lower percentage (76%) of cells expressing the HN at the surface (Table 3). The mean fluorescence intensities ranged from 60 to 220 % of the parental rBC virus. Cells infected with the rG4 virus had a reduced percentage of mean fluorescence intensity than that of the parental rBC virus. The surface expression of the HN proteins of rG1, rG2 and rG3 viruses was higher than that of the rBC virus. These results indicated that all glycosylation mutant HN proteins retained the ability to be transported to the cell surface except rG4 virus, which had a greatly reduced rate of transport.

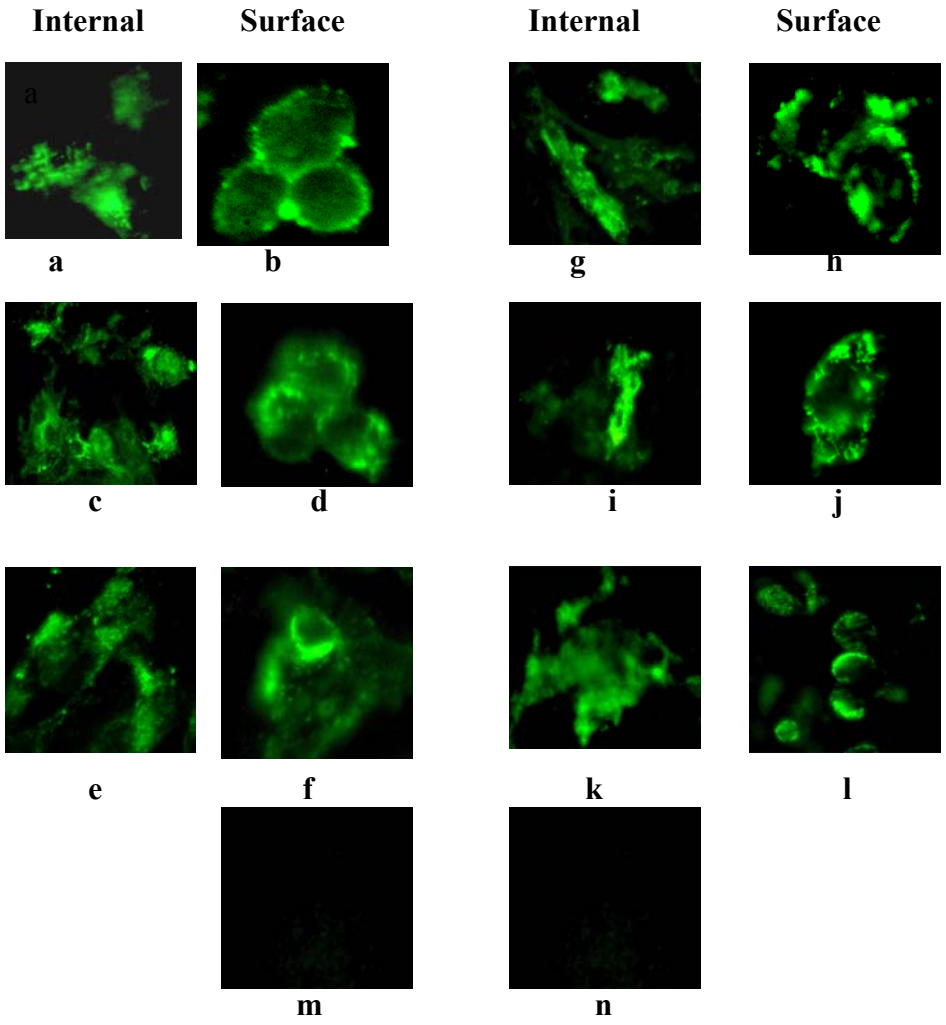


Figure 17. Detection of cell surface immunofluorescence of parental and mutant HN proteins. Virus-infected DF1 cells (0.1 MOI) were washed with PBS at 48h post-infection and fixed with 3% paraformaldehyde in PBS for cell surface fluorescence (b, d, f, h, j, l and n), or fixed and then permeabilized with 0.1% Triton X-100 in PBS for intracytoplasmic fluorescence (a, c, e, g, i, k and m). HN proteins were stained with anti-HN monoclonal antibody cocktail, followed by affinity-purified fluorescein-labeled goat anti-mouse immunoglobulin (KPL, Gaithersburg, MD). (a,b) rBC; (c, d) rG1; (e, f) rG2; (g, h) rG3; (i, j) rG4; (k, l) rG12 and (m,n) negative control. Cells were photographed at a magnification of 200X.

Table 3. Surface expression of HN proteins of glycosylation mutants.

Virus	Surface expression efficiency of HN protein	
	% of positive cells	Relative mean fluorescence intensity
rBC	87	1.00
rG1	90	1.60
rG2	96	2.20
rG3	97	2.00
rG4	76	0.60
rG12	88	1.00
Mock-infected cells	-	0.01

Cell surface expression of HN was determined by flow cytometry. DF1 cells were infected at an MOI of 1 with each virus. Surface expression of the HN proteins was assessed by flow cytometry at 18h post-infection with a cocktail of NDV HN-specific monoclonal antibodies followed by a fluorescein-isothiocyanate secondary antibody. Surface immunoflurescence was quantitated by FACS analysis using the FACS Vantage flow cytometer (Becton Dickinson, CA, USA). Uninfected DF1 cells were used as negative controls. Values shown are averages of results from three independent experiments.

4.5.6 Biological activities of glycosylation mutant viruses.

The results described above show that glycosylation at sites 2 and 4 affects the formation of a mature HN molecule. But, the cell surface expression of virus missing site 2 was similar to the parental virus. However, proteins missing oligosaccharides at sites 1, 3 singly or sites 1 and 2 in combination have no effect on antigenic site formation or cell surface expression. To explore the role of these carbohydrates in the biological activities of the HN protein, cells infected with the recombinant viruses were assayed for NA, HAd and fusion activities (Fig. 18A). Our results showed that the NA and HAd values of all glycosylation mutant viruses were similar to that of parental virus, indicating that glycosylation probably does not play a role in NA and HAd activities of NDV. However, differences were observed in fusion promotion activity of the HN protein. The fusion indices of rG1, rG2 and rG3 viruses were similar to that of the parental virus, rBC. Surprisingly, the rG12 virus showed more than 1.8-fold higher fusogenicity, and the fusion promotion activity of rG4 virus was approximately one-third of the parental virus. The rG4 virus also showed reduced surface expression. The extents of fusion of parental and mutant viruses are shown in Fig. 18B.

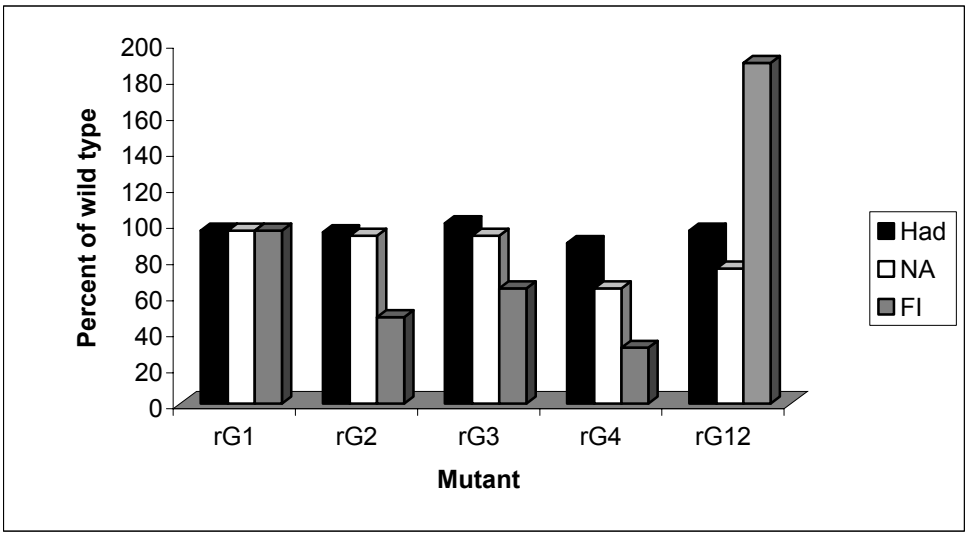


Figure 18A. Biological activities of the HN glycosylation mutants. The hemadsorption (HAd), neuraminidase (NA) and fusogenicity of HN glycosylation mutants were examined. HAd was measured as the percentage of the hemoglobin released compared to the parental rBC virus. The NA activities and fusion indices (FI) of the mutant viruses were also shown in comparison with these activities of the rBC virus. NA activity of purified viruses was measured by a fluorometric assay. The FI is the ratio of the total number of nuclei to the number of cells in which the nuclei were observed (i.e., the mean number of nuclei per cell). The average of three experiments is shown.

