ABSTRACT

Title of Document: ROLE OF NONCODING REGIONS IN NEWCASTLE DISEASE VIRUS REPLICATION AND PATHOGENESIS

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The roles of the intergenic sequences (IGS) and untranslated regions (UTR) in Newcastle disease virus (NDV) transcription and pathogenesis are not clear. By our established reverse genetics system, we investigated the role of these noncoding regions in NDV life cycle.

The infectious recombinant viruses containing increased/decreased length of F-HN and HN-L IGS were recovered and the transcription and pathogenicity of mutants were characterized. Our studies indicated that increased F-HN or HN-L IGS length reduced the downstream gene transcription. Morever, all IGS mutants were attenuated in chickens and the level of attenuation was increased as the length of IGS increased.

The mutant viruses with modified 5' and 3' UTR of HN mRNA were also recovered. The transcription, translation and pathogenecity of these recombinant viruses were characterized. Our studies indicated that the UTRs are not essential for NDV replication *in vitro*. Complete deletions of 5' HN UTR down regulated its transcription, translation levels and incorporation of HN protein into virus particle, therefore, attenuated the pathogenicity of NDV in chickens.

Moreover, studies on the HN UTRs replaced with corresponding NP UTRs virus suggested that UTRs can be exchanged between NDV mRNAs without affecting the replication of virus *in vitro* and *in vivo.*

In summary, my research identifies for the first time the role of noncoding regions in NDV replication and pathogenesis and provides novel methods for the development of attenuated live vaccines for NDV.

ROLE OF NONCODING REGIONS IN NEWCASTLE DISEASE VIRUS REPLICATION AND PATHOGENESIS

By

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Dedication

I would like to dedicate this work to my parents and husband for their love and support.

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List of Abbreviations

APMV	avian paramyxovirus
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
DNA	deoxyribonucleic acid
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	Hemagglutination assay
HDV	hepatitis delta virus
HI	hemagglutinin inhibition
HN	hemagglutinin-neuraminidase
hPIV3	human parainfluenza virus 3

ICPI	intracerebral pathogenicity index
IGS	intergenic sequence
IRES	internal ribosome entry site
IVPI	intravenous pathogenicity index
kD	kilodaltons
L	large polymerase
M	matrix
mRNA	message 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

RER	rough endoplasmic reticulum
RNA	ribonucleic acid
rNDV	recombinant Newcastle disease virus
RNP	ribonucleoprotein
RSV	Respiratory syncytial virus
RT-PCR	reverse transcription PCR
SeV	Sendai virus
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SPF	specific pathogen free
SV5	simian virus 5
UTR	untranslated region
VLP	virus like particle
VSV	vesicular stomatitis virus

Chapter 1

1.1 Title

General Introduction

1.2 Introduction

Newcastle disease is a highly contagious fatal disease in chickens that affects many domestic and wild avian species, causing severe economic losses to the poultry industry in the world. The infected birds may develop respiratory signs, like nasal discharge, coughing and sneezing, or nervous signs, like lameness, circling and complete paralysis. In some cases, sudden death occurs without any clinic signs.

In the United States, the virulent form of the disease is known as Exotic Newcastle disease (END). There were several END outbreaks in the US recently. In 1971, a major outbreak occurred in Southern California and almost 12 million birds were destroyed (http://www.lapublichealth.org/vet/newcastle.htm). This outbreak threatened the nation's entire poultry and egg supply. The most recent END outbreak was reported in California during 2002 to 2003. This outbreak, which first occurred in a small flock of backyard birds, resulted from illegal importation of game birds and later spread to commercial poultry. During this outbreak, 4 million birds were destroyed at a lost of $160 million dollars (Pedersen *et al*., 2004; Nolen, 2003 a&b).

The causative agent, Newcastle disease virus (NDV), is a member of the genus *Avulavirus* in the family *Paramyxoviridae*. NDV strains can be classified into three major pathotypes based

on severity of the disease produced in chickens. Avirulent, intermediate virulent and highly virulent strains are termed as lentogenic, mesogenic and velogenic strains, respectively. Lentogenic strains, such as LaSota, B1, Ulster, D26, may only cause mild or inapparent respiratory disease. Some lentogenic strains, such as LaSota and B1, are usually used as live vaccines in the field. Mesogenic strains, such as Kimber, Roakin and Beaudette C, cause respiratory or nervous signs with moderate mortality. Velogenic strains cause severe intestinal and/or neurologic disease resulting in up to 100% mortality. The velogenic strains can be further classified into neurotropic, such as Cormorant, AV and Texas GB, and viscerotropic, such as Herts, Milano and Fontana.

The genome of NDV is a nonsegmented, single-stranded, negative-sense RNA of 15,186 nucleotides (nt). The genome contains six genes, encoding the nucleocaspid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large RNA-dependent RNA polymerase protein (L). Two additional proteins, V and W, may be produced by RNA editing during P gene transcription. The RNA genome is tightly encapsidated by the NP protein. This ribonucleoprotein (RNP) complex serves as the template for transcription and replication by viral RNA polymerase proteins, which are L and P proteins. The NDV genes are arranged on genomic RNA in the order 3'-NP-P-M-F-HN-L-5'. Flanking the genes are 3' and 5' extracistronic sequences, known as the leader and trailer, respectively. These leader and trailer regions are cis-acting regulatory elements involved in transcription, replication, and packaging of the genomic and antigenomic RNAs. The beginning and end of each gene are conserved transcriptional control sequences, known as gene start (GS) and gene end (GE), respectively. Between the gene boundaries are non-coding intergenic sequences (IGSs), which vary in length from 1 to 47 nt. Between GS and each gene open reading frame (ORF), or between

each gene ORF and GE, there are untranslated regions (UTRs). These UTRs are not conserved in sequence composition and length between each gene and also vary among NDV strains. The functions of viral proteins coding sequences are well known, but very little is known about the function of noncoding regions. For example, the role of IGS and UTR in NDV transcription, replication, and pathogenesis remains unknown. Therefore, in this study, we have examined the role of IGS and UTR in the NDV life cycle using reverse genetics.

1.3 Research objectives:

(1) Determine the role of IGS in NDV transcription and pathogenesis.

Infectious recombinant viruses were constructed that contained deletion/addition of nucleotides into IGS between F-HN and/or HN-L. The transcription, replication, and pathogenicity of these viruses were examined in chickens.

(2) Analyses the role of UTR in NDV transcription, replication and pathogenesis.

Several recombinant viruses were constructed that contained deletion/modification in the UTR of HN mRNA. These mutant viruses were examined for their *in vitro* replication abilities and pathogenicity in chickens.

Chapter 2

2.1 Title

Review of Literature

2.2 Classification

Newcastle Disease virus (NDV) is a member of the genus *Avulavirus* in the family *Paramyxoviridae* of the order *Mononegavirales* (de Leeuw *et al.*, 1999). The family *Paramyxoviridae* is divided into two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. The subfamily *Paramyxovirinae* is further subdivided into five genera: *Avulavirus, Henipavirus, Morbillivirus, Respirovirus and Rubulavirus*. The genus *Avulavirus* contains nine serotypes of avian paramyxoviruses (APMV-1-9). NDV represents type 1(APMV-1). Besides NDV, the family *Paramyxovirudae* includes other important animal pathogens such as rinderpest virus and bovine respiratory syncytial virus, and some human pathogens such as mumps virus and measles virus.

2.3 Viral Morphology

The typical NDV is a pleomorphic, enveloped particle with diameter between 100-300nm (Shnyrova *et al*., 2007) (Fig.2.1). The envelope of NDV is derived from host cell membrane. Two glycoproteins, fusion (F) and hemagglutinin neuraminidase protein (HN) form the spike-

like protrusions on the outer surface of the virions. The F protein is required for fusion of virion into the host cell membrane through pH-independent mechanism. The HN protein is responsible for attachment of the virus to host cell. Under the envelope is a Matrix protein, which is thought to play a major role in mature virus budding. Inside the envelope is the ribo-nucleocapsid core structure which is the template for virus RNA synthesis. The core structure is formed by nucleocapsid protein (NP) tightly bound to the genomic RNA, to which phosphoprotein (P) and large polymerase protein (L) are attached.

2.4 Genome

The genome of NDV is a nonsegmented, single-stranded, and negative sense RNA of 15,186 nucleotides (nt) in length (Fig.2.2). The genome contains six genes: nucleocaspid protein (NP) gene, phosphoprotein (P) gene, matrix protein (M) gene, fusion protein (F) gene, hemagglutinin neuramindase protein (HN) gene, and large RNA-dependent RNA polymerase protein (L) gene. These genes are arranged in a linear order 3'-NP-P-M-F-HN-L-5'. The RNA genome and antigenome are tightly encapsidated by the NP protein. Flanking the genome are 3' and 5' extracistronic sequences, known as the leader and trailer, respectively. These leader and trailer regions are cis-acting regulatory elements involved in replication, transcription and packaging of the genomic and antigenomic RNAs. The beginning and end of each gene are conserved transcriptional control sequences, known as the gene start (GS) and gene end (GE), respectively. Between the gene boundaries are non-coding intergenic sequences (IGS), which vary in length from 1 to 47 nt. Each mRNA or open reading frame (ORF) is flanked by 5' and 3' untranslated region (UTR). The mRNAs are capped and have poly (A) tails.

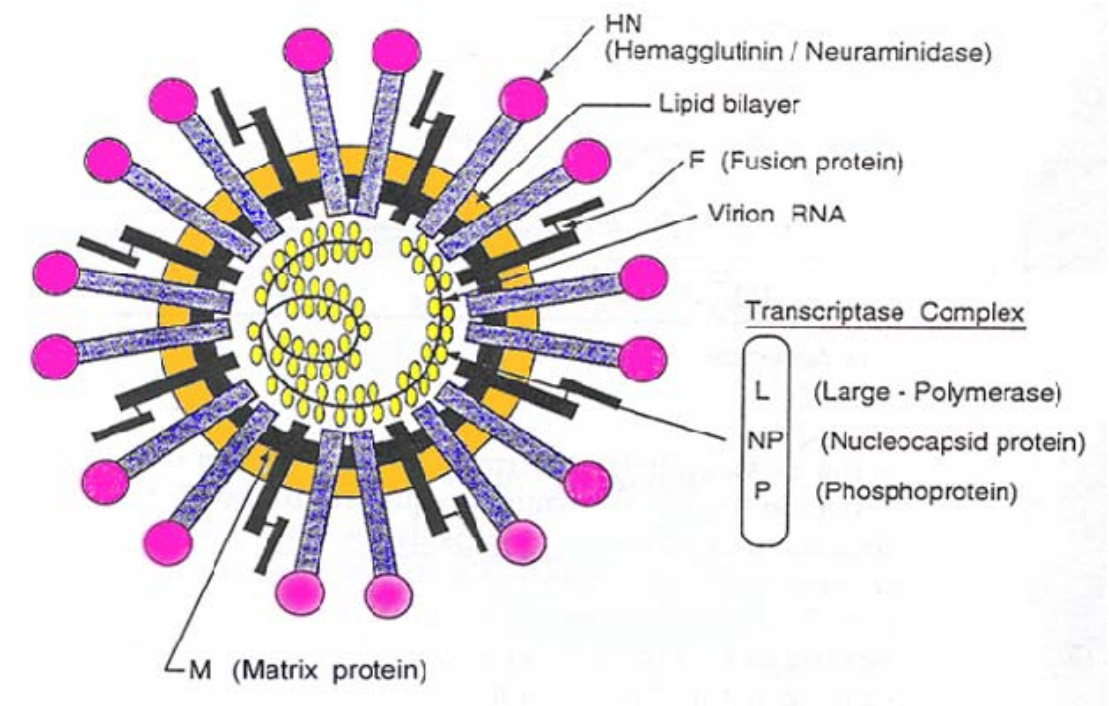


FIG.2.1 Schemtic of Newcastle disease virus particle.

The F and HN proteins form spike-like instruments on the virion envelope. The M protein is under the envelope. Insider envelope the NP protein is tightly bound to genome RNA, forming necleocapsid complex, The L, and P proteins are loosely bound to necleocaspid complex.

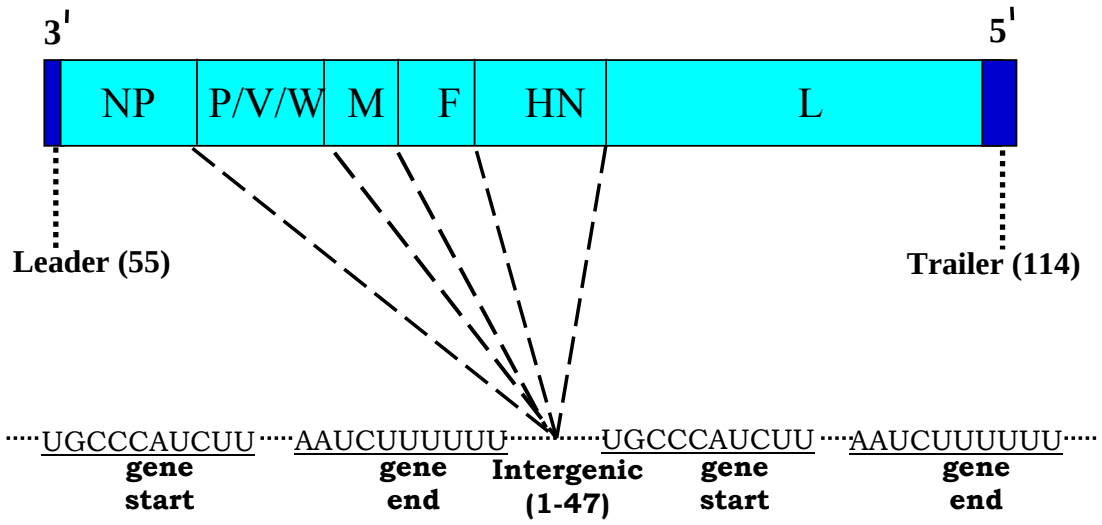


FIG.2.2 Genetic map of genome RNA of NDV.

The single-stranded, negative-sense genome RNA is 15,186 nt. in length. The length of leader, trailer is shown in parentheses. Each gene is flanked by conserved gene start and gene end. Between each gene boundary, nontranscribed intergenic sequences present which vary 1-47 nt in length.

2.5 Viral proteins

The genome of NDV encodes six major structural proteins: nucleocaspid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin neuraminidase protein (HN), and large RNA-dependent RNA polymerase protein (L). Two more proteins W and V are produced by P gene editing during mRNA transcription. The genome RNA together with NP, P, and L proteins forms the ribonucleoprotein complex (RNP), which serves as the template for RNA transcription and replication. On the envelope, two membrane glycoproteins, HN and F proteins, form the spike-like protrusions.