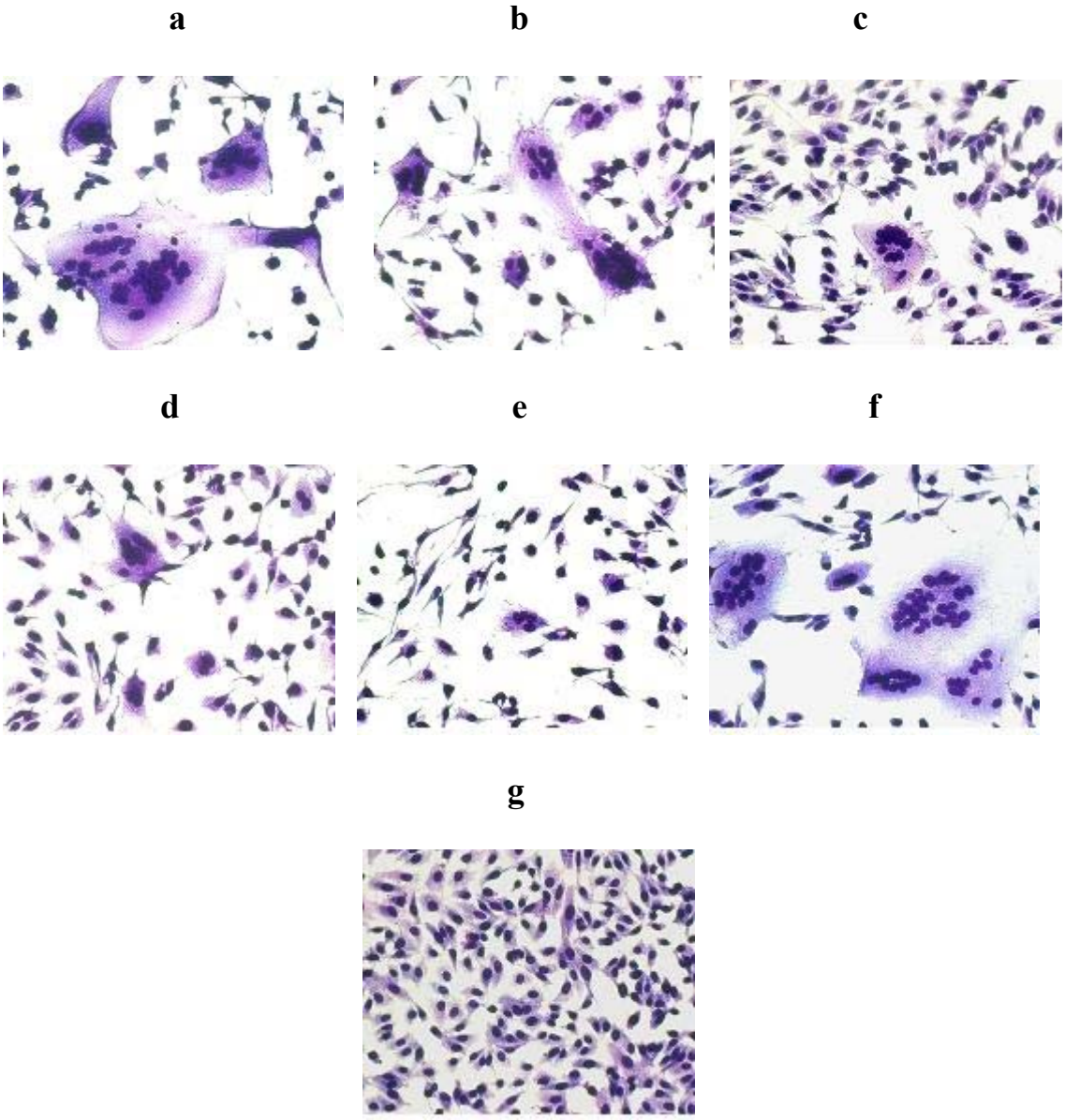


Figure 18B. Fusion of HN glycosylation mutants.The fusogenicity of the mutant viruses in Vero cells in comparison to the parental rBC virus. Infected cells were fixed at 48h post-infection and stained with hematoxylin-eosin. The extent of fusion of the parental rBC (a) and the mutant viruses rG1, rG2, rG3, rG4 and rG12 (b, c, d, e and f) in Vero cells are captured at 200X magnification in a Nikon Eclipse TE 300 microscope. Uninfected Vero cell monolayer (g) is included as a negative control for comparison.

4.5.7 The effect of loss of glycosylation from the HN protein on virulence

Three internationally-accepted tests were used to assess the degree of virulence of NDV isolates: the MDT test in 9-11-day-old embryonated SPF chicken eggs; the ICPI test in 1-day-old chicks, and the IVPI test in 6-week-old chickens. We examined the virulence of the parental and glycosylation mutant viruses by all three tests (Table 4). The MDT result showed a significant increase in the time required by rG4 virus (70h) to kill the chicken embryos when compared to the time required by the parental rBC virus (62 h), indicating a reduction in virulence of rG4 *in vivo*. The rG1 and rG3 viruses had MDT values of 60h each; whereas, rG2 and rG12 had MDT values of 64h each. The ICPI values of all glycosylation mutants were lower compared to the parental virus (Table 3). The ICPI value of parental rBC virus was 1.50; whereas, the ICPI values of rG1, rG2, rG3, rG4 and rG12 viruses were 1.12, 0.87, 1.07, 0.66 and 0.76, respectively. The results obtained by IVPI tests in 6-week-old chickens showed that all glycosylation mutants had lower IVPI values compared to that of the parental virus. There was a discrepancy between the ICPI and IVPI values for rG1 and rG2 viruses. These viruses showed higher ICPI values when compared to the rG12 virus. However, they showed lower IVPI values when compared to the IVPI value of the rG12 virus. The IVPI value of parental rBC virus was 1.32; whereas, the IVPI values of rG1, rG2, rG3, rG4 and rG12 viruses were 0.37, 0.44, 0.75, 0.31 and 0.67, respectively. These results indicated that loss of glycosylation in the HN protein leads to reduction in virulence of NDV. They further indicated that loss of glycosylation at residue 481 (G4) or at residues 119 and 341 (G12) significantly reduced the virulence of NDV.

Table 4. HN glycosylation mutants of NDV and virus pathogenicity *in vivo*.

Virus	MDT[a]	ICPI[b]	IVPI[c]
rBC	62	1.50	1.32
rG1	60	1.12	0.37
rG2	64	0.87	0.44
rG3	60	1.07	0.75
rG4	70	0.66	0.31
rG12	64	0.76	0.67

The virulence of the mutant and parental viruses was evaluated by intracerebral pathogenicity index (ICPI) in day-old-chicks, intravenous pathogenicity index (IVPI) in six-week-old chickens, and mean death time (MDT) in eggs. [a] MDT in hours for the minimum lethal dose to kill 9-day-old embryonated chicken eggs by allantoic route of inoculation.

Highly-virulent viruses take under 60h to kill the embryos, whereas, avirulent viruses take more than 90h to kill the embryos. [b] ICPI was determined by inoculating 10^3 PFU of each virus/bird, into groups of 10 one-day-old SPF chicks, *via* the intracerebral route. The inoculation was done using a 27-guage needle, attached to a 1ml stepper syringe

dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were observed daily for 8 days and at each observation, scored 0 if normal, 1 if sick, and 2 if dead. The ICPI value is the mean score per bird, per observation. Highly-virulent viruses give values approaching 2 and avirulent viruses give values approaching 0. [c] IVPI was determined by inoculating 10^3 PFU of each virus/ bird, intravenously, into groups of 10 six-week old chickens. The dispenser was set to dispense 0.1 ml of inoculum per inoculation. The birds were observed daily for a period of 10 days for clinical symptoms and mortality. They were scored 0 if normal, 1 if sick, 2 if paralyzed and 3 if dead. The weighted sums were then added and divided by the total number of observations (10 birds x 10 days = 100 observations), which provided the mean score per bird, per observation. Both ICPI and IVPI studies had mock-inoculated controls that received a similar volume of sterile PBS by the respective routes.

4.6 Discussion

Carbohydrates play important roles for a variety of structural and functional activities of glycoproteins (100, 101, 112,114,123,143). Previous studies have shown that *N*-linked carbohydrates are important for folding, transport and biological activities of the HN glycoprotein of NDV (30, 60, 81). We have characterized *N*-linked glycosylation mutants of NDV HN protein in terms of their biological activities and virulence. By using reverse genetics, we analyzed the role of individual *N*-linked oligosaccharides on the pathogenicity of NDV in its natural avian host.

It has been reported that only four of the six putative *N*-linked glycosylation sites in the NDV HN sequence are normally used for carbohydrate addition (81). Our results confirmed this finding. Further, we have shown that all four glycosylation sites in the HN glycoprotein could be mutated individually without adversely affecting the recovery of replication-competent NDV. A double HN glycoprotein mutant virus lacking carbohydrates in sites 1 and 2 was also recovered. Similar to other virus systems (61, 115), conserved *N*-linked oligosaccharides of NDV HN protein are dispensable for viral replication, and loss of individual glycosylation sites in the HN protein has minimal effects on the biological activities of NDV.

The effect of substitution of single, or combinations of several, amino acid residues on HN protein expression and function has been reported for NDV (30, 60, 81),

but none of the studies looked at the role of individual *N*-glycans in viral replication. We recovered viruses with individual mutations at glycosylation sites 1, 2, 3, and 4, and a combined mutation at glycosylation sites 1 and 2, and tested the effects of these substitutions on viral replication in DF 1 cells. Surprisingly, viruses with mutations at site 4 (rG4) and sites 1 and 2 (rG12) had decreased growth rates and smaller plaque sizes than the other mutants or the parental virus. Each of the individual sites is dispensable for virus replication, although all sites appear to contribute optimal replication efficiency. Glycosylation sites 1-4 are highly conserved in the HN protein of 13 different strains of NDV (119), which suggests that these sites are used and that all four side chains serve important biological functions (81). But conserved glycosylation sites in NDV are found to be largely dispensable for virus replication. Conserved N-linked glycans of human immunodeficiency virus type 1 gp41 envelope protein were found to be dispensable for viral replication (61). The differences in replication and the severe defects in replication displayed by G4 and G12 mutants may be the result of inefficient processing of the HN protein. At least for G4 virus, we demonstrated that the cell surface expression was compromised in comparison to the other mutants.

Antibody-selected *N*-glycan addition to HN protein of NDV decreased the ability of HN protein to complement the viral F protein in the promotion of cellular fusion (30). In our study, even though the *in vitro* growth rate of rG12 virus was lower, its fusogenicity was significantly higher when compared to the parental virus. Its cell surface expression was similar to the parental virus. This result is in agreement with a previous study by McGinnes and Morrison (1995), who demonstrated higher fusogenicity of G12

double mutant virus when compared to the parental virus. Increased fusion by rG12 may be due to the location of the first glycosylation site (residue119) on the HN protein. It is located near the Cys-123 residue in the stalk region of the HN protein. This cysteine residue plays an important role in the disulfide linkage of the HN homodimers (80, 85, 109). Elimination of glycosylation at residue 119 may result in better covalent linkage by increasing the efficiency of participation of the adjacent cysteines in the covalent bond, as indicated by McGinnes and Morrison (81). Alternatively, it is possible that the simultaneous elimination of carbohydrates at residues 119 and 341 may result in better interaction of the HN mutant G12 with the F protein, leading to increased fusogenicity of rG12 virus. This may result in the formation of a very stable tetrameric HN protein which can interact more efficiently with the fusion (F) protein of the virus and, thus, cause increased fusion when compared to the parental virus. Interaction of the HN and F proteins as requirements for cell fusion has been reported previously (129, 138).

In vivo studies with rG12 virus showed loss of virus virulence in chickens. It is possible that removal of these glycosylation sites (residues 119 and 341) increases cell-cell fusion, resulting in rapid spread and destruction of the host cell monolayers. The antigenic reactivity of rG12 virus to the MAb B79 is higher than that of rBC virus. This mutant may thus cause an early and enhanced immune response in infected chickens, and hence, would be cleared by neutralizing antibodies of the immune system in the chickens during the initial stages of infection. This would prevent its spread in the chickens, making rG12 virus less virulent *in vivo*. Thus, the presence of carbohydrates at residues 119 and 341 may serve to modulate viral infection and increase the survival time of the

virus in the host. Our ICPI results corroborate this inference, as chicks inoculated with G12 mutant survived longer than the other mutants. There have been reports of an increase in virulence of a neurovirulent influenza virus strain A/NWS/33 in mice (146) and the H5N2 influenza virus in chickens (63), with the loss of a carbohydrate from the HA protein of the viruses. Thus, loss of a glycosylation site(s) can influence the pathogenicity of viruses by either decreasing or increasing their virulence.

Carbohydrates masking neutralization epitopes on the viral surface glycoproteins, thus forming a shield that protects the virus from immune recognition, has been reported for many viruses (3, 4, 28, 58, 127, 135). We have shown that removal of carbohydrate side chains resulted in exposure or masking of the antigenic sites in NDV HN protein. The reactivity to neutralizing NDV monoclonal antibodies increased when the glycosylation at sites 1 (G1), 1 and 2 together (G12), and 3 (G3) were removed. On the other hand, loss of glycosylation at sites 2 and 4, lead to reduction of reactivity of rG2 and rG4 viruses, respectively, to the monoclonal antibodies. Previous studies have defined residues 193 to 201, 345 to 353, 494, 513 to 521, and 569 as the domains on the HN protein of NDV that are involved in neutralization (56, 58, 59).

The bulky hydrophilic carbohydrate chains in *N*-glycosylated proteins may aid protein folding by preventing inappropriate hydrophobic interactions. The role of individual oligosaccharides in the maturation of SV5, measles and sendai viruses has been studied. Those studies showed, as did ours, that the loss of some oligosaccharides influenced folding more than the loss of others. In simian virus 5 (SV5), removal of

oligosaccharides from the F_1 subunit of the F protein of SV5 resulted in delayed intracellular transport and decreased stability of the protein (5). In measles and SV5 viruses, glycosylation has been shown to be necessary for folding of proteins into their native conformation, which, in turn, affects the protein stability and intracellular transport (52, 97). In Sendai virus, the loss of glycosylation sites 3 and 4 affected the transport of the HN protein to the cell surface (128). In our study, glycosylation site 4 of the HN protein of NDV is found to be necessary for correct folding, as the misfolded HN protein is incorrectly processed and transported to the cell surface inefficiently. Loss of this oligosachharide reduces the formation of antigenic sites to approximately one-third the level of the parental virus. The cell surface expression of this virus was also only 60% of the parental virus. Loss of oligosaccharides at site 2 had some effect on formation of antigenic sites, while mutants with deletion of sites 1, 3 and 1and 2 together, had little or no effect on the formation of antigenic sites, but had either increased or similar levels of cell surface expression as that of the parental virus. The glycosylation site 4 is located in the globular head and is likely to contribute to the conformation of the protein. Our results are in agreement with those of McGinnes and Morrison (1995), who also observed that oligosaccharides at residue 481 had the most significant effect on folding of the HN protein of NDV. Since the HN protein mediates the attachment of the virus to host cells, a misfolded HN protein in the mutant virus would result in loss of infectivity and virulence of the virus. We have demonstrated that the loss of glycosylation at site 4 decreases the biological activities of the HN glycoprotein and attenuates the virus considerably. It is noteworthy, that we were also able to recover the mutant virus with loss of glycosylation at site 4 (rG4), even though the production and cell surface

expression of the HN protein in this virus was lower when compared to the other mutants and the parental viruses.