2.5.1 Ribonucleoprotein complex

The RNA genome of NDV is tightly encapsidated by the NP protein to form the NP: RNA complex which serves as the template for transcription and replication. Together with P and L proteins, NP: RNA complex is the minimal transcription unit (Hamagachi *et al.*, 1983).

NP protein: The NP protein of NDV is 489 amino acids long and is highly conserved. The molecular weight of NP is around 54 kilodaltons (kD) (Samson *et al.*, 1988). The genome and antigenome of paramyxoviruses are never found as free RNA instead they are tightly associated with the viral NP protein to form a ribonucleoprotein core. This core structure severs as the transcription and replication template. The level of unbounded NP protein is the key factor to control the switch of genome from transcription to replication. The NP protein regulates transcription and replication of the viral genome by interacting with P alone, with P and L, or with itself (NP-NP interaction) (Mebatsion *et al*., 2002). The NP protein of NDV is highly

immunogenic in nature and has been used as antigens for diagnostic purposes (Errington *et al.,* 1995).

Phosphoprotein: The molecular weight of P protein is 53-56 kD (Samson *et al.*, 1988). The amino terminus of the P protein acts as a chaperone for preventing uncontrolled encapsidation of non-viral RNA by NP protein (Curran *et al.*, 1995; Errington *et al*., 1997). Like other paramyxoviruses, P gene mRNA of NDV has been modified during transcription by the addition of nontemplated G residues at position 484; as a result, two additional viral proteins, V and W, are produced from alternative ORF within the P gene during RNA transcription (Steward *et al*., 1993).

Large polymerase protein: The L gene is 6704 nt long and the predicted molecular mass of the polypeptide is 242 kD (Yusoff *et al*., 1987). The L protein is the largest viral protein and contains all the catalytic activities associated with the viral RNA dependent RNA polymerase (Lamb and Parks, 2007). In the virions, the L protein is the least abundant of the structural proteins (about 50 copies per virion). The L protein is responsible for capping and polyadenylation of mRNAs. L protein of NDV is a determinant of virulence (Rout and Samal, 2008).

2.5.2 Matrix protein

The M gene of NDV is 1241 nt long and its predicted molecular mass is 38-40 kD (Chamber *et al*., 1986; Samson *et al*., 1988). The M protein is the most abundant protein in the virion and is under the envelop serving as a bridge to interact with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer and the nucleocapsid. The M protein is considered to be the central organizer of viral morphogenesis. The self-association of M protein and its affinity

to interact with the nucleocapsid is the driving force in forming a budding virus particle (Peeples, 1991).

2.5.3 Membrane glycoproteins

The envelope of NDV virions contains two transmembrane glycoproteins, HN and F. They form spike-like protrusions on the outer surface of the virions. The F protein exists on the surfaces of virions and infected cells as a homotrimeric spike, whereas the HN protein exists as a tetrameric spike (Russell *et al*., 1994; Thompson *et al*., 1988; Melanson and Iorio, 2006; Mirza *et al*., 1993). F protein of NDV requires the presence of the HN protein for the fusion activity (Sergel *et al*., 1993).

Fusion protein: Like other paramyxovirus F proteins, the F protein of NDV is a type I membrane protein. It is synthesized as an inactive precursor F_0 which consists of 553 amino acids. The F_0 must be cleaved into two biological active disulfide-linked F1 and F2 subunits by host-cell protease (Lamb and Parks, 2007). The F_1 and F_2 polypeptides are derived from the carboxyl-terminus and amino-terminus, respectively (Lamb and Parks, 2007). The sequences composition at the cleavage site is one of the primary determinants of virulence. For the velogenic and mesogenic strain of NDV, The F cleavage site consists of multiple basic amino acid (^{112}(R/K)RO(R/K)RF^{117}), while for the lentogenic strains, the sequences at the cleavage site are ^{112}(G/E)(K/R)Q(G/E)RL^{117} without multiple basic amino acids (Kawahara *et al*., 1992; Sakaguchi *et al*., 1991). The F protein of virulent viruses can be cleaved by host proteases which are found in a wide range of cells and tissues, whereas the F protein of lentogenic viruses can be cleaved only by trypsin-like enzymes which are only found in the respiratory and intestinal tracts (Garten *et al*., 1980; Nagai *et al*., 1976). Thus, the infection from virulent viruses usually results

in severe clinic signs in nervous system and high mortality. The cleavage site of F_0 protein is a major determinant of NDV virulence (Peeters *et al*., 1999; Panda *et al*., 2004b).

The F protein of NDV directs fusion of the virion envelope with the plasma membrane of the host cell (Romer-Oberdorfer *et al*., 2003). Paramyxovirus infection can occur at the host cell membrane, and thus, the fusion is pH independent (Lamb and Parks, 2007).

Paramyxovirus F protein consists of several domains that are involved in fusion. The amino terminus of F_0, termed fusion peptide, is thought to insert into the target cell membrane. In the F_1 peptide, two heptad repeats (HR1 and HR2) have identified as important to fusion. HR1 is located just carboxyl terminal to the fusion peptide; and the HR2 is located adjacent to the transmembrane domain. The HR2 domain has a leucine zipper motif which is thought to play a role in fusion (Buckland and Wild, 1989; Chamber *et al*., 1990; Hernandez *et al*., 1996). NDV F protein has this leucine repeat motif which extends for four heptads. A single amino acid change in this leucine repeat motif of F protein will alter the requirement for HN protein in fusion in NDV (Sergel *et al*., 2000). The fully glycoslated F protein of NDV contains a 470-amino-acid extracellular domain, a transmembrane domain near C-terminal, and a 29-amino-acid cytoplasmic tail (Chen *et al*., 2001). The molecular weights of F_0, F_1 and F_2 are 67 kD, 55 kD and 12 kD, respectively (Samson *et al*., 1988). The F protein is the major immunogenic protein of NDV.

HN protein: HN protein is a type II membrane glycoprotein with N-terminus exposed to the cytoplasm while the C-terminus is in the lumen of the endomembrane compartment.

The HN protein is a multifunctional enzyme necessary for attachment, fusion, and maturation of NDV. First, during the attachment of virion to host cell, it serves to recognize the sialic acid-containg receptor on host cell surface. Secondly, the HN protein promotes fusion in

concert with the F protein. Most paramyxovirus F protein requires coexpression of the HN protein to promote membrane fusion during fusion process. The third function of HN protein is to serve as neuraminidase. It cleaves sialic acid on the cell surface and facilitates release (Crennell *et al*., 2000). Therefore, HN protein is one of the major protective antigens of NDV.

The biologically active HN protein is formed by cleavage of a small glycoslated fragment from the precursor, HN_0, which contains 616 aa (Sato *et al*., 1987; Garten *et al*., 1980). The HN protein of most NDV strains includes 577 or 571 aa, since terminator codon of HN ORF is located before the HN_0 stop codon (Romer-Oberdorfer *et al*., 2003). The length of HN ORF is one of the factors for the virulence of different NDV strains. For example, the shortest HN ORF (571) has only been found in some velogenic strains (e.g. Miyadera and Herts) to the date; whereas, avirulent strains (e.g. Queensland, Ulster and D26) produce a 616 aa HN_0 precursor which requires proteolytic cleavage to become a biological active HN protein. A 577-amino-acid HN protein can be found in lentogenic strains (e.g. Clone-30 and B1), mesogenic strains (e.g. Beaudette C), or virulentic strains (e.g.Texas) (Romer-Oberdorfer *et al*., 1999; Gorman *et al*., 1988&1992).

The HN protein of NDV contains a 26-amino-acid cytoplasmic tail, a 22-amino-acid hydrophobic signal-transmembrane region, and a 522-amino-acid translocated carboxy-terminal domain that contains six potential N-linked glycosylation sites (residues 119,341,433,481,508 and 538) (McGinnes *et al*., 1987). These N-linked glycosylated sites are important for NDV pathogenesis. The loss of single glycosylated site in the globular head or multiple sites from the stalk and globular head regions will result in attenuating virulence of NDV (Panda *et al*., 2004a). A hydrophobic region near N-terminus functions as both a signal sequence and a membrane anchor (Markoff, *et al*., 1984; Spiess *et al*., 1986). The molecular weight of HN is 72-75 kD

(Samson *et al*., 1988).

2.5.4 V and W proteins

Members of subfamily *Paramyxovirinae* encode multiple proteins from the P gene by RNA editing during mRNA transcription. P gene editing occurs by inserting one or two nontemplated G residues at the conserved editing site (UUUUUCCC) on the genome. Three mRNAs, P mRNA (unedited), V mRNA (with a +1 frame shift) and W mRNA (with a +2 frame shift) are produced at the ratio as 68:29:2 (Mebatsion *et al*., 2001&2003; Huang *et al*., 2003). V and W proteins are present in the infected cells but absent in viral particles. The N-terminal domains of these chimeric proteins are encoded by the P ORF upstream of the editing site and the C-terminal domains are encoded by alternative ORF downstream of the editing site; therefore all three proteins are conserved at amino terminus but vary at their carboxyl terminus in length and amino acid composition.

The sequences of V protein vary among the NDV strains. The carboxyl-terminal domain of V protein contains an IFN inhibitor; therefore it functions as an alpha interferon antagonist. Due to its ability to counteract the antiviral effects of interferon, V protein is considered as a virulence factor of NDV in its natural host, birds.

Sequences of W protein vary among the strains of NDV. In the case of Ulster strain, the length of W protein ORF is 181 aa; whereas, for those of D26, Beaudette C, and AV are of 141, 221, and 228 aa, respectively (Steward *et al*., 1993). The function of the W protein of NDV has not been established, and work with other virus suggests that it may be an inhibitor of replication (Curran *et al*., 1991).

2.6 Nonconding regions

Besides coding regions, which encode viral proteins, some noncoding regions also present on the viral genome of NDV. Between each gene boundary, there are nontranscribed sequences termed intergenic sequences (IGSs). The lengths of IGS vary from 1 to 47 in NDV strains. In mesogenic strain Beaudette C, the first three 3' end IGS are single nucleotide; whereas, the F-HN and HN-L IGSs are 31 and 47 nt, respectively (Chambers *et al*., 1986a&b&c; Krishnamurthy and Samal, 1998). During transcription, the viral RNA polymerase bypasses each IGS without synthesis and reiniates transcription at the next downstream gene start signal. Occasionally transcribes across IGS to generate readthrough polytranscripts (Collins, 1991).

The leader and trailer regions are not encoded as polypeptides, but they contain the promoters for viral RNA replication. Other noncoding regions on the viral genome are untranslated regions (UTR). Between each gene ORF and GS or GE, there are 5' or 3' UTR, respectively. The length and sequence compositions vary between each gene and among NDV strains. The role of IGS and UTR regions in NDV transcription, replication, and pathogenesis is not clear.

2.7 Life cycle of NDV

The replication strategy employed by NDV is similar to that of other nonsegmented negative-sense RNA viruses (Fig.2.3). The initial step of replication is the adsorption of the virus to the cell surface receptor followed by fusion with the host cellular membrane. The fusion causes the viral genome to be released into the cytoplasm. The transcription and replication of NDV

genome occurs in the cytoplasm. At the end of the replication cycle, the viral proteins are packaged and the progeny viruses mature by budding through the plasma membrane.

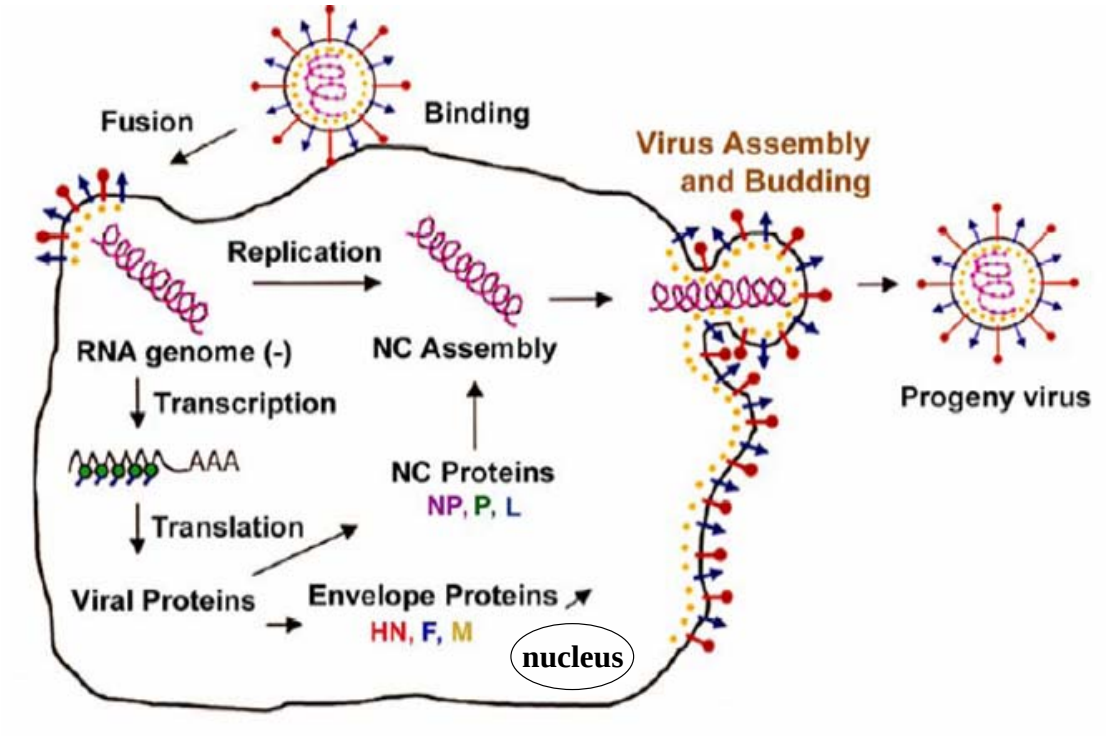


FIG.2.3 The life cycle of NDV.
(Figure modified from

http://www.urmc.rochester.edu/SMD/mbi/education/courses/MBI456files/ParamyxovirusII.pdf)

2.7.1 Virus adsorption and entry

The initial step of virus entry requires the binding of the HN protein into the sialic acid-containing receptor on the host cell surface. Following the attachment, the envelope of the virus fuses with the host cell plasma membrane. The fusion process is mediated by F protein. Upon fusion, disruption of matrix-nucleocaspid occurs and the viral nucleocapsid is released into the host cell cytoplasm. The entry of NDV into cells is believed to occur by direct fusion at the plasma membrane through a pH-independent mechanism. Recent study also suggests that NDV may infect the cells through an alternative route: caveolae-dependent endocytic pathway (Cantin *et al*., 2007).