A number of studies have shown that *N*-linked glycans influence proper folding, disulfide bond formation, and oligomerization of proteins, and affect the conformation of proteins (5, 52, 80, 81, 97). Whether this conformational change affects the ability of the N-linked glycosylation mutants to function in NDV virions has not been tested so far. Our results provide evidence that *N*-linked glycans are important for the functional integrity of the virions. The loss of an individual glycosylation site in the globular head region of NDV or a combined loss of glycosylation sites from stalk and globular head region of NDV resulted in attenuating the virulence of NDV.

These studies indicate that by eliminating certain glycosylation sites found on the HN glycoprotein of NDV, attenuated NDV strains can be engineered. This approach can be used to generate improved recombinant vaccines for controlling Newcastle disease in poultry. It would be interesting to evaluate the effects of glycosylation on the functional activities of the fusion glycoprotein of NDV, another important determinant of NDV virulence.

CHAPTER 5

5.1 Title

Mutational analysis of key amino acids on the HN protein of Newcastle disease virus

5.2 Abstract

The recent report of the crystal structure of the globular head of the hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) has helped in the location of a number of residues that are predicted to form the receptor-recognition and neuraminidase active sites. A site-directed mutagenesis procedure was adopted to change these amino acid residues near the active site in the HN protein of Beaudette C, a virulent strain of NDV. These residues were made similar to the ones present in LaSota, an avirulent strain of NDV. The role of these residues in the functional activity of NDV was assessed by measuring hemadsorption, neuraminidase and fusion activities. The importance of these sites in the virulence and infectivity of the virus was assessed with the mean death time (MDT) study in 9-day-old embryonated eggs. Our results indicated that the residue R416 is important in receptor-recognition and neuraminidase activities and residues E495 and N568 play a role in fusion promotion activity of the HN protein. These studies also show the importance of these sites in virus infectivity and virulence.

5.3 Introduction

The HN protein of NDV is a type II homotetramer glycoprotein. It is a multifunctional protein and is responsible for receptor-recognition by binding to sialic acid receptors on host cells. It also acts as a neuraminidase and cleaves virus from infected cells, by hydrolyzing sialic acid from progeny virion particles, thus, preventing viral self-agglutination. Finally, it helps in fusion by specifically interacting with the fusion protein of NDV (71, 121, 122). The HN protein of NDV consists of a 26 amino acid amino-terminal cytoplasmic domain and a 22 amino acid transmembrane domain (88). The ectodomain of the HN protein consists of a 95 amino acid long stalk, supporting a 428 amino acid terminal globular head, which contains the receptor recognition, neuraminidase (NA) and antigenic sites (86, 142). The recent report of the crystal structure of the globular head of NDV throws tremendous light on the location of the sites active in receptor recognition, NA and fusion promotion activities (24). Previous studies have proposed conserved residues R174, I175, D198, K 236, R416, Y526 and E547 as being important in receptor recognition and NA activities and residues R174 and E547 to influence the fusion promotion activity of the HN protein of NDV (23). Although, mutations in the transmembrane and stalk regions of the HN protein can affect the activities of the protein (79, 139), the ectodomain region of the HN protein of NDV is thought to play a key role in influencing virus infectivity. All of the antigenically and functionally important residues in HN are localized in the globular head (142). Therefore, our goal was to examine the role of certain key amino acid changes in the globular head region. Comparisons of the HN protein sequence between LaSota and BC

viruses identified only 17 amino acid differences. Three of the amino acid differences were in the transmembrane domain, two were in the stalk region, and twelve of the differences were in the globular head region. In this study besides examining the role of the conserved residue R416 (found near the sialic-acid binding site of the HN protein of NDV), in the biological activities of the HN protein of NDV, we also examined certain residues (I219, S494, E495 and N568), which were different between a avirulent strain of NDV, LaSota and a virulent strain of NDV, Beaudette C (BC). Examination of the amino acid changes using the HN crystal structure showed that residues T214, I219 and N568 are in the region where mutations resulted in a decrease or an abolition of fusion (personal communication, Dr. Allen Portner, St. Jude's Children Research Hospital, Memphis, TN). Two other amino acids, S494 and E495 are also in close proximity to this region.

We tested the effect of the mutations done on these residues in virus infectivity by *in vitro* and *in vivo* assays. The growth characteristics, neuraminidase, hemadsorption and fusion activities of the mutant viruses were compared with those of the parental BC virus.

Our results indicate the importance of the residue R416 in hemadsorption and NA activity and virus infectivity, thus, influencing NDV virulence. Fusion promotion activity of viruses with mutations at residues E495 and N568 was also altered indicating the importance of these sites in fusion promotion. Identification of specific sequences involved in virulence will give insights into the mechanisms involved in NDV pathogenesis.

5.4 Materials and Methods

5.4.1 Cells and Virus

DF1 cells were maintained in DMEM and Vero and HEp2 cells were maintained in EMEM media. NDV strain, Beaudette C (BC) was received from the National Veterinary Services Laboratory, Ames, IA. The virus was propagated in the allantoic cavity of embryonated chicken eggs and the virus was purified from the allantoic fluid as described previously (Chapter 3).

5.4.2 Construction and recovery of active site NDV HN mutants

Full-length antigenomic cDNA of BC virus was cloned into a low-copy number plasmid vector, pBR322 (69). It was designated as pBC. Reverse genetics procedures were used to rescue the recombinant virus rBC, as described elsewhere (69). The *Mlu*I – *Age*I fragment HN gene from pBC was subcloned into pGEM 7Z(+), as described previously in Chapter 4. Using site-directed mutagenesis (9), a panel of 5 HN mutants were generated. All PCR mutagenesis studies were performed using high fidelity pfu DNA polymerase (Stratagene). Based on the specific amino acid residue that was mutated, these clones were designated as I219V, R416Q, S494G, E495V, and N568D. The amino acid residues 219, 494, 495 and 568 present on the mesogenic BC HN protein were mutated into the corresponding amino acid residues found on the HN protein of the lentogenic LaSota virus. Sequence analysis of the HN protein of BC and LaSota viruses revealed that the amino acid residue 416, present near the active sialic-acid binding site

(23) was similar in both the viruses. The residue R416 was mutated to glutamine (Q) to study the effect of the mutagenesis on the biological activities of the HN protein and on viral infectivity. Listed below are the set of primers used to mutate the BC HN cDNA clone, resulting in the generation of 5 HN mutagenized clones in the PGEM 7Z (+) vector. Bold and italicized alphabets represent mutated nucleotides present on the HN gene of BC:

HN 219 5′ ***G***T***C***TTCTTTTCTACTCTGCGTTCC 3′

HN219/R 5′ CCTCCCTGTTGCAGTTGTCCG 3′

HN 416 5′ C***AG***GGGTCATCATACTTCTCT 3′

HN416/R 5′ CTGATACAAGAAATGAGATGTCCC 3′

HN 494 5′ ***G***G***A***GAACAAGCAAGAC 3′

HN 494/R 5′ ATCAAGCATTGTCCCGAATACC 3′

HN 495 5′ G***TG***CAAGCAAGACTTAATCC 3′

HN495/R 5′ ACTATCAAGCATTGTCCCGA 3′

HN 568 5′ ***G***A***C***GATGGGGTTAGAGAAGCCAGG 3′

HN 568/R 5′ TTTGAGGATCTCAACTAGTAACGGG 3′

All the mutated HN genes were sequenced entirely before introducing into the full-length NDV cDNA clone. Each mutagenized HN gene was then inserted into the full-length BC cDNA clone (pBC). These full-length cDNA clones were transfected into HEp2 cells and 5 mutant viruses were recovered using the recovery procedures mentioned earlier (69). Based on the HN mutagenized clones I219V, R416Q, S494G, E495V, and N568D, the recovered recombinant viruses were called rI219V, rR416Q,

rS494G, rE495V, and rN568D, respectively. The mutant HN gene in each of the recovered viruses was sequenced by RT-PCR to confirm the presence of the substituted amino acid.

5.4.3 RNA extraction and RT-PCR of recovered chimeric viruses

The recovered recombinant were grown in the allantoic cavity of 9-day-old embryonated chicken eggs. After 2 days, the allantoic fluid was harvested and clarified and virus was purified as described previously. Viral RNA was extracted from the recovered viruses using TRIzol (Invitrogen, USA), according to the manufacturer's instructions. Reverse transcription was done with the extracted RNA, using the Thermoscript RT kit (Invitrogen, USA) to synthesize the first strand cDNA. The genomes of recovered chimeric viruses were entirely sequenced after RT-PCR to confirm the presence of the substituted gene.

5.4.4 Growth characteristics of HN mutant viruses

The growth kinetics of the recombinant HN mutant viruses were performed by multi-step growth curves in DF1 cells. The viruses were inoculated to confluent monolayers of DF1 cells at a m.o.i. of 0.01. Supernatant was collected at 8 h intervals until 56 h post infection (P.I). The virus titer in the cell culture supernatant was assessed by plaque assay on DF1 cells as described previously (Chapter 3).

5.4.5 Biological functions of recombinant viruses

Biological functions of the recovered viruses were assessed by hemadsorption, fusion and neuraminidase assays. These were done as described earlier in Chapter 3.

5.4.6 Mean Death Time (MDT) in chicken embryos

The virulence of the recovered viruses was determined by the mean death time (1) in embryonated SPF chicken eggs. A series of 10-fold dilutions of infected allantoic fluid was made in sterile PBS, and 0.1 ml of each dilution was inoculated into the allantoic cavity of each of the five 9-day old embryonated eggs. The eggs were incubated at 37^{o}C and examined four times daily for 7 days. The time that each embryo was first observed dead was recorded. The highest dilution at which all embryos died was considered the miimum lethal dose. The MDT was recorded as the mean death time in hours for the minimum lethal dose to kill the embryos. The MDT has been used to classify NDV strains into velogenic (taking under 60 h to kill); mesogenic (taking between 60 h to 90 h to kill) and lentogenic (taking more than 90 h to kill).

5.5 Results

5.5.1 Construction of HN mutant cDNAs and recovery of mutant viruses

The recovery of recombinant NDV from infectious cDNA clones, derived from a mesogenic strain of NDV, BC, and a lentogenic NDV strain, LaSota, have been reported earlier from our laboratory (54, 69). In this study, we used the established reverse genetics system to examine the role certain key amino acids present in the globular

ectodomain (Fig.19) of the HN protein in the biological activities of the HN protein and in the virulence of NDV. A cDNA subclone of the HN gene (*Mlu*I-*Age*I fragment) of NDV strain BC was used for site-directed mutagenesis to generate five HN mutant clones. Each of the mutagenized HN gene was then inserted into a full-length cDNA clone of BC, thus generating five full-length BC cDNA clones carrying the specific HN mutations. NDV strain BC HN amino acid residue I219 isoleucine (**A**T**A**) was mutated to valine (**G**T**C**), R416 arginine (C**GA**) to glutamine (C**AG**), S494 serine (**A**G**T**) to glycine (**G**G**A**), E495 glutamic acid (G**AA**) to valine (G**TG**) and N568 asparagine (**A**A**T**) to aspartic acid (**G**A**C**). Each amino acid residue was changed by mutating at least two of its nucleotides to ensure the stability of the mutation and to prevent the mutation from reverting back to the original residue. Based on the amino acid residues mutated on the HN protein of BC, the cDNA clones were designated as I219V, R416Q, S494G, E495V and N568D (Fig. 20). To ensure the presence of the intended mutations, the entire HN gene from each of the full-length cDNA clones was sequenced using Big dye terminator (Applied Biosystems) method. Each full-length cDNA clone was used for transfection and recovery of a HN mutant virus using reverse genetics procedures (69). The supernatants from transfected HEp2 cells were passaged two times in DF1 cells to recover infectious NDV. Virus-positive supernatants were used to inoculate the allantoic cavities of 9-11-day-old embryonated SPF eggs. The allantoic fluid harvested after embryo deaths were analyzed in a hemagglutination (HA) assay. Allantoic fluid with a positive HA titer was used for the isolation of viral RNA, followed by a sequence analysis of an RT-PCR fragment that covered the mutated HN gene region. Nucleotide sequencing confirmed that presence of intended mutations in the recovered viruses.

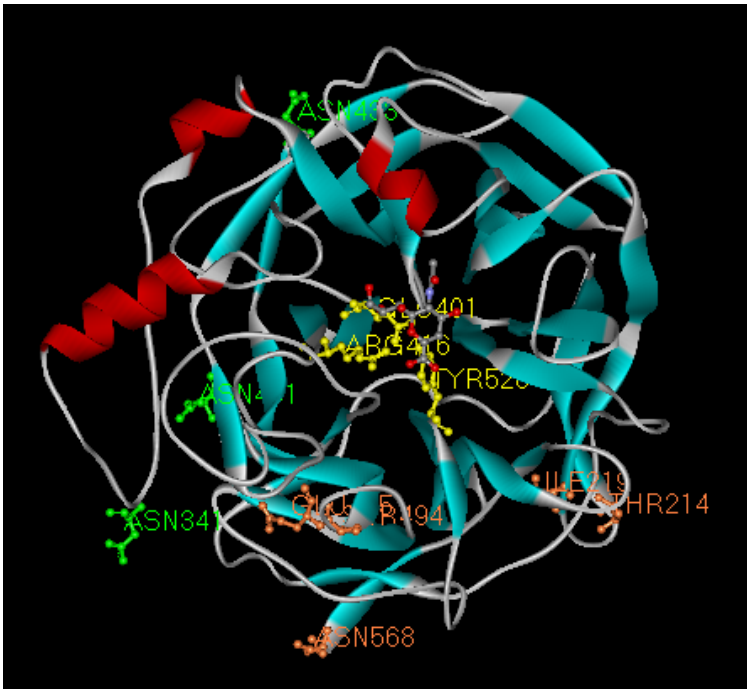
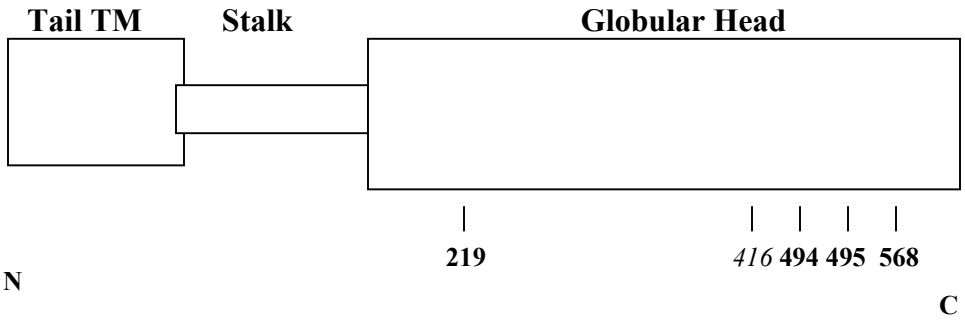


Figure 19. Three-dimensional structure of the HN protein monomer derived from the crystal structure of NDV HN reported by Crennell *et al*. (24) showing location of key amino acid residues (in orange) involved in the biological activities of the HN protein. These locations were predicted using the Acceryls Viewer Pro 4.2 software. The central region (amino acids in yellow) is the active site for receptor binding and neuraminidase activities as reported by Crennell *et al*. (24). Glycosylation sites are indicated by amino acids in green.



Amino acid	Beaudette C	LaSota	Mutant
219	I	V	I219V
494	S	G	S494G
495	E	V	E495V
568	N	D	N568D
416	*R*	*R*	*R416Q*

Figure 20. Schematic representation of the generation of HN mutants in the globular head region of HN of NDV. The numbers in the figure and table represent the amino acid residues that were mutated using site-directed mutagenesis on a cDNA clone of the HN gene of the NDV strain Beaudette C (BC). Amino acids 219, 494, 495 and 568 were changed on the BC HN clone to the corresponding amino acids present in the HN gene of NDV strain LaSota. Amino acid residue 416 which is the same in LaSota and BC was mutated to a different amino acid.

The recovered recombinant viruses were designated as rI219V, rR416Q, rS494G, rE495V, and rN568D. The HN mutations in these viruses were stable and were seen even after ten sequential passages of these viruses in DF1 cells (data not shown).