2.7.2 Transcription

The mRNA synthesis begins at the 3' end of the genome of NDV (Fig.2.4). Since the uninfected host cell lacks the RNA dependent RNA polymerase, the viral RNA polymerase has to first transcribe the positive-stranded leader RNA at the 3' end promoter. The first gene, NP, is transcribed at the NP gene start (GS) and is terminated at the NP gene end (GE). This results in release of the first capped and polyadenylated mRNA, NP mRNA. The transcription of paramyxovirus follows the "start-stop" mechanism until the last mRNA, L mRNA, is synthesized. That is, the polymerase stops at the upstream GE and reiniates synthesis of the next mRNA at the next GS. Some polymerase falls down at the intergenic sequences (IGS) region, which is located between the upstream GE and downstream GS signals. Some polymerase may bypass the IGS, and the readthrough transcripts are formed. This "start-stop" transcription results in the gradient of mRNA abundance that reduces according to the relative distance of the location of the individual gene from the 3' end promoter. Since the NP gene is the closest to the 3'end promoter,

the NP mRNA is produced at the most amount; whereas, the L gene, which is located the farthest to the 3' end promoter, produces the least amount of L mRNA.

For efficient transcription and replication, most paramyxoviruses follow the "Rule of Six". That is, the length of genome has to be a multiple of six. The hexamer rule is most likely that NP subunit of the nucleocapsid is associated with exactly six nucleotides. Apart from NDV, other paramyxoviruses, such as Sendai virus and measles virus, also follow this "Rule of Six"; but the members of pneumoviruses do not follow the "Rule of Six" (Samal and Collins, 1996).

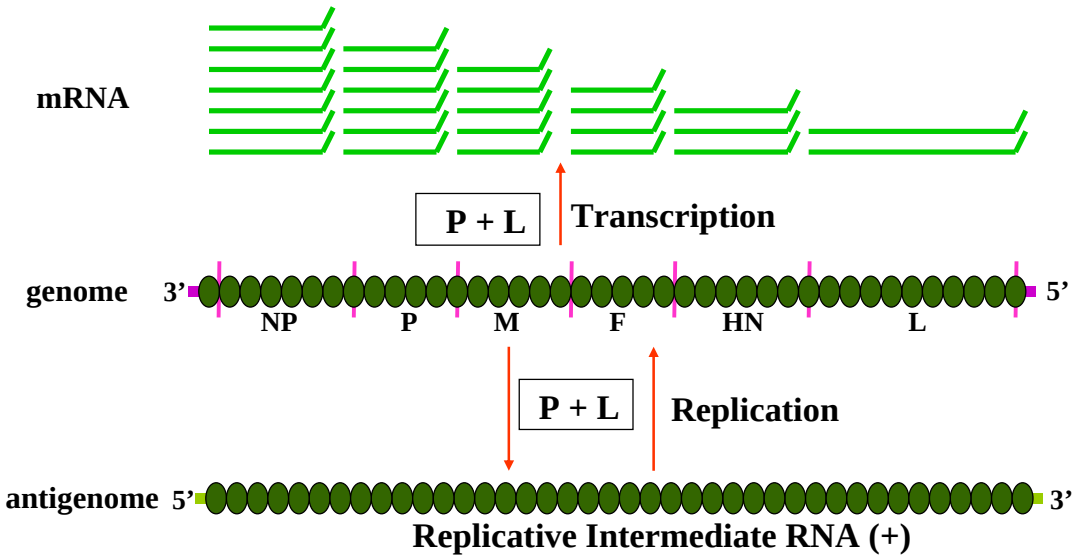


FIG.2.4 Schematic diagram of Paramyxovirus transcription and replication.
Transcription occurs in a sequential start-stop manner during which the polymerases transcribe the genome to produce mRNA. There is a polar attenuation of transcripts in which each downstream gene is transcribed less than its mediate upstream gene. Vertical line in the genome indicate gene junction; Oval shapes indicate nucleocapsid protein; Horizontal lines with slanted side bar lines indicate mRNA; and wavy lines indicate viral proteins. (Figure modified from Collins *et al*., 1996. Respiratory Syncytial virus, *In:* Foelds, B.N.Knipe, D.M.and Howley, P.M.. Virology 3rd ed. Raven Press, New York.)

2.7.3 Replication

The processes of replication and transcription of NDV are tightly regulated. The switch from transcription to replication is controlled by the NP protein. When free NP protein in the cytoplasm is limiting, the viral polymerase is preferentially engaged in mRNA synthesis, transcription, resulting in the increased free NP protein and other viral proteins. Once the amount of free NP protein is sufficient, the viral polymerase ignores the gene junction signal, such as GS and GE, and switches to replication. The full-length complementary copy, known as antigenome (+), is first synthesized to serve as the template for replication of negative sense genome RNA. Both the genome and antigenome are packaged into encapsidated nucleocapsid. Leader and trailer of the genome contain the specific signals for encapsidation (Blumberg *et al*., 1981a&b).

2.7.4 Virus assembly and release

Recent studies suggest that the assembly and release of infectious NDV particles take place at the membrane lipid rafts (Laliberte *et al*., 2006; Dolganiuc *et al.,* 2003).

The first step in viral assembly is the encapsidation of genome/antigenome RNA into nucleocapsid. The nucleocapsid is thought to be assembled in the cell cytoplasm. First, the free NP proteins are tightly associated with the genome RNA to form the ribonucleoprotein (RNP) core structure; secondly, the P and L proteins are loosely bound to RNP, forming transcriptase complex (Kingsbury *et al*., 1978).

The assembly of viral envelope takes place at the cell surface. The membrane proteins, F and HN proteins are synthesized on the rough endoplasmic reticulum (RER) and transport to the cell surface through the secretory pathway. Before transportation, the proteins undergo stepwise conformational maturation. Folding and maturation occur inside the ER with the help of many

molecular cellular chaperones. Only the correctly folded proteins are transported out of the ER to the Golgi apparatus for further post translation modifications including carbohydrate chain modification of HN protein and cleavage of F_0 protein at the multiple basic cleavage site to form functional F_1 and F_2 proteins (Doms *et al*., 1993). Finally, the mature F and HN proteins are transported to the cell plasma membrane through vesicles.

The M Proteins are thought to play the major role in taking the assembled RNP to the plasma membrane to form budding virions. Study on NDV virus-like particle (VLP) suggests that M-HN and M-NP interactions are responsible for incorporation of HN and NP proteins into VLPs and the F protein is incorporated indirectly due to interactions with NP and HN protein (Pantua *et al*., 2006). The assembled virions at the plasma membrane are then released by budding from host cell membrane.

2.8 Reverse genetics

Reverse genetics technique is the method which allows generating the infectious virus from cloned cDNA of the viral genome. It is useful to manipulate the viral genome RNA at the cDNA level.

Reverse genetics system for nonsegment, single-stranded, negative-sense (NSN) RNA viruses were not available before 1994. One of the reasons is that genome RNA itself is not infectious and requires the viral polymerase protein for the first round of mRNA synthesis. Rabies virus was the first NSN RNA virus to be rescued entirely from cloned cDNA in 1994 (Schnell *et al*., 1994). In this system, intracellular expression of antigenome positive-sense RNA

and polymerase complex, NP, P and L proteins, from plasmids are transcribed by T7 RNA polymerase.

Since then, many negative-sense RNA viruses in the family *Paramyxoviridae,* such as simian virus 5 (He *et al*., 1997), human respiratory syncytial virus (Collins *et al*., 1995), Sendai virus (Garcin *et al*., 1995; Kato *et al*., 1996), and measles virus (Radecke *et al*., 1995), have been recovered from cloned cDNAs.

To date, reverse genetics systems have been established in several laboratories for NDV strains including: Lentogenic strains LaSota (Huang *et al*., 2001; Romer-Oberdorfer *et al*., 1999; Peeters *et al*.,1999), B1(Nakaya *et al*., 2001); mesogenic strain Beaudette C (Krishnamurthy *et al*., 2000) (Fig.2.5) and velogenic strain Herts/33 (de Leeuw *et al*., 2005). The availabilities of the reverse genetics system for NDV and other negative sense RNA viruses have provided a useful tool for basic studies of molecular biology and pathogenesis, leading to design of new generations of live vaccines.

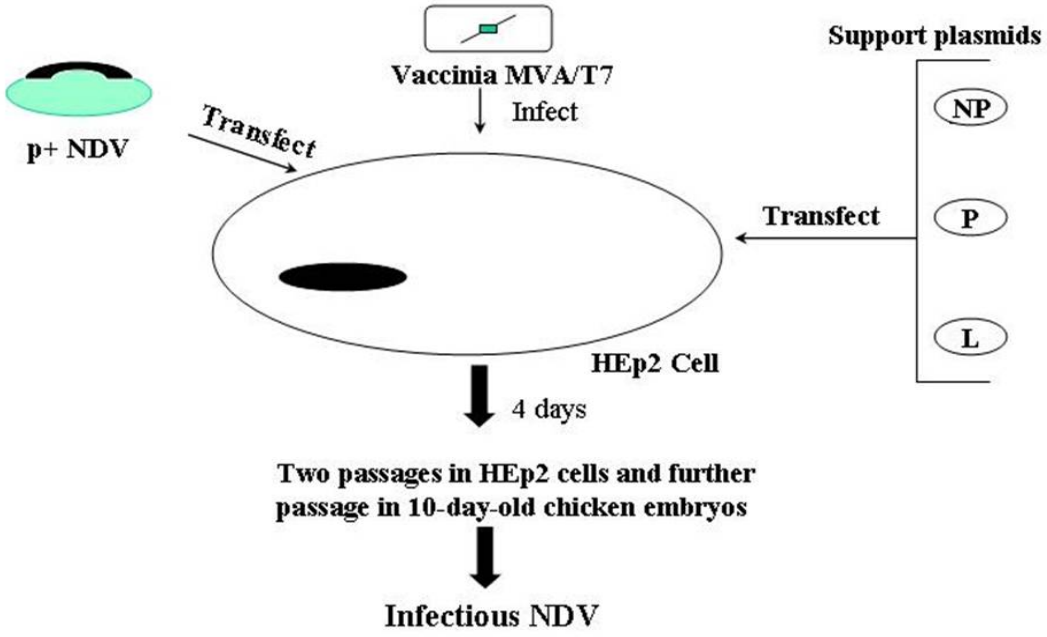


FIG.2.5 Schematic diagram for the recovery of infectious NDV from cDNA.
Cloned full length cDNA which is complementary to genomic RNA and plasmids which encode NP, P, and L proteins were co-transfected into HEp-2 cells. All plasmids are under the control of T7 RNA polymerase promoter. Simultaneously, HEp-2 cells were infected with recombinant vaccine MVA/T7 virus capable of expression T7 RNA polymerase. Infectious NDVs were amplified in 10-day-old chicken embryos. The figure was modified from Krishnamurthy *et al.*, 2000.

Chapter 3

3.1 Title

Role of Intergenic Sequences in Newcastle Disease Virus RNA Transcription and Pathogenesis

(Previously published as: Yan Y and Samal SK. 2008. J.Virol. 82:1323-31.)

3.2 Abstract

Newcastle disease virus (NDV), a member of the family *Paramyxoviridae*, has a non-segmented negative-sense RNA genome consisting of six genes (3'-NP-P-M-F-HN-L-5'). The first three 3' end intergenic sequences (IGSs) are single nucleotide (nt), whereas the F-HN and HN-L IGSs are 31 and 47 nt, respectively. To investigate the role of IGS length in NDV transcription and pathogenesis, we recovered viable viruses containing deletions or additions in the IGSs between the F and HN, and the HN and L genes. The IGS of F-HN were modified to contain an additional 96 nt or more, or deletion of 30 nt. Similarly, the IGS of HN-L were modified to contain an additional 96 nt or more, or deletion of 42 nt. The level of transcription of each mRNA species (NP, F, HN, and L) was examined by Northern blot analysis. Our results showed that NDV can tolerate an IGS length of at least 365 nt. The extended length of IGS down-regulated the transcription of downstream gene and suggested that 31 nt in the F-HN IGS and 47 nt in the HN-L IGS are required for efficient transcription of the downstream gene. The effect of IGS length on pathogenicity of mutant viruses was evaluated in embryonated chicken eggs, 1-day-old chicks and 6-week-old chickens. Our results showed that all IGS mutants were attenuated in chickens. The level of attenuation increased as the length of IGS increased.

Interestingly, decreased IGS length also attenuated the viruses. These findings can have significant applications in NDV vaccine development.

3.3 Introduction

Newcastle disease virus (NDV) causes a highly-contagious respiratory, neurologic, or enteric disease in chickens, which leads to severe economic losses in the poultry industry worldwide. NDV isolates display a spectrum of virulence in chicken, ranging from an inapparent infection to 100% mortality. Strains of NDV are classified into three major pathotypes based on the severity of the disease produced in chickens. Avirulent, intermediate virulent and highly virulent strains are termed as lentogenic, mesogenic and velogenic strains, respectively.

NDV is a member of genus *Avulavirus* in the family *Paramyxoviridae*. The genome of NDV is a nonsegmented, single-stranded and negative-sense RNA of 15,186 nucleotides (nt) (de Leeuw and Peeters, 1999; Krishnamurthy and Samal, 1998; Phillips *et al*., 1998). The genome contains at least six genes which encode nucleocaspid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuramindase protein (HN), and large RNA-dependent RNA polymerase protein (L). Two additional proteins, V and W, may be produced by RNA editing during P gene transcription (Steward, 1993). The RNA genome is tightly encapsidated by the NP protein. This ribonucleoprotein (RNP) complex serves as the template for transcription and replication by viral RNA polymerase proteins, which are L and P proteins. The NDV genes are arranged on genomic RNA in the order 3'-NP-P-M-F-HN-L-5'. Flanking the genes are 3' and 5' extracistronic sequences, known as the leader and trailer, respectively. These leader and trailer regions are cis-acting regulatory elements involved in replication, transcription and packaging of the genomic and antigenomic RNAs. The beginning and end of each gene are conserved

transcriptional control sequences, known as the gene start (GS) and gene end (GE), respectively (Galinski and Wechler, 1991; Lamb and Parks, 1996). Between the gene boundaries are non-coding intergenic sequences (IGSs), which vary in length from 1 to 47 nt. Each of the first three IGSs, NP-P, P-M and M-F gene junctions, has only one nucleotide, while the other two IGSs, F-HN and HN-L gene junctions, are 31 nt and 47 nt, respectively (Chambers *et al*., 1986; Krishnamurthy and Samal,1998). The lengths of IGS are generally conserved in most NDV strains, and one of the exceptions is that the IGS in NP-P gene junction of strain D26 is 2 nt. However, the sequences of IGSs vary among NDV strains (Ishida *et al*., 1986).