5.5.2 Growth of the recombinant viruses *in vitro*

The growth characteristics of the parental and HN mutant viruses were assessed by multi-step growth curves (Fig. 21). The differences in the growth kinetics of only the HN mutant R416Q was markedly different form the parent BC virus. The growth rate of the recombinant virus R416Q was two-fold lower than the BC virus during time points 8 and 16h PI. After 20h PI the R416Q virus titers were comparable to its parental BC virus titers. All other HN mutant viruses grew to titers similar to that of the BC virus at all time points. For comparison the titers of LaSota virus is also shown in the figure. These studies showed the relevance of the amino acid residue R416 at the active site of the HN protein of NDV.

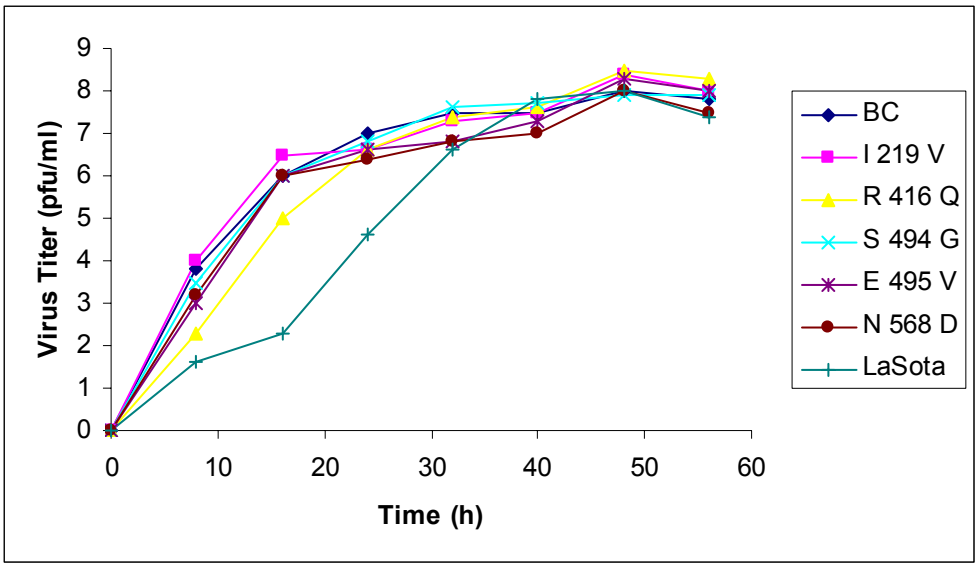
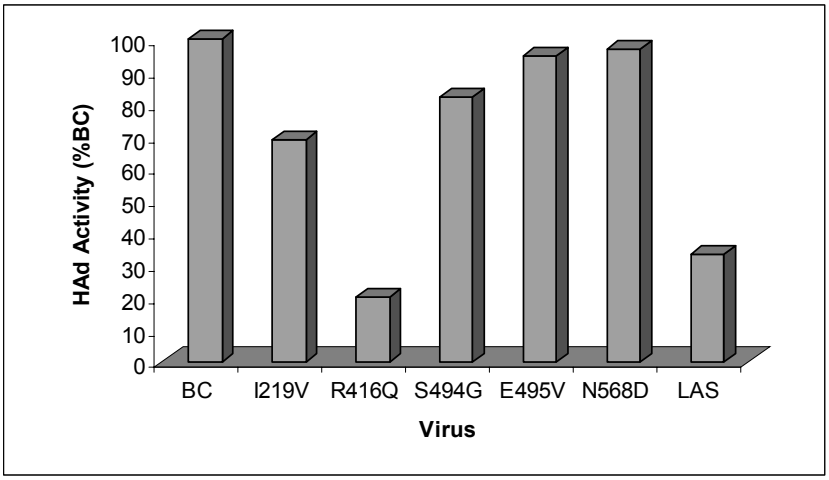


Figure 21. Growth kinetics of HN mutant viruses in tissue culture. Multicycle growth of parental and HN glycosylation mutant viruses in chicken embryo fibroblast (DF1) cells. Cells were infected at an MOI of 0.01 with the indicated parental or chimeric virus. Samples were taken at 8h intervals and virus titers were determined by plaque assay. Values are averages from three independent experiments.

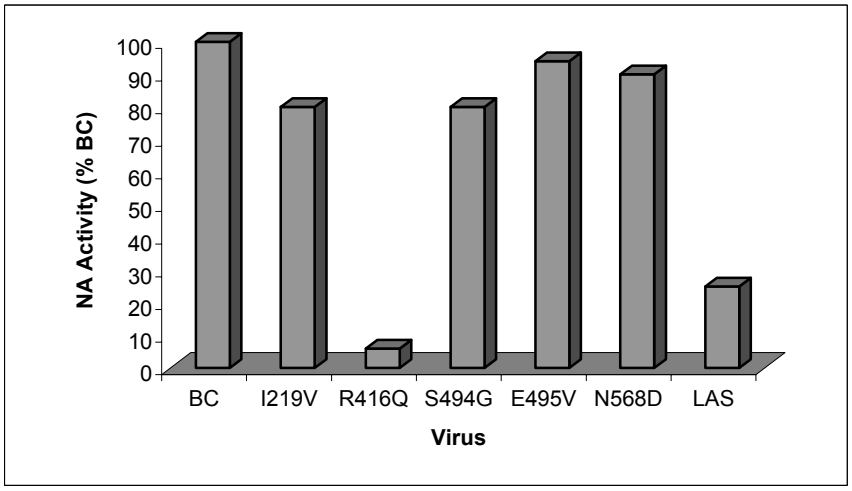
5.5.3 Biological activities of HN mutant viruses

We analyzed whether the mutations in the HN protein modulated the biological activities of NDV. The NA, HAd and fusion activities of the parental and HN mutant viruses are shown (Fig. 22A). The percent biological activity of each virus is shown relative to the rBC virus, whose biological activities were considered to be 100%. The HN mutant virus R416Q had the most significant differences in the NA, HAd and fusion promotion activities when compared to its parent rBC virus. The NA value of rR416Q was less than 10% of rBC virus and its HAd activity was only 20% of rBC. Its fusion promotion activity was only 50% of the parental rBC virus. The HN mutants I219V, S494G, E495V, and N568D did not have significant differences in their NA and HAd activities when compared to the BC virus. Fusion promotion activities of E495V (65% of BC), and N568D (approximately 75% of BC) mutant viruses were diminished when compared to the BC virus. (Fig. 22B). The fusogenic ability of the HN mutant viruses I219V and S494G did not vary significantly from that of rBC virus. These studies emphasize the importance of mutations in certain key amino acid residues on the HN protein in the attachment and neuraminidase functions of NDV in the context of a viral infection. These differences in the attachment and elution may translate into differences in viral growth kinetics *in vivo*.

Hemadsorption



Neuraminidase



Fusion

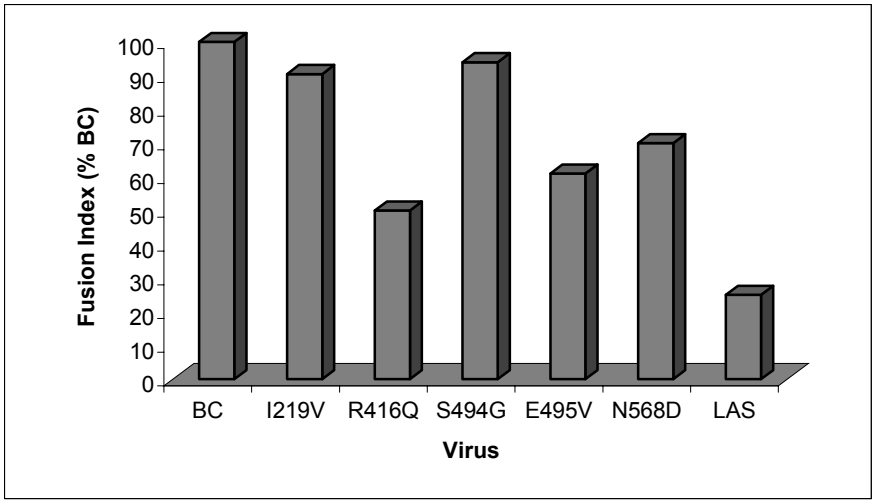


Figure 22A. Biological activities of the HN mutants. The hemadsorption (HAd), neuraminidase (NA) and fusogenicity of HN mutants were examined. HAd was measured as the percentage of the hemoglobin released compared to the parental rBC virus. The NA activities and fusion indices (FI) of the mutant viruses were also shown in comparison with these activities of the rBC virus. NA activity of purified viruses was measured by a fluorometric assay. The FI is the ratio of the total number of nuclei to the number of cells in which the nuclei were observed (i.e., the mean number of nuclei per cell). The average of three experiments is shown.

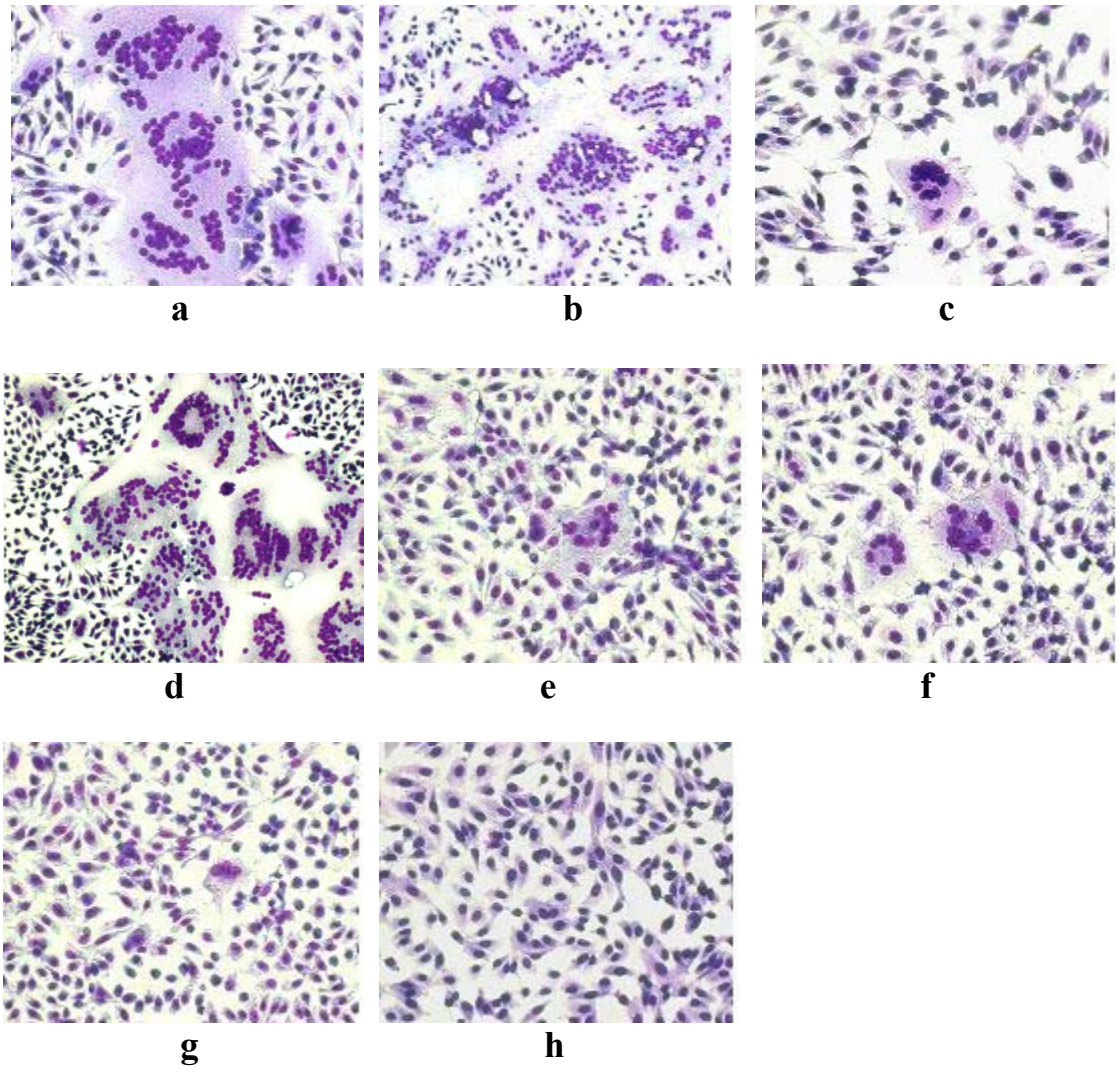


Figure 22B. Fusion of HN mutants.The fusogenicity of the mutant viruses in Vero cells in comparison to the parental rBC virus. Infected cells were fixed at 48h post-infection and stained with hematoxylin-eosin. The extent of fusion of the parental rBC (a) and the mutant viruses rI219V, rR416Q, rS494G, rE495V, rN568D and rLaSota (b, c, d, e, f, and g) in Vero cells are captured at 200X magnification in a Nikon Eclipse TE 300 microscope. Uninfected Vero cell monolayer (h) is included as a negative control for comparison.

5.5.4 Pathogenicity studies of HN mutant viruses

To determine whether the differences in *in vitro* biological characteristics of the chimeric viruses would translate into increased or decreased virulence in chicken embryos *in vivo*, the mean death time (MDT) pathogenicity test was carried out with the recovered recombinant HN mutant viruses (Table 5). The HN mutant virus rR416Q took 70h to kill 9-day-old chicken embryonated eggs, which was longer than the time taken by the parental BC virus (62h). This indicated a loss of virulence of the R416Q virus *in vivo*. These results corroborated with the *in vitro* growth characteristics and biological activities of the R416Q virus. The MDT of the I219V virus was 60 h, S494G virus was 66 h, E495V virus was 68 h. These were comparable to the MDT of parental virus BC.

Table 5. HN mutants of NDV and virus pathogenicity *in vivo*.

Virus	MDT[a] (h)
rBC	62
rI219V	60
rR416Q	***70***
rS494G	66
rE495V	64
rN568D	68
rLaSota	96

The virulence of the mutant and parental viruses was evaluated by intracerebral pathogenicity index (ICPI) in day-old-chicks, intravenous pathogenicity index (IVPI) in six-week-old chickens, and mean death time (MDT) in eggs. [a] MDT in hours for the minimum lethal dose to kill 9-day-old embryonated chicken eggs by allantoic route of inoculation.

Highly-virulent viruses take under 60h to kill the embryos, whereas, avirulent viruses take more than 90h to kill the embryos.

The MDT of the avirulent NDV strain LaSota was 96h. None of the mutant viruses took as long as the LaSota virus to kill the embryos indicating that virus infectivity *in vivo* is a multifactorial phenomenon. Although mutating key amino acids residues such as R416 present at the receptor-binding site of the HN protein, has significant effects on the biological functions of the HN protein, it does not alter viral infectivity drastically.

5.6 Discussion

The HN protein of NDV is a multifunctional spike protein, which serves as the attachment protein and has three functions: attachment, neuraminidase and fusion promotion activities (71). A recent report of the crystal structure of the globular head region of the HN protein of NDV has provided important clues about the location of the receptor binding and the sialic acid binding sites, (otherwise known as the "active site") present on the HN protein (24). For the initiation of fusion promotion activity of the HN protein, a type-specific functional interaction between F and HN proteins of paramyxoviruses is required (53). Studies of hybrid HN proteins by three different laboratories have all implicated the membrane-proximal ectodomain of the HN protein in virus-specific fusion promotion activity (31, 141, 144). In an attempt to study the importance of specific amino acid residues present at the active site of the HN protein, on the virulence of NDV, we performed the following study. Using site-directed mutagenesis, on a cDNA clone of BC (a mesogenic strain of NDV), amino acid residue R416 present at the active site of NDV was mutated by changing the arginine residue to glutamine. Mutational analysis of certain other amino acid residues (219, 494, 495, and