The transcription process for NDV is similar to other non-segmented negative-sense RNA viruses (Lamb and Kolakofsky, 1996). The viral RNA polymerase first transcribes the leader RNA at the 3'end of the genome. Synthesis of the leader sequence terminates at the boundary of the first gene (NP), and proceeds with the NP mRNA synthesis at the GS of the NP gene. Transcription terminates at the GE of the NP genes. The transcription complex probably passes over the NP-P IGS and begins transcription of the P gene. Therefore, IGSs are not copied into mRNAs. Transcription continues in this start-stop manner until the mRNA of the last gene, L, is synthesized. The mode of transcription leads to a gradient of mRNA abundance that is reduced according to the distance of the location of a particular gene from the 3'end of the genome. Replication occurs when the polymerase complex ignores the transcription stop signal at the 3' end of each gene (Lamb and Parks, 2007). NDV RNA replication follows "the Rule of Six"; that is, efficient replication occurs only if the genome size is a multiple of six nucleotides (de Leeuw and Peeters, 1999).

There are two different IGS groups among members of the Order *Mononegavirales*. One group has short, conserved or semi-conserved IGS. Members of the genera, Respirovirus,

Morbillivirus, and Henipavirus, have IGS that are a conserved trinucleotide. For example, Sendai virus (SeV), human parainfluenza virus type 3 (hPIV3) and Hendra virus have conserved trinucleotide (GAA), with the exception of the HN-L IGS of SeV which is GGG (Skiadopoulos *et al*., 2000; Wang *et al*., 2000). Rhabdovirus vesicular stomatitis virus (VSV) has semi-conserved dinucleotide GA or CA as the IGS (Hinzman *et al*., 2002). The IGS in the other virus group vary significantly in sequence and length. The IGS of human parainfluenza virus type 2 (hPIV-2) vary in length from 4 to 45 nt (14). For simian virus 5 (SV5) and respiratory syncytial virus (RSV), the IGS vary in length from 1-22 to 1-56 nt, respectively (Bukreyev *et al*., 2000; Collins *et al*., 1986; Hardy *et al*., 1999; Rassa and Parks, 1999). NDV belongs to the latter group.

The role of IGS in transcription, replication and viral pathogenesis of the members of *Mononegavirales* is unclear. Studies using infectious recombinant RSV that vary in IGS length from 16 to160 nt showed that there was no significant difference in transcription or replication *in vitro* and *in vivo* (Bukreyev *et al*., 2000). Studies using VSV minigenome system showed that some nucleotide changes in the IGS resulted in higher levels of read-through transcription. This indicated that IGS plays a role in transcription termination in VSV (Hinzman *et al*., 2002). Studies with SV5 IGS showed that the length of IGS alone is not a determining factor in transcription termination/ polyadenylation (He and Lamb, 1999). The roles of NDV IGS in viral transcription, replication, and pathogenicity have not been studied. It is not known why the first three 3'end IGSs are only 1 nt long; whereas the next two IGSs are always 31 and 47 nt long.

In this chapter, we have investigated the role of the length of IGS in NDV transcription and virulence. We have made several constructs which have either addition or deletion of nucleotides in F-HN and/or HN-L IGS. The length of IGS changes were made based on the "Rule of Six". Recombinant NDVs were recovered from their respective cDNA clones by reverse genetics

technique (Krishnamurthy *et al*., 2000). The effect of IGS length on upstream and downstream gene transcription was quantified. The data indicated that addition of nucleotides to the IGS length down-regulated the transcription of the downstream gene, but did not affect transcription of the upstream gene. The virulence of these recombinant viruses was examined by mean death time (MDT) in 9-day-old embryonated chicken eggs, by intracerebral pathogenicity index (ICPI) in 1-day-old chicks and by intravenous pathogenicity index (IVPI) in 6-week-old chickens. Our *in vivo* studies indicated that addition or deletion of nucleotides to IGS length decreased the virulence of mutant viruses.

3.4 Materials and Methods

3.4.1 Cells and viruses. DF1 cells (chicken embryo fibroblast cell line) were maintained in Dulbecco's minimal essential medium (DMEM) with 5% fetal bovine serum (FBS). HEp2 cells (human epidermoid carcinoma cell line) were maintained in Eagle's minimal essential medium (MEM) with 5% FBS. A moderately pathogenic (mesogenic) NDV strain Beaudette C (BC) and recombinant viruses generated from BC virus were grown in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The modified vaccinia virus Ankara recombinant that expresses the T7 RNA polymerase (a generous gift of Dr. Bernard Moss, National Institutes of Health) was grown in primary chicken embryo fibroblast (CEF) cells.

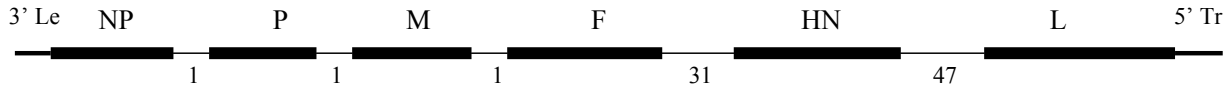
3.4.2 Constructions of NDV cDNAs with modified F-HN IGS and HN-L IGS. Full-length antigenomic cDNA of NDV strain BC was cloned into plasmid pBR 322 and designated as pBC (Krishnamurthy *et al*., 2000). F-HN IGS and HN-L IGS were modified in pBC. All constructs

were confirmed by dideoxynucleotide sequencing. The mutant IGS include deletion of 30 nt in F-HN IGS; deletions of 30 nt or 42 nt in HN-L IGS; additions of 96 nt, 210 nt, and 318 nt in F-HN and HN-L IGS, and a double deletion of 18 nt in F-HN IGS and 30 nt in HN-L IGS, as summarized in Table 3.1 and shown in Fig.3.1.

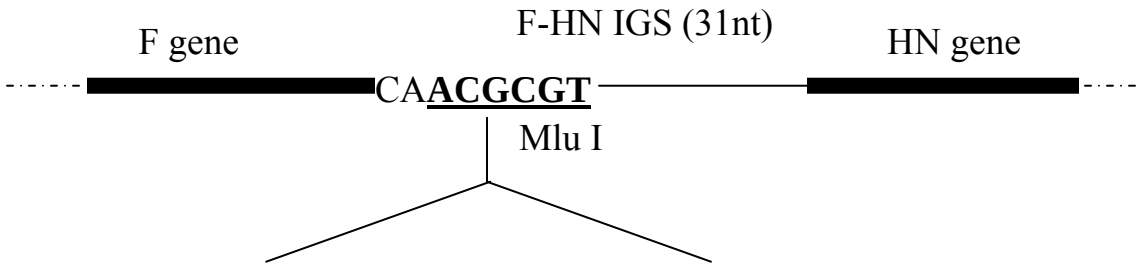
TABLE 3.1 Summary of mutant viruses with modified IGS

Gene junction	No. of nucleotides change	Virus name
F-HN	30 nt deleted	rBC-FHNΔ30
F-HN	96 nt inserted	rBC-FHN96
F-HN	210 nt inserted	rBC-FHN210
F-HN	318 nt inserted	rBC-FHN318
HN-L	30 nt deleted	rBC-HNLΔ30
HN-L	42 nt deleted	rBC-HNLΔ42
HN-L	96 nt inserted	rBC-HNL96
HN-L	210 nt inserted	rBC-HNL210
HN-L	318 nt inserted	rBC-HNL318
F-HN and HN-L	18 and 30 nt deleted	rBC-FHNΔ18HNLΔ30

A

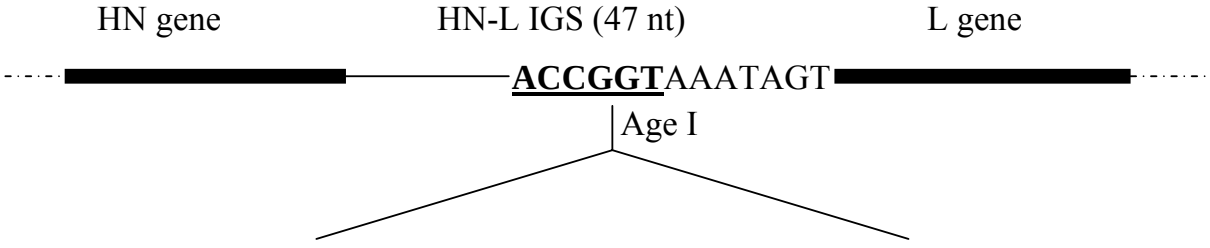


B



rBC-FHN96	90 nt from AMPV SH-G IGS and 6 nt of MluI
rBC-FHN210	204 nt from AMPV N gene 88 to 291 nt and 6 nt of Mlu I
rBC-FHN318	312 nt from AMPV N gene 88 to 399 nt and 6 nt of Mlu I

C



rBC-HNL96	90 nt from AMPV SH-G IGS and 6 nt of Age I
rBC-HNL210	204 nt from AMPV N gene 88 to 291 nt and 6 nt of Age I
rBC-HNL318	318 nt from AMPV N gene 88 to 399 nt and 6 nt of Age I

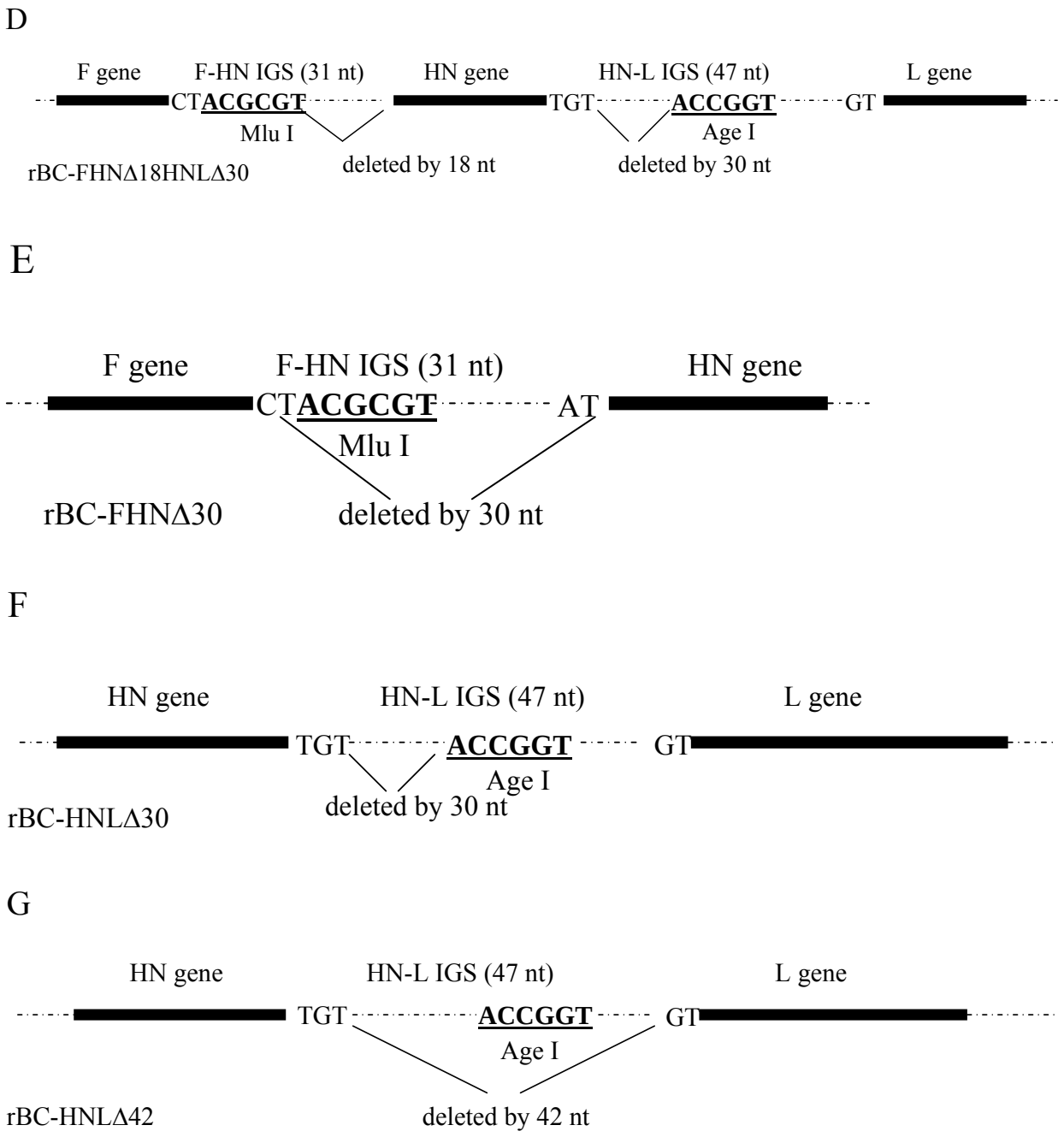


FIG.3.1 Structure of modified IGS between F and HN, HN and L genes of mutant NDVs.
(A) Structure of NDV genome order. Between each gene, IGS was indicated as thin line, and length of each IGS is shown under the line. (B) Structures of IGS between the F and HN genes of rBC-FHN96, 210 and 318. The 31 nt represents the length of wild type F-HN IGS. (C) Structures

of IGS between the HN and L genes of rBC-HNL96, 210, and 318. The 47 nt represents the length of wild type HN-L IGS. (D) Structure of IGS between the F and HN, and HN and L genes of rBC-FHNΔ18HNLΔ30. The 18 nt and 30 nt were deleted from F-HN and HN-L IGS, respectively. (E) Structure of IGS between the F and HN genes of rBC-FHNΔ30. The 30 nt were deleted from F-HN IGS. (F) Structures of IGS between the HN and L genes of rBC-HNLΔ30. The 30 nt were deleted from HN-L IGS. (G) Structure of rBC-HNLΔ42. The 42 nt were deleted from HN-L IGS.

For F-HN IGS insertion, different lengths (90 nt, 204 nt or 312 nt) of avian metapneumovirus (AMPV, GenBank accession No. AY590688) sequences were amplified by PCR with Mlu I site at both ends. The amplified sequences were digested with Mlu I and inserted into the full-length cDNA clone pBC, which contains a unique Mlu I site in IGS between F and HN genes. AMPV sequences were chosen because it is a closely related avian paramyxovirus. The amplified AMPV sequences were: 90 nt sequences were from AMPV SH-G IGS; 204 nt sequences were from AMPV N gene 88 to 291 nt; and 312 nt sequences were from AMPV N gene 88 to 400 nt.

For deletion of nucleotides in the F-HN IGS, three rounds of PCR were performed. In the first round PCR, template pBC was amplified by primers Not I (+) (5'- CAA ATA ACA GCG GCC GCA GCT C -3') and HN6340 (-) (5'- CTC TTA CCG TTC TAC CCG TGT TTT TTC TAA ACT CTC CGA -3'). This PCR product contained 30 nt deletion in the F-HN IGS and a Not I site, which is present in the upstream gene F. In the second round PCR, template pBC was amplified by primers HN6272 (+) (5'-TCG GAG AGT TTA GAA AAA ACA CGG GTA GAA CGG TAA GAG -3') and HN/L (-) (5'- AAG GCC TTG TCT GCT GAG AAT GAG GTG -3'). This PCR product contained Age I site, which is present in the HN-L IGS and 30 nt deletion in the F-HN IGS. In the third round PCR reaction, the first and second PCR products were used as templates, and primers, Not I (+) and HN/L (-), were used to amplify the templates. This PCR product was digested with Not I and Age I and then cloned into pBC, and designated as pBC-FHNΔ30.

For HN-L IGS insertion, different length (90 nt, 204 nt or 312 nt) sequences were amplified with Age I site on the both ends by PCR. These HN-L IGS insertion sequences were the same as F-HN IGS inserted sequences. The amplified sequences were digested with Age I

and then inserted into full-length cDNA clone pBC, which contained a unique Age I site in IGS between HN and L genes.

For HN-L IGS deletion, pBC-HNLΔ30, primers F6202 (+) (5'- GTG AAC ACA GAT GAG GAA CG - 3') and HNL8366 (-) (5'-TTT ACC GGT TAC ATT TTT TCT TAA TCG AGG GAC TAT TGA C-3') were used to amplify the template pBC. The PCR product was digested with Mlu I and Xba I and then cloned into pBC. For pBC-HNLΔ42, three rounds of PCR were performed. In the first round PCR, primers HN7513 (+) (5'- CGC ATA CAG CAGGCTATCTTA -3') and HNL 8390 (-) (5'- GAG CTC GCC ATG TCC TAC CCG TAC ACA TTT TTT CTT AAT CGA GGG ACT ATT GAC - 3') were used to amplify the template pBC. This PCR product contained Spe I site, which is present in the HN gene and a 42 nt deletion in the HN-L IGS. In the second round PCR, primers HNL8300(+) (5'- GTC AAT AGT CCC TCG ATT AAG AAA AAT GTG TAC GGG TAG GAC ATG GCG AGC TC -3') and XbaI (-) (5'- AGT ACT CCG GTT ATT CTA GAA TTG TGG TTG - 3') were used to amplify template pBC. This PCR product contained Xba I site and a 42 nt deletion in the HN-L IGS. In the third round PCR, primers HN7513 (+) and XbaI were used to amplify templates from the first and second round PCR reactions. The PCR product was digested with SpeI and XbaI and then cloned into pBC.

For the double deletion construct which contained deletions of 18 nt in F-HN IGS and 30 nt in HN-L IGS (pBC-FHNΔ18HNLΔ30), primers HN6290(+) (5'- ACT ACG CGT GAT ATA CGG GTA GAA CGG TAA GAG AGG CCG - 3') and HN8366(-) (5'- TTT ACC GGT TAC ATT TTT TCT TAA TCG AGG GAC TAT TGA C - 3') were used to amplify pBC, and the product contained deletions of 18 nt in F-HN IGS and 30 nt in HN-L IGS. The PCR product was digested with Mlu I and Age I, and then cloned into pBC.

All manipulated regions in the NDV cDNAs were sequenced to confirm the presence of desired mutations.

3.4.3 Recovery of recombinant NDV. Recombinant NDVs were recovered by co-transfection of each NDV cDNA mutant plasmid with support plasmids encoding NP, P and L proteins into HEp-2 cells. Simultaneously, HEp-2 cells were infected with recombinant vaccinia virus (MVA/T7), which is capable of synthesizing T7 RNA polymerase. Three or four days after transfection, the cell culture supernatant was used to recover the recombinant NDV by either passaging in DF-1 cells until a virus-specific cytopathic effect (CPE) appeared or injecting into the allantoic cavities of 9-day -old embryonated chicken eggs until the allantoic fluid showed HA positive (Krishnamurthy *et al*., 2000).

3.4.4 Reverse transcription PCR (RT-PCR) and sequence analysis of modified IGS. Total RNAs were isolated from the mutant NDVs infected allantoic fluid in the 9-day-old SPF chicken embryos using RNeasy Mini Kit (Qiagen) according to manufacturer's recommendations. Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen). The positive-sense primers used for RT reaction were F6202 (+) for F-HN IGS mutants; and HN/L (+) (5'-TCC GCG ACA CCA AGA ATC AAA C-3') for HN-L IGS mutants. For F-HN IGS mutants, the generated cDNA products were PCR amplified using primers, F 6202 (+) and HN 6414(-) (5'-CAT GAC TGA GGA CTG CTG-3'). For HN-L IGS mutants, the primers, HN/L (+) and HN/L (-), were used for the PCR amplification. The RT-PCR products spanning the F-HN and HN-L IGS regions were separated on 1% agarose gel, and the sequences were confirmed by sequencing.

3.4.5 Characterization of recombinant NDVs. The growth kinetics of recombinant mutant viruses was evaluated by multiple-step growth assay. DF1 cells in duplicate wells of six-well plates were infected with viruses at a multiplicity of infection (M.O.I.) of 0.01 plaque forming unit (PFU).After one hour adsorption, the cells were washed with DMEM and then covered with DMEM containing 5% FBS at 37^{o}C in 5% CO_2. Supernatant was collected and replaced with an equal volume of fresh medium at 8 h intervals until 64 h post infection. The titer of virus in the sample was quantified by plaque assay in DF1 cells. All plaque assays were performed in six-well plates. Briefly, monolayers of DF1 cells were infected with 0.2 ml of 10-fold diluted fresh virus. After 1 h adsorption, cells were covered with DMEM containing 2% FBS, 1% methylcellulose, and incubated at 37^{o}C in 5% CO_2. Four days later, the cells were fixed with methanol and stained with crystal violet. The average plaque diameter was calculated by measurement of ten plaques for each virus.

3.4.6 Northern blot hybridization. The total intracellular RNAs were isolated from virus-infected DF1 cells using RNeasy mini Kit (Qiagen). The RNAs were electrophoresed in denaturing 1.5% agarose gels containing 0.5 M formaldehyde, transferred onto a nitrocellulose membrane, and then hybridized with a P^{32} labeled double-stranded cDNA probe specific to NDV NP, F, HN or L gene. The bands corresponding to the individual NDV mRNAs were quantified by Fuji phosphor imager.

3.4.7 Pathogenicity Studies. The pathogenicity of the mutant viruses was determined by three different internationally accepted pathogenicity tests (Alexander 1989). These include mean death

time (MDT) test in 9-day-old embryonated chicken eggs, intracerebral pathogenicity index (ICPI) test in 1-day-old chicks and intravenous pathogenicity index (IVPI) test in 6-week-old chickens.

Briefly, for MDT, a series of 10-fold (10^{-6}-10^{-9}) dilutions of fresh infective allantoic fluid were made in sterile PBS, and 0.1 ml of each diluent was inoculated into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs (BEE eggs company, PA) and incubated at 37^{o}C. Each egg was examined 3 times daily for 7 days, and times of any embryo death were recorded. The minimum lethal dose is the highest virus dilution that causes all the embryos inoculated with the dilution to die. The MDT is the mean time in hours for the minimum lethal dose to kill all the inoculated embryos. The MDT has been used to classify NDV strains into the following groups: velogenic strains (taking less than 60 h to kill); mesogenic strains (taking 60 -90 h to kill); and lentogenic strains (taking more than 90 h to kill).

For ICPI, 0.05 ml (1:10 dilution) of fresh infective allantoic fluid of each virus was inoculated into groups of ten 1-day-old SPF chicks via the intracerebral route. The inoculation was done using a 27-gauge needle attached to a 1 ml stepper syringe dispenser that was set to dispense 0.05 ml of solution 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 8 h for a period of 8 days. At each observation, the birds were scored: 0 if normal, 1 if sick and 2 if dead. ICPI is the mean score per bird per observation over the 8-day period. Highly virulent (velogenic) viruses give values approaching 2, and avirulent (lentogenic) viruses give values close to 0.

For IVPI, 0.1 ml 1:10 dilution of fresh infective allantoic fluid of each virus was inoculated intravenously into groups of ten 6-week-old SPF chickens. The birds were observed for clinical symptoms and mortality once every 8 h for a period of 10 days. At each observation, the birds

were scored: 0 if normal, 1 if sick, 2 if paralyzed and 3 if dead. IVPI is the mean score per bird per observation over 10-day period. Highly virulent (velogenic) viruses give values approaching 3 and avirulent (lentogenic) viruses give values close to 0.

Each experiment had mock-inoculated controls that received a similar volume of sterile PBS by the respective routes. The MDT, ICPI and IVPI values were calculated as described by Alexander (Alexander, 1989).

3.5 Results

3.5.1 Construction of rNDVs with F-HN and HN-L IGS of different lengths. We manipulated the length of IGS between F and HN genes and HN and L genes, in a full-length NDV strain Beaudette C antigenomic cDNA clone, pBC. The reason for selecting the F-HN and HN-L IGS is that these two IGSs include 31 nt and 47 nt, respectively, but the other three IGSs (NP-P, P-M, and M-F) have only one nucleotide. We wanted to know why the IGS of F-HN and HN-L are longer than other IGSs of NDV, and what effect the IGS length has on expression level of mRNA transcription, virus replication and pathogenicity of the virus. We made a 30 nt deletion in F-HN IGS, 30 nt and 42 nt deletions in HN-L IGS, and a double deletion that included 18 nt deletion in F-HN IGS and 30 nt deletion in HN-L IGS. We also made 6 additional constructs in which 96 nt, 210 nt and 318 nt were added to F-HN and HN-L IGS. The numbers of deletion or addition nucleotides were adjusted so that the entire genome length of NDV obeyed the "Rule of Six" (Phillips *et al*., 1998). The schematic and details of the constructs are described in Fig.3.1 and Table 3.1, respectively.

The recombinant viruses were recovered by reverse genetics technique as described previously (Krishnamurthy *et al*., 2000). The sequences of modified IGS were confirmed by RT-PCR and sequencing. There was no noticeable difference in the recovery of recombinant viruses from different constructs.

3.5.2 The modified IGSs are stable in rNDVs. Mutant viruses were passed five times in 9-day-old embryonated chicken eggs to examine the stability of the modified IGS length. Allantoic fluid from each passage was collected and analyzed by RT-PCR using primers that flanked the F-HN and HN-L IGS. The lengths of PCR product from rNDV modified F-HN and HN-L IGS are shown on the agarose gel (Fig.3.2). The integrity of the RT-PCR product was confirmed by sequence analysis. Our results did not show any change in length and sequence of rNDVs IGS even after 5 passages, indicating that the modified F-HN and HN-L IGS were stable.

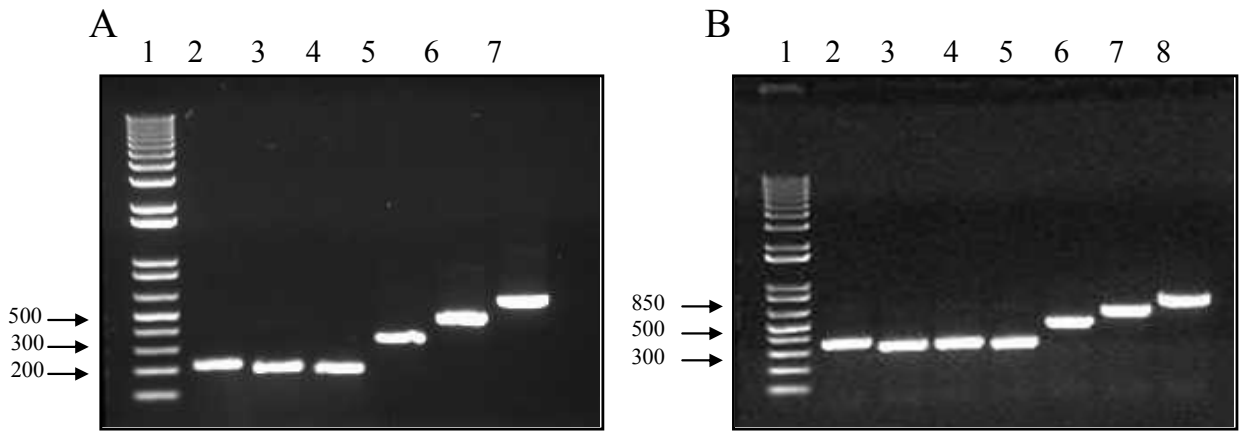


FIG.3.2 RT-PCR analysis of the F-HN and HN-L IGS from mutant NDVs.
Total RNAs isolated from allantoic fluid after the fifth passage of mutant NDVs in the 9-day-old SPF chicken embryos were analyzed by RT-PCR using primers spanning F-HN and HN-L IGS. The RT-PCR products were separated on 1% agarose gel. (A) RT-PCR of recombinant viruses for F-HN IGS. Lanes 1: 1kb DNA ladder; 2: rBC; 3: rBC-FHNΔ18HNLΔ30; 4: rBC-FHNΔ30; 5: rBC-FHN96; 6: rBC-FHN210; and 7: rBC-FHN318. (B) RT-PCR of recombinant viruses for HN-L IGS. Lanes 1: 1 kb DNA ladder; 2: rBC; 3: rBC-FHNΔ18HNLΔ30; 4: rBC-HNLΔ30; 5: rBC-HNLΔ42; 6: rBC-HNL96; 7: rBC-HNL210; and 8: rBC-HNL318.

3.5.3 Increased IGS length affects viral replication in vitro and results in reduced plaque size. Multi-step growth kinetics was performed in order to analyze the replication of rNDV *in vitro*. DF-1 cell monolayers were infected at an MOI of 0.01 PFU per cell, and supernatant samples were taken at 8 h intervals till 64 h post infection. Samples were then quantified by plaque assay. There was no significant difference in growth kinetics between the various mutant viruses and the parental virus rBC, with the exception that rBC-HNL318 showed slightly delayed growth kinetics (Fig.3.3).

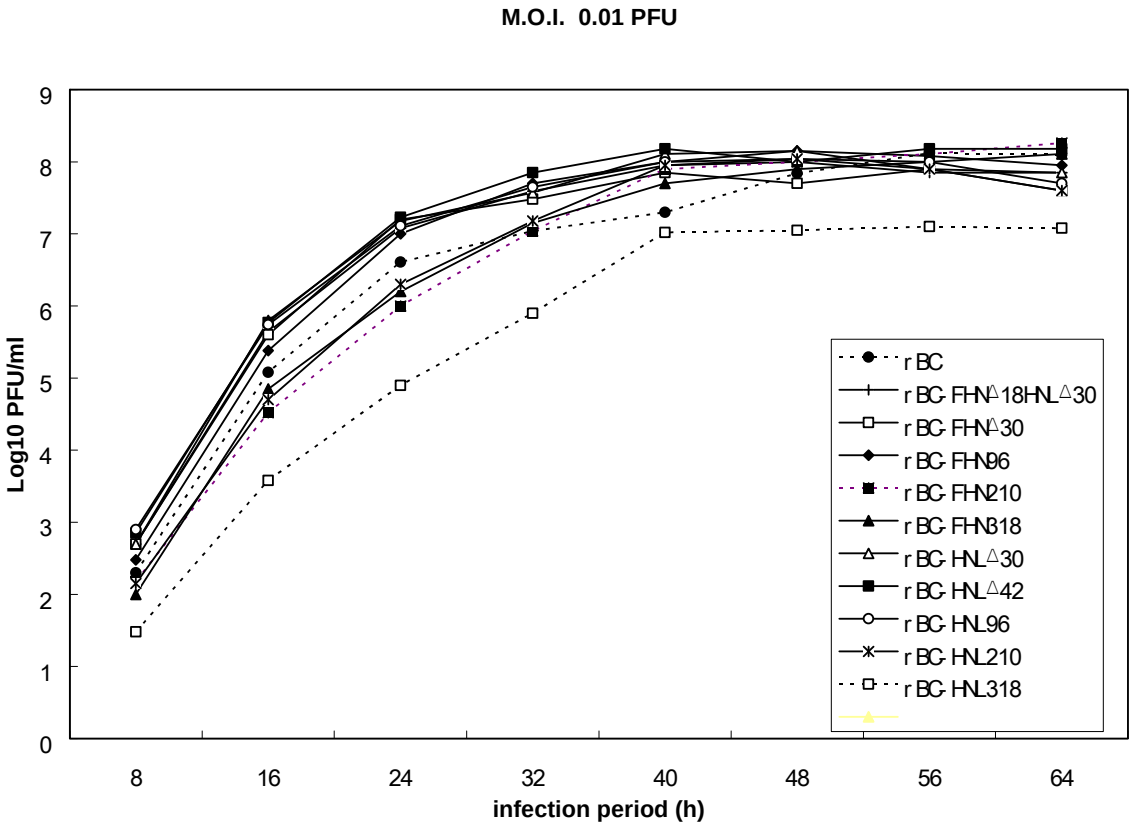


FIG.3.3 Kinetics of replication of mutant NDVs in DF-1 cells.

DF-1 cell monolayers were infected in duplicate with the indicated viruses at an M.O.I. of 0.01 PFU per cell for 1 h. The cells were washed with DMEM and then covered with DMEM containing 5% FBS at 37^0C in 5% of CO2. Aliquots of the supernatant medium were taken at 8 h intervals till 64 h post infection, replaced with an equal volume of fresh medium, flash-frozen, and analyzed in a single plaque assay.

The plaques formed by the various rNDVs in the DF-1 cell monolayer were visualized by staining with crystal violet (Fig.3.4). The plaque size was measured in several independent experiments. Viruses that had deletions either in F-HN or HN-L IGS were indistinguishable from rBC on the basis of plaque size. Interestingly, the double deletion virus, rBC-FHNΔ18HNLΔ30, produced slightly larger size of plaques than those of the parental rBC. The viruses, with the addition of 96 nt or more in either F-HN or HN-L IGS, produced progressively smaller size of plaques compared to those of rBC. The average sizes of ten plaques of various rNDVs in a typical experiment were as follows: rBC ,1.48 mm, (standard deviation, SD, 0.19); rBC-FHNΔ18HNLΔ30, 1.54 mm (SD 0.16); rBC-FHNΔ30, 1.44 mm (SD 0.24); rBC-FHN96, 1.25 mm (SD 0.24); rBC-FHN210, 0.96 mm (SD 0.19); rBC-FHN318, 0.68 mm (SD 0.21); rBC-HNLΔ30, 1.41 mm (SD 0.23); rBC-HNLΔ42, 1.40 mm (SD 0.24); rBC-HNL96, 1.19 mm (SD 0.26); rBC-HNL210, 0.93mm (SD 0.08); rBC-HNL318, 0.66 mm (SD 0.12).

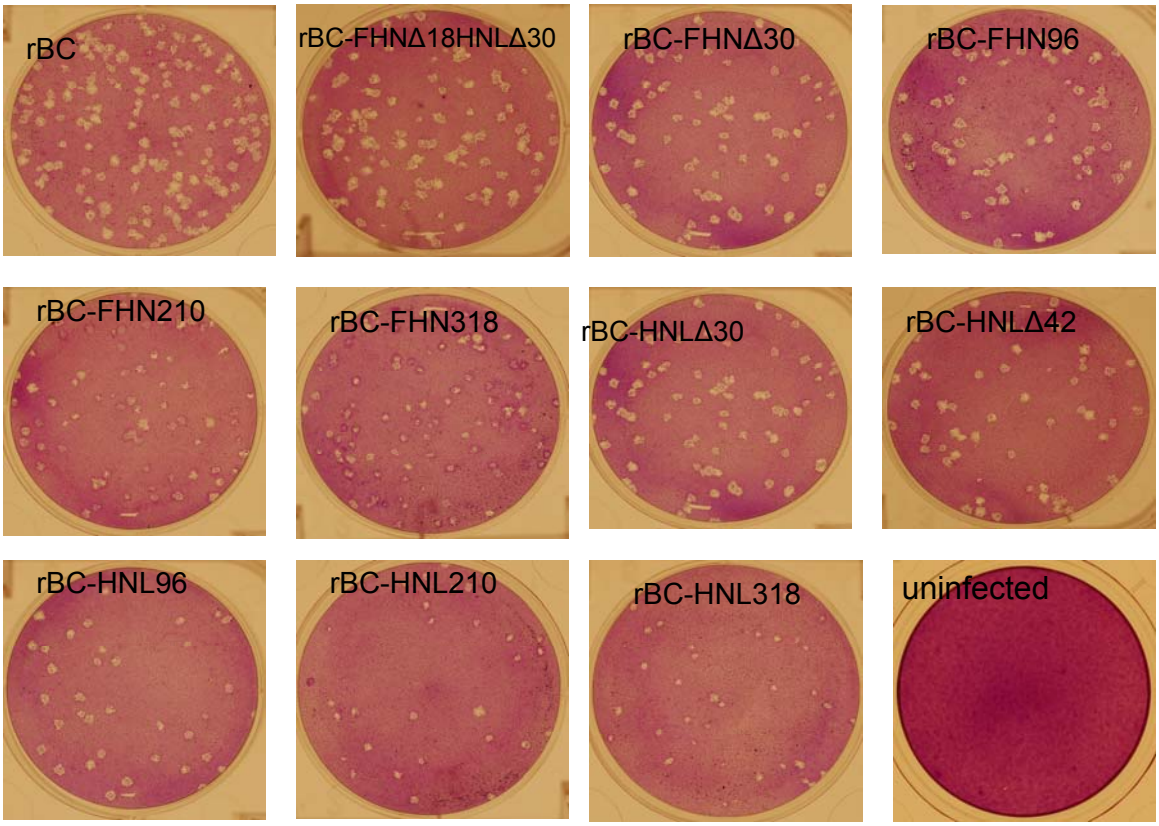


FIG.3.4 Plaque morphology of mutant NDVs containing increased or decreased length of IGS in F-HN and HN-L gene junctions.

Recovered viruses were titrated in duplicate in 6-well plates. Supernatant collected from virus-inoculated samples was serially diluted, and 0.2 ml of each diluent was added into confluent DF1 cells per well. After 1 h of adsorption, cells were overlaid with DMEM containing 2% FBS and 1% methylcellulose, and incubated at 37^0C for 4 days. The cells were then fixed with methanol and stained with crystal violet.

3.5.4 Transcription of the downstream gene is down-regulated by increased IGS. Transcription of the upstream or downstream gene of the recombinant viruses was analyzed by Northern blot hybridization. DF-1 cells were infected with viruses at an MOI of 5 PFU per cell and harvested at 24h post infection. The total intracellular RNA was isolated and analyzed by Northern blot hybridization with a double-stranded radiolabeled cDNA probe specific to NP, F, HN or L gene. The results are shown in Fig.5 and accumulation of transcripts of the downstream and upstream genes was quantified by Fuji Phosphor Imager. F-HN IGS mutants, rBC-FHN210 and rBC-FHN318, showed decreased transcription of the downstream gene, HN (panel A, lanes 5and 6); but no significant change in the transcription level of the upstream gene, F (panel C, lane 5 and 6). Similarly, HN-L IGS mutants, rBC-HNL210 and rBC-HNL318, showed decreased transcription of the downstream gene, L (panel B, lanes 10 and 11); but no significant change in the transcription level of the upstream gene, HN (panel A, lanes 10 and 11). These results indicated that extending IGS length probably affected initiation of downstream gene transcription, but probably had no effect on the transcription of the immediate upstream gene. In addition, there was no difference in the level of NP gene expression between the viruses, indicating that increasing IGS length has no effect on upstream gene transcription. Our studies also indicated that increased the length of F-HN IGS decreased the level of F-HN dicistronic read-through mRNA production. However, we were unable to clearly quantify HN-L dicistronic mRNA level in the gel due to its high molecular weight.

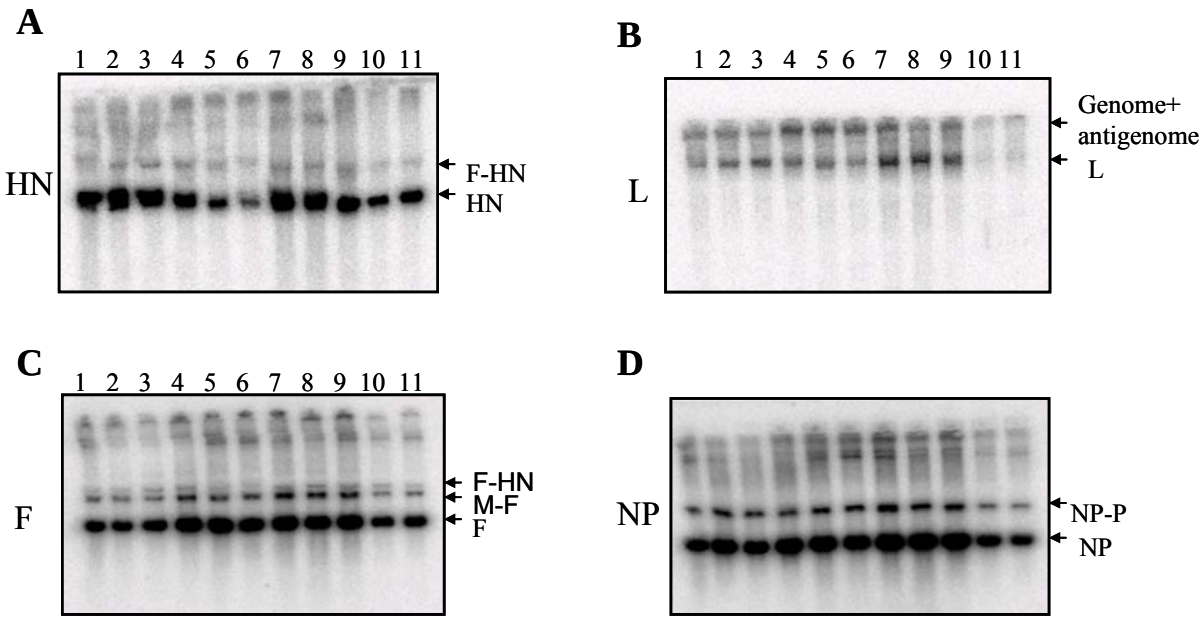


FIG.3.5 Northern blot analysis of RNAs synthesized by mutant NDVs bearing modified F-HN and HN-L IGS.

(A-D) DF-1 cells were infected with the indicated virus (M.O.I. of 5 PFU), incubated for 24 h, and harvested; and then total intracellular RNA was extracted. The RNA was separated by electrophoresis in formaldehyde agarose gel, transferred onto a nitrocellulose membrane, and hybridized with a P^{32} labeled double-stranded cDNA probe specific to the HN, L, F, or NP gene, as indicated. Positions of the specific gene, read-through mRNA, genomic, and antigenomic RNAs are indicated. Lanes: 1, rBC ; 2, rBC- FHNΔ18HNLΔ30; 3, rBC- FHNΔ30; 4, rBC-FHN96; 5, rBC-FHN210; 6, rBC- FHN318 ; 7, rBC-HNLΔ30; 8, rBC- HNLΔ42; 9, rBC-HNL96; 10, rBC- HNL210; and 11, rBC- HNL318.

3.5.5 Pathogenicity studies of rNDVs containing decreased or increased F-HN and HN-L IGS lengths. We wanted to determine the effect of increased or decreased IGS length on pathogenicity of the recombinant viruses. At present, a definitive assessment of NDV virulence is based on the following *in vivo* tests: MDT in embryonated SPF chicken eggs, ICPI test in 1-day-old chicks, and IVPI test in 6-week-old chickens. We examined the virulence of parental rNDV and rNDVs containing modified IGS length by all these tests (Table 3.2).

TABLE 3.2. Pathogenicity of mutant viruses in chicken embryos, chicks and chickens

Virus	MDT (h)[a]	ICPI[b]	IVPI[c]
rBC	60	1.49	2.06
rBC-FHNΔ18HNLΔ30	60	1.28	0.72
rBC-FHNΔ30	58	1.36	1.04
rBC-FHN96	59	1.44	0.34
rBC-FHN210	60	1.31	0.20
rBC-FHN318	60	1.06	0.14
rBC-HNLΔ30	64	1.45	0.99
rBC-HNLΔ42	59	1.44	0.44
rBC-HNL96	58	1.11	0.54
rBC-HNL210	60	0.61	0.00
rBC-HNL318	68	0.47	0.00

[a] Mean death time in 9-day-old embryonated chicken eggs (h)

(>90: lentogen; 60-90: mesogen; <60: velogen; Alexander, 1989).

[b]Intracerebral pathogenicity index in 1-day-old chicks

(0.0-0.5: lentogen; 1.0-1.5: mesogen; 1.5-2.0: velogen; Alexander, 1989).

[c]Intravenous pathogenicity index in 6-week-old chickens

(0.0: lentogen; 0.0-0.5: mesogen; 2.0-3.0: velogen; Alexander, 1989).

The MDT test results showed that the parental rBC took 60 h to cause embryo mortality. All recombinant viruses showed MDT similar to that of rBC, except rBC-HNLΔ30 and rBC-HNL318, which took 4 h and 8 h longer, respectively, in causing the death of embryos (Table 3.2).

The ICPI value of parental rBC was 1.49 (out of a maximum 2.00) (Table 3.2). The ICPI values of rNDVs containing decreased IGS length (rBC-FHNΔ30, rBC-HNLΔ30, and rBC-HNLΔ42) were similar to that of the parental rBC. However, the ICPI value of rBC-FHNΔ18HNLΔ30 was 1.28, which was lower than that of the parental rBC. Interestingly, the ICPI values of recombinant viruses decreased as the length of the IGS increased. In the case of increased F-HN IGS length, the ICPI values for rBC-FHN96, rBC-FHN210, and rBC-FHN318 were 1.44, 1.31, and 1.06, respectively. In the case of increased HN-L IGS length, the ICPI values of rBC-HNL96, rBC-HNL210, and rBC-HNL318 were 1.11, 0.61 and 0.47, respectively. These results indicated that increased HN-L IGS length reduces the pathogenicity of rNDV more than that of viruses with the increased F-HN IGS length. To determine the amount of each recombinant virus required to cause the death of 1-day-old chicks by intracerebral route, the brain tissues from the dead chicks were plaque assayed in DF-1 cells. Our results showed that all recombinant NDVs grew to similar titer (10^4-10^5 PFU/gm brain tissue) in the brain at the time of chick death, but the time to cause the death was longer in viruses with increased IGS length. The viruses recovered from the brain tissues of dead chicks were sequenced to determine the stability of the modified IGS. Our results showed that the modified IGSs in rNDVs were stable after their growth in the chick brain.

The results obtained by IVPI tests in 6-week-old chickens for the mutant viruses were quite surprising (Table 3.2). The parental rBC virus had an IVPI value of 2.06 (out of a maximum

of 3.00), but the IVPI values of all mutant viruses were much lower (ranging from 0.00 to 1.04), indicating that both decreased and increased IGS length reduced the virulence of rNDVs. All rNDVs containing decreased IGS length showed reduced virulence. The IVPI values for rBC-FHNΔ18HNLΔ30, rBC-FHNΔ30, rBC-HNLΔ30, and rBC-HNLΔ42 were 0.72, 1.04, 0.99, and 0.44, respectively. Again interestingly, the IVPI values of rNDVs decreased as the length of the IGS increased. In the case of increased F-HN IGS length mutants, the IVPI values for rBC-FHN96, rBC-FHN210 and rBC-FHN318 were 0.34, 0.20 and 0.14, respectively. In the case of increased HN-L IGS length mutants, the IVPI values for rBC-HNL96, rBC-HNL210 and rBC-HNL318 were 0.54, 0.00 and 0.00, respectively. These results confirmed that increased HN-L IGS length reduced the pathogenicity of rNDVs in adult chickens more than that of the viruses with increased F-HN IGS length. Furthermore, our results showed that the IVPI test is more sensitive than the ICPI test in determining the pathogenicity of NDV strains. MDT test is the least sensitive test to assess pathogenicity of NDV.

3.6 Discussion

The length and sequence of IGSs vary among the member of *Mononegavirales*. In some viruses, such as VSV, Sendai virus and measles virus, the IGSs are conserved dinucleotide or a trinucleotide, whereas in some other viruses, such as NDV, RSV and SV5, the IGS vary in length and in sequence composition. Previous studies have indicated that in virus with conserved IGS sequences, the IGS plays an important role in termination of upstream mRNA transcription and initiation of downstream mRNA synthesis (Barr *et al*., 1997; Stillman and Whitt, 1998; Tordo *et al*., 1986). On the contrary, in some other virus, such as RSV, IGS has little effect on either upstream or downstream mRNA transcription (Bukreyev *et al*., 2000; Kuo *et al*., 1996). In NDV,

the first three 3'-proximal IGSs are single nucleotide, whereas, the last two 5'-proximal F-HN and HN-L IGSs are non-conserved 31 and 47 nt, respectively. In this study, we analyzed the effect of decreasing or increasing length of F-HN or HN-L IGS on mRNA transcription and on the eventual pathogenicity of the virus in its natural host, chicken.

Increasing either the F-HN or HN-L IGS had no significant effect during multi-step growth kinetics of the mutant NDVs in cell culture except rBC-HNL318 virus showed decreased growth kinetics. However, the sizes of plaque varied among the mutant viruses. Based on plaque size, the level of attenuation of *in vitro* growth increased as length of IGS increased. Our research confirmed previous observation with the RSV IGS study that plaque size was remarkably sensitive to small changes in IGS length (Bukreyev *et al*., 2000). It is unknown whether the effect of increased *in vitro* attenuation was due to the effect of increased genome length of the recombinant NDV or due to alteration of mRNA transcription level resulting from the increased IGS length.

Decreasing either the F-HN or HN-L IGS had no discernible effect during multi-step growth kinetics of mutant NDVs in cell culture. However, when the plaque size of IGS deletion viruses were compared, we found that the plaque sizes of single IGS deletion viruses were similar or slightly smaller than that of the parental virus. This result indicated that the decreased plaque size observed with viruses of increased or decreased IGS length were probably not due to the effect of simple increase in genome length but could be due to the alteration of mRNA transcription level. Surprisingly, the plaque size of the double IGS deletion virus rBC-FHNΔ18HNLΔ30 was increased. Although it is unclear why the plaque size was increased, it is possible that the augmentation effect was due to ratio of mRNAs produced as a result of double 5'-proximal IGS deletions.

In the present study, we demonstrated that NDV can tolerate an IGS length of at least 365 nt. The IGS length was stable after five passages in chicken and chicken embryos. Among the members of *Mononegavirales*, the IGSs of Ebola virus vary from 3 to 143 nt (Sanchez *et al*., 1993), Rabies virus has a 423 nt long G-L intergenic region which is thought to be a pseudogene (Tordo *et al*., 1986), and human metapneumovirus IGSs vary from 2 to 126 nt (Ishiguro *et al*., 2004). Recombinant RSV was shown to tolerate an IGS length of 160 nt (Bukreyev, 2000). Therefore, to our knowledge this is one of the largest IGS in *Mononagavirales*.

During virus recovery studies, we came across an interesting observation. When the F-HN IGS was replaced with random non-viral sequences we were unable to recover viable NDV even after five attempts. In all those attempts the parental virus and the viruses, in which F-HN or HN-L IGS were replaced with avian metapneumovirus sequences, were recovered. Although these experiments need to be repeated with additional viral and non-viral random sequences, our research indicated that certain non-viral sequences can not be used to replace NDV IGS. It is possible that the NDV polymerase recognizes sequences from a distantly related virus but fails to recognize randomly generated non-viral sequences.

Our observations of diminution of plaque size with either increased or decreased IGS length indicated that it was probably not due to the result of change in genome length but associated with a change in the efficiency of sequential transcription. As expected, our Northern blot analysis showed that increased F-HN or HN-L IGS length resulted in decreased transcription of the downstream gene. We were unable to detect any significant effect on the transcription of the upstream gene. Our results were consistent with the study of hPIV3 (Skiadopoulos *et al*., 2000). hPIV3 has a conserved trinucleotide GAA or GCG as IGS. Studies showed that insertion of an additional nucleotide into the IGS can decrease the efficiency of transcription of

downstream gene (Skiadopoulos *et al*., 2000). On the contrary, for RSV in which IGSs vary in length and in sequence composition, studies with minigenome and infectious recombinant virus showed that RSV IGS did not modulate transcription (Bukreyev *et al*., 2000; Kuo *et al*., 1996).

The pathogenicity studies showed that all IGS mutants were attenuated by ICPI and IVPI tests. The results showed that the level of attenuation increased as the length of IGS increased. For example, rBC HNL210 and rBC-HNL318 were completely attenuated by the IVPI test. These results are in agreement with the studies of RSV, where increasing IGS length affected viral pathogenicity in mice (Kuo *et al*., 1996). Interestingly, our results showed that decreasing the length of IGS also affected viral pathogenicity. These findings suggested that either increasing or decreasing the length of IGS alters the level of downstream gene transcription, which changes the ratios of viral transcripts, and probably these ratios are very important for virus growth and pathogenicity. It is possible that the most effective ratios of transcripts in NDV are obtained by having 1 nt in the first three 3' end IGS and 31 and 47nt in the last two IGS. Therefore, NDV has maintained these IGS lengths over the years without any changes. We also observed that the HN-L IGS was more amenable to attenuation than the F-HN IGS. This result indicated that the level of L protein is probably more important for virus growth and any minor change in its level can have a dramatic effect on virus growth and pathogenicity.

Previously, it has been suggested that the ability of NDV strains to produce large size plaques is related to virulence for chicken (Reeve and Poste, 1971). Seemingly, our data differ from this suggestion in that the double IGS deletion virus rBC-FHNΔ18HNLΔ30 produced a slightly larger size plaque compared to that of the parental rBC virus, despite its attenuation in chickens. Therefore, plaque size may not always be a correct indicator of NDV virulence.

Our pathogenicity studies showed that the IVPI test was more sensitive than the ICPI test which was in turn more sensitive than the MDT test to assess the pathogenicity of NDV IGS mutants. These results indicated that the immune system of the host also plays an important role in the pathogenicity of NDV. The virus has to overcome a mature immune system in a 6-week-old chicken in the IVPI test than a less developed immune system in a 1-day-old chick in the ICPI test or in a 9-day-old embryo in the MDT test. Therefore, any minor difference in the pathogenicity of a NDV mutant could be amplified in the IVPI test than in the ICPI or the MDT test.

The results presented in this paper can have significant applications in the development of live attenuated NDV vaccines. One of the major problems encountered in the current live attenuated NDV vaccines is the undesirable pathogenicity of the vaccine virus. Since field strains of low virulence are currently used as NDV vaccines, the pathogenicity of these strains can not be changed. There is a great need to develop a live NDV vaccine that is immunogenic but less pathogenic. Our research showed a new method to attenuate NDV strains. We hope that by changing the length of IGS the pathogenicity of an NDV strain can be adjusted to the desired level without affecting the immunogenicity of the vaccine virus, which will greatly benefit the poultry industry throughout the world.

Chapter 4

4.1 Title

The untranslated regions of the Newcastle disease virus hemagglutinin neuraminidase gene play essential roles in virus replication and pathogenesis

4.2 Abstract

Newcastle disease virus (NDV) has a non-segmented negative-sense RNA genome consisting of six genes. Each gene is flanked by a conserved transcriptional control sequence, gene start (GS) and gene end (GE). Between the GS and open reading frame (ORF) and between the ORF and GE, there are untranslated regions (UTRs). To investigate the role of the UTRs of Hemagglutinin Neuraminidase (HN) gene in replication and pathogenesis, several NDVs with deletions in the 5' and 3' UTRs of HN gene were generated. The levels of HN mRNA transcription, HN protein expression in infected cells, and HN protein incorporation into viral particles were examined. Pathogenicity of the recombinant NDVs was evaluated in chickens. Our results showed that the UTRs of HN did not noticeably affect *in vitro* replication of recombinant viruses. Complete deletions of 5' or 3' UTRs of HN gene decreased accumulations of HN mRNA in infected cells; whereas, complete deletions of HN 5' UTR affected translation of HN protein and incorporation of HN protein into virus particles. In addition, complete deletion of 5' UTR of HN mRNA attenuated the pathogenicity of NDV mutants in 1-day-old chicks and 2-week-old chickens. These results suggest that the UTRs of NDV genes may not be absolutely required for replication *in vitro* but are essential for replication and pathogenicity *in vivo*.

Replacement of the HN 5' and 3'UTRs with the corresponding UTRs of NP gene showed that the functions of HN UTRs could be fulfilled by the NP UTRs, indicating that the requirement of UTR sequence is nonstringent.

4.3 Introduction

Newcastle disease virus (NDV) causes a highly-contagious respiratory and neurological disease in chickens (Taylor, 1964). The disease is prevalent worldwide and causes the great economic loss to poultry farmers. All known strains of the virus are of a single serotype, but they differ widely in virulence. Strains of NDV are classified into three pathotypes depending on the severity of disease produced in chickens. Lentogenic strains do not cause disease and are termed, "avirulent", viruses of intermediate virulence are termed, "mesogenic", while virulent strains that cause high mortality are termed, "velogenic" (Alexander, 1989).

NDV is a member of the genus Avulavirus in the family Paramyxoviridae. The genome of NDV is a nonsegmented, single-stranded, negative-sense RNA of 15,186 nucleotides (nt) (Krishnamurthy *et al*., 2000). The genome contains six genes (3'-NP-P-M-F-HN-L -5'), which encode six structural proteins: nucleoprotein (NP), phosphate protein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) (de Leeuw and Peeters, 1999). Two additional proteins, V and W, are produced from the P gene by RNA editing (Steward *et al*., 1993). Like other paramyxoviruses, the entire length of the genome and antigenome of NDV are always tightly bound by the NP protein (Lamb and Parks, 2007). The genome of NDV is flanked by 3' leader and 5'trailer regions, which are cis-acting regulatory elements involved in transcription, replication, and packaging of the genomic and antigenomic RNAs. Between each gene boundary, there are intergenic sequences (IGS), which

vary in length and sequence among genes. The IGS are not copied into mRNAs. Each gene is flanked by conserved transcriptional initiation and termination control sequences, known as gene start (GS) and gene end (GE) signals, respectively (Lamb and Parks, 2007).

The mechanism of transcription and replication of NDV is similar to those of other paramyxoviruses (Lamb and Parks, 2007). Transcription begins at a single promoter at the 3' leader end and the genes are copied in a sequential manner into individual mRNAs by a start-stop mechanism guided by GS and GE signals. Genome replication occurs by synthesis of complementary positive-sense copies of the genome called antigenomes, which serve as templates for synthesis of progeny genomes.

In paramyxoviruses, mRNAs generally contain open reading frames (ORFs) which are flanked by 5' and 3' untranslated regions (UTRs). These UTRs are neither conserved in sequence nor in length among different mRNAs. Very little is known about the functions of these UTRs in transcription, translation and pathogenesis. In Measles virus (MeV), the M and F mRNAs have unusually long 3' and 5' UTRs, respectively (Barrett *et al*., 1991). These long UTRs occupy as much as 6.4% of the virus genome. It has been known that these long UTRs in MeV are not essential for viral replication, but they play an important role in replication and pathogenicity of the virus. The long 3'UTR of M mRNA increases its translation, promoting virus replication, whereas, the long 5' UTR of F mRNA inhibits the F protein production thus inhibiting virus replication and greatly reducing cytopathogenicity (Cathomen *et al*., 1995; Takeda *et al*., 2005). However, the functions of normal paramyxovirus 5' and 3' UTRs in the context of virus replication and pathogenicity remain unknown.

In this study, we have analyzed the roles of 5' and 3' UTRs of the HN mRNA of NDV in replication and pathogenicity. We chose the HN mRNA because the HN protein is not only

involved in important biological functions in the virus life cycle, but also in inducing a high level of NDV-specific neutralizing antibody in chickens. In NDV mesogenic strain Beaudette C, the 5' and 3' UTRs of HN mRNA are 81 and 166 nt, respectively. In order to investigate the functions of these UTRs, we have used a reverse genetics technique to generate a series of recombinant NDVs carrying deletions in the 5' and 3' UTRs of the HN mRNA. We have also generated a recombinant NDV in which both the 5' and 3' UTRs of HN mRNA were replaced by the corresponding UTRs of the NP mRNA. The effects of these alterations in viral transcription, translation, replication and pathogenesis were examined. Our results showed that mutation of the UTRs affected transcription and translation of HN protein, leading to decreased incorporation of HN protein into virus particles and reduced pathogenicity.

4.4 Materials and Methods

4.4.1 Cells and viruses. DF1 cells (chicken embryo fibroblast cell line) were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM with 2% FBS. HEp2 cells (human epidermoid carcinoma cell line) were grown in Eagle's minimal essential medium (MEM) containing 10% FBS and maintained in MEM with 2% FBS. A moderately-pathogenic (mesogenic) NDV strain Beaudette C (BC) and other recombinant viruses generated from BC virus were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The modified vaccinia virus strain Ankara that expresses the T7 RNA polymerase (a generous gift from Dr. Bernard Moss, National Institutes of Health) was grown in primary chicken fibroblast (CEF) cells in DMEM with 10% FBS.

4.4.2 Construction of NDV cDNAs with altered 5' and 3' UTRs in the HN gene. Full- length antigenome cDNA of NDV strain BC was cloned into plasmid pBR322 and designated pBC (Krishnamurthy *et al*. 2000). The 5' and 3' UTRs of HN gene in pBC were modified. All constructs were confirmed by dideoxynucleotide sequencing. The mutant UTRs included deletion of 6 or 78 nt in 5' UTR; deletion of 6, 24, or 162 nt in 3' UTR; double deletions of 78 nt in 5' UTR and 162 nt in 3' UTR; and replacement of HN 5' and 3' UTRs with the corresponding UTRs of NP mRNA (Fig.4.1). To construct pBC-5UTRΔ1-6, forward primer HNGS1F (+) (5'-CT**A CGC GT**T GTA GAT GAC CAA AGG ACG ATA TAC GGG TAG AAG AGA GGC CGC CCC TCA ATT -3'), which contains Mlu I site and 6 nt deletions at the 5'UTR of HN, and the reverse primer Age I (-) (5'- TT**A CCG GT**A GCT GTT TTG CCT TGT ATC TCA TTG CCA -3'), which contains Age I site, were used to amplify the desired fragment on the template full-length pBC. The PCR product was digested with Mlu I and Age I, and then inserted into pBC.

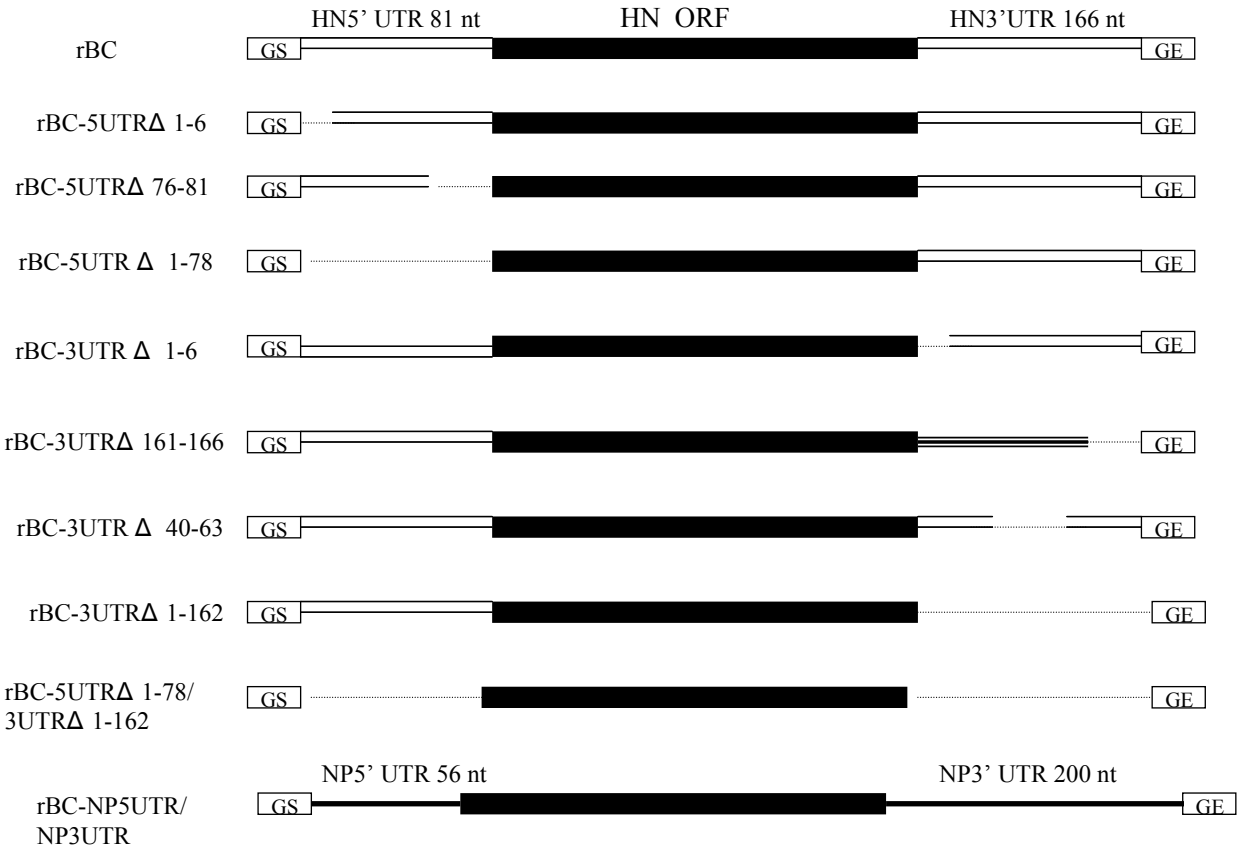


FIG.4.1 Constructs of recombinant viruses containing deletions in the 5' and 3' UTRs of HN mRNA.

Solid bars represent HN mRNA of rBC; double lines represent 5' and 3' UTR; thin square dots represent deletions of UTR; and the single lines represent 5' and 3' UTR of NP mRNA. The lengths of HN or NP UTR are marked on the top of the bars. Briefly, viruses rBC-5UTRΔ1-6, rBC-5UTRΔ76-81, and rBC-5UTRΔ1-78 include 1st to 6th nucleotide deletions, 76th to 81st nucleotide deletions, and 1st to 78th nucleotide deletions from the 5'UTR of HN MRNA,

respectively. Viruses rBC-3UTRΔ1-6, rBC-3UTRΔ40-63, rBC-3UTRΔ161-166, and rBC-3UTRΔ1-162 include 1st to 6th, 40th to 63rd, 161st to 166th, and 1st to 162nd nucleotide deletions in the 3' UTR of HN mRNA, respectively. The virus rBC-5UTRΔ1-78/3UTRΔ1-162 includes 1st to 78th nucleotide in 5'UTR of HN and 1st to 162nd nucleotides in 3' UTR of HN mRNA deletions. The virus rBC-NP5UTR/NP3UTR designate that the 5'and 3' HN UTRs were replaced by the NP 5' and 3' UTRs.

TABLE 4.1 Primers used to construct pBC-5UTRΔ76-81, pBC-3UTRΔ1-6, pBC-3UTRΔ161-166 and pBC-3UTRΔ40-63

Plasmids	**Primers**
pBC-5UTRΔ 76-81	HNGS2F(+) (5'-ATG GAC CGC GCA GTT AGC CGA GTT GCG TTA GAG -3') HNGS2R(-) (5' - GGA CTG CTG TCG GTG AAG CGG TAG AAC GGA GGT - 3')
pBC-3UTRΔ 1-6	HNGE1F(+) (5' - CAA CTA TGA AAG AGC TGG GAA GAT GGC ATT GTA -3') HNGE1R(-) (5'- CTA ACC AGA CCT GGC TTC TCT AAC CCC ATC -3')
pBC-3UTRΔ 161-166	HNGE2F(+) (5'- ATT AAG AAA AAA TGT AAG TGG CAA TGA GAT ACA AGG CAA AAC -3') HNGE2R(-) (5'- ACT ATT GAC AAG ACT TGA TCT GAT CGC ATG AGC -3')
pBC-3UTRΔ 40-63	HNGE3F(+) (5'- AAT CAA ACT GAA TGC CGG TGC GAG CTC GAA TTC -3') HNGE3R(-) (5'- TAC AAT GCC ATC TTC CCA GCT CTT TCA TAG -3')

To construct pBC-5UTRΔ1-78, forward primer HNGS3F(+) (5’ - CT**A CGC GT**T GTA GAT GAC CAA AGG ACG ATA TAC GGG TAG AAG TCA TGG ACC GCG CAG TTA - 3’), which contains Mlu I site and 78 nt deletions at 5’UTR of HN, together with the reverse primer Age I (-), was used to amplify the desired fragment on the full-length pBC. The PCR product was digested with Mlu I and Age I, and then inserted into pBC.

To construct pBC-5UTRΔ76-81, pBC-3UTRΔ1-6, pBC-3UTRΔ161-166 and pBC-3UTRΔ40-63, plasmid pGEM-HN was used as a PCR template. Forward and reverse primers were designed to face away from each other and space the required distance apart relative to the template (Table 4.1). The primers were phosphorylated with T4 polynucleotide kinase (New England, BioLabs), and the plasmid pGEM-HN was then amplified with platinum Pfx DNA polymerase (Invitrogen). The PCR products were cloned into plasmid pGEM-HN and sequenced to their entirety. Cloned HN genes were digested with Mlu I and Age I, and then inserted into full-length pBC.

To construct pBC-3UTRΔ1-162, forward primer F6202(+) (5’- GTG AAC ACA GAT GAG GAA CC -3’), together with reverse primer HNGE4R(-) (5’- TAT TT**A CCG GT**A GCT GTT TTG CCT TGT ATC TCA TTG CCA CTT ACA TTT TTT CTT AAT CGA CTA ACC -3’), which contains Age I site and a deletion of 162 nt at 3’ UTR of HN, was used. The PCR product was digested with Mlu I and Age I, and then inserted into pBC.

To construct pBC-5UTRΔ1-78/3UTRΔ1-162, forward primer HNGS3F(+), which contains Mlu I site and a deletion of 78 nt at 5’UTR , together with reverse primer HNGE4R(-) which contains Age I site and a deletion of 162 nt at 3’ UTR , was used to amplify the desired fragment

on the template pBC-5UTRΔ1-78. The PCR product was digested with Mlu I and Age I, and then cloned into pBC.

For rBC-NP5UTR/3UTR, both the 5' and 3' UTRs of HN were substituted with NP 5' and 3' UTRs. Briefly, replacement of 3'UTR of HN gene with 3' UTR of NP was performed by PCR on the template pBC with the two primers, F- spe-NP3UTR (5'-TTA CTA GTT GAG ATC CTC AAA AAT GAT GGG GTT AGA GAA GCC AGG TCT GGT TAG TTG ACA AAA CCC AGC TTG CTT CCA CAA AAT CAT CCC AAT ATC CTC ACC CGT AGT CGA CCC CTC GA-3') and R-age-NP3UTR (5'-TTA CCG GTA GCT GTT TTG CCT TGT ATC TCA TTG CCA CTT ACA TTT TTT CTT AAT TTC TCC TCG GCT CTG TTT TGA TTG TTA GTG AGC CGC ATT GTG CCT GTG GTA CCT AGG GCG TGC G-3'). The PCR product was digested with Spe I and Age I, and then cloned into pBC, and named pBC-NP3UTR. Replacement of HN 5UTR with NP 5 UTR was performed by PCR on the template pBC-NP3UTR with two primers: F-mlu-NP5UTR (5'-CTA CGC GTT GTA GAT GAC CAA AGG ACG ATA TAC GGG TAG AAG GTT GTG AAT CTC GAG TGC GAG CCC GAA GCA CAA ACT CGA GAA AGC CTT CTG CCA ACA TGG ACC GCG CAG TTA GCC GAG TTG CGT TAG AGA ATG ATG AAA GAG AGG CAA AAA ATA CA-3') and Age I (-). The PCR product containing both NP 5' and 3' UTRs was then digested with Mlu I and Age I, then cloned into pBC, and named pBC-NP5UTR/NP3UTR. All manipulated regions in the NDV cDNAs were sequenced to confirm the presence of the desired mutations.

4.4.3 Recovery of recombinant NDVs. Recombinant NDVs (rNDVs) were recovered by co-transfection of each mutant full-length plasmid along with support plasmids encoding NP, P, and L proteins into HEp2 cells. Simultaneously, HEp2 cells were infected with recombinant vaccinia

virus (MVA/T7) capable of synthesizing T7 RNA polymerase. Three days after transfection, the cell culture supernatant was used to recover the rNDV by either passage in DF1 cells or injection into the allantoic cavities of 9-day-old embryonated chicken eggs until the allantoic fluid became hemagglutination (HA) positive (Krishnamurthy *et al*., 2000).

4.4.4 Sequence analysis of modified HN gene UTRs in recombinant NDVs. Total RNAs were isolated 24 h post-infection from virus-infected DF1 cells, using RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendation. Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen). The positive-sense primer for RT reaction was F6202(+). The cDNA products were amplified by PCR, using F6202(+) as a positive-sense primer and a negative-sense primer Xba (-) (5'- AGT ACT CCG GTT ATT CTA GAA TTG TGG TTG -3'). The modifications in 3' and 5' UTRs of HN gene were confirmed by sequencing of the RT-PCR products.

4.4.5 Characterization of recombinant NDVs. The growth kinetics of recombinant mutant viruses was evaluated by multiple-step growth assay. DF1 cells in duplicate wells of six-well plates were infected with viruses at a multiplicity of infection (M.O.I.) of