568) present near the active site of the HN protein of BC was also carried out. These amino acid residues are different in BC when compared to the corresponding amino acid residues present in the HN protein of an avirulent NDV strain LaSota. We mutated the amino acids present in the HN protein of a virulent NDV strain BC, to the ones present in an avirulent NDV strain LaSota to examine the role of these residues in modulating virulence of the virus. Using reverse genetics procedures (69) five mutant viruses were recovered and the HN gene of each recovered virus was entirely sequenced to ensure the presence of the intended mutations. These mutations were stable after ten sequential passages of the recovered viruses in tissue culture cells and one passage in 9-day-old chicken embryonated eggs. The effect of these mutations on the biological activities of the HN protein of each mutant virus was evaluated with hemadsorption, neuraminidase and fusion promotion studies *in vitro*. The effect of the mutations on virus infectivity *in vivo*, was studied by infecting 9-day-old embryos with each of the mutant viruses and recording the time taken for each virus to kill the embryos (mean death time study). The most significant finding of this study was the near abolition of the hemadsorption and neuraminidase activities of the R416Q mutant. This reflected its important role in virus attachment and neuraminidase activity. This mutant was also less virulent when compared to the parental BC virus as indicated by the MDT study. The mutant R416Q took 70 hours to kill the infected 9-day-old embryos (as opposed to 62 hours taken by the parental BC virus), to kill the embryos. Another interesting finding was the role of residues E495V and N568D in fusion promotion activity of the HN protein. These mutants exhibited lower rates of fusion promotion when compared to the parental BC virus. However, their hemadsorption and neuraminidase activities were not significantly

different from the BC virus. *In vivo* studies (MDT) with these mutants did not reveal any significant difference from the parental BC virus in viral infectivity. These residues may play a role in modulating fusion promotion activity of the HN protein of NDV. It will be interesting to create mutant viruses with multiple mutations of such key amino acids and study the combined effect of these mutations on viral infectivity. This can be done by incrementally placing additional amino acid changes into the original mutant viruses and testing the infectivity of the virus *in vitro* and *in vivo*. Besides the residue R416, the other amino acids forming the triarginyl cluster at the receptor-binding domain of the HN protein are residues R174 and R498 (23). Residues E401 and Y526 have been reported by Connaris *et al*, to be important for receptor binding (23). To test the effect of mutations of these amino acids on virus infectivity, further studies are being carried out. Although the amino acid changes in and around the active site pocket or on the outer surface of the HN molecule are important to the functions of the protein, the amino acid changes in other areas of the protein can also have an indirect effect on the functions of the protein. Comparisons of the HN protein sequence between LaSota and BC viruses identified only 17 amino acid differences. Therefore, we plan to examine the role of all of the amino acid differences using a chimeric approach. To find out whether virulence determinants lie in the globular head, stalk or transmembrane regions of the HN protein, chimeric viruses can be constructed using these regions of the HN gene of virulent and avirulent NDV viruses. With these studies we will be able to better understand of the molecular biology of paramyxoviruses in general, using a reverse genetics approach.

CHAPTER 6

6.1 Title

Conclusions and prospects

6.2 Conclusions and Prospects

Newcastle disease is a highly contagious and fatal viral disease that affects all species of birds. It is an economically important avian virus that can cause huge economic losses to the poultry industry (2). The causative agent of the disease is the Newcastle disease virus (NDV). NDV is an enveloped, negative-sense, single stranded RNA virus, belonging to the *Paramyxoviridae* family and genus *Avulavirus* (78, 89). The *Paramyxoviridae* family also includes other important pathogens such as the mumps virus, human parainfluenza virus, sendai virus, simian virus 5 and recently emerging nipah and hendra viruses. Nipah and hendra viruses may prove to be agents of bioterrorism (50). Highly virulent strains of NDV have been categorized as potential agents for agroterrorism (51). Understanding the molecular biology and molecular basis for pathogenesis for these viruses is thus, of great importance.

Results from the various studies done in this project indicate the importance of the HN protein of NDV in virus pathogenesis. By exchanging the HN gene between virulent and avirulent viruses for the first time, we established a direct role of the HN protein in

tissue tropism and NDV virulence. Further studies were done using site-directed mutagenesis, to eliminate each of the functional glycosylation sites on the HN protein of NDV. This investigation indicated that glycosylation sites of the HN protein play an important role in modulating NDV pathogenesis. We also explored the importance of certain key amino acid residues in the globular head ectodomain of the HN protein with a mutational analysis approach. The study indicated the key role of some of these residues in the biological activities of the HN protein and in influencing NDV pathogenesis. These studies provide the basis for further investigations to understand the overall role of the HN protein in NDV virulence. It will be interesting to study the molecular mechanism by which the HN gene determines tropism and virulence.

Reverse genetics techniques have revolutionized the study and molecular understanding of RNA viruses. This procedure allows production of infectious RNA virus entirely from cloned cDNA. Using this system, genetic manipulation of RNA viral genomes at the cDNA level can be done. Studies with this plasmid-based approach help in introducing defined mutations anywhere in the cDNA genome and studying their effects on characteristics of the generated recombinant virus. Although live, attenuated virus vaccines against NDV are available currently, the need for a more stable and efficacious recombinant vaccine is still in demand. With the help of the reverse genetics system, we have investigated the role of the HN protein in NDV pathogenesis. Since HN is a multifunctional protein of NDV, which is actively involved in virus infectivity, studying the role of the HN gene in virus pathogenesis is important to understand the basis for molecular pathogenesis of the virus. The HN protein is the main target of

immune response for NDV (82, 87). Since HN possesses immunogenic properties, further studies on this protein may prove to be useful for the development of NDV vaccines.

With the current study on the HN protein of NDV, we could generate an attenuated virus by abolishing glycosylation site 4 and sites 1 and 2 in combination (Chapter 4). Additional studies on the HN protein may pave the path towards generation of an attenuated recombinant NDV vaccine. The introduction of reverse genetics system to the molecular biology of NDV has opened several doors in the development of recombinant NDV vaccines. Generation of a recombinant chimeric HN vaccine that allowed serological differentiation between vaccinated and infected animals has been reported (107). Recombinant NDV has also been used as a vaccine vector (95). Identification of specific sequences in the HN protein responsible for NDV virulence will give insights into the mechanisms involved in NDV pathogenesis. With the availability of an established reverse genetics system for avirulent and virulent NDV strains, it would be of special interest to study the importance of various regions of the HN protein in NDV virulence using a chimeric approach.

Molecular biology studies of the NDV HN protein of NDV should also prove useful in the development of NDV as antineoplastic agents. Recent research (152) has shown that the HN protein of NDV may be responsible for induction of interferon-alpha and tumor necrosis factor-related apoptosis. Promising results on using NDV as an antitumor agent have already been shown in many clinical trials (25, 96,124). NDV possesses many qualities that make it an excellent antineoplastic agent. It has good cell-

binding properties, it binds specifically to tumor cells, it replicates only in tumor cytoplasm, and it is relatively safe (99, 125). A more detailed study of the HN protein may thus provide some insights into use of NDV in cancer therapy.

Previous research has indicated a strong and definitive interaction between the F and HN protein of NDV in initiating and influencing extent of viral infection (138). It would be interesting to exchange both F and HN proteins between virulent and avirulent NDV viruses to study the effect of this swap in viral pathogenesis. Since HN is an immunogenic protein, it would also be intriguing to include an additional HN gene of NDV into the full-length DNA genome of NDV and studying the effect of this gene addition in viral immune response. Insertion of a foreign gene into NDV cDNA has been reported earlier (69). Also, generation of chimeric HN viruses would throw more light on the role of specific domains of the HN protein in NDV virulence. Another interesting area to explore would be the importance of the glycosylation sites of the F protein in virus infectivity. Armed with the knowledge of basic studies on the virus, genetically stable live attenuated NDV viruses can be made for vaccine development.

NDV can be used as an ideal model for understanding the molecular basis of pathogenesis of other paramyxoviruses, since various NDV strains are found in nature which cause a continuous spectrum of disease in birds, ranging from inapparent to fatal disease. Moreover, the experiments involving the study of pathogenesis of NDV can be carried out directly in chickens, the natural hosts of NDV.

A deeper understanding of NDV molecular biology may not only be applied to the control of Newcastle disease but may also be exploited to understand the molecular biology and basis of pathogenesis for other paramyxoviruses, many of which are hazardous to human health and well being. Identification of viral factors and molecular mechanisms involved in NDV pathogenesis will enhance our understanding in paramyxoviruses in general. Future work with NDV will also be applicable to the study of pathogenesis of other RNA viruses.

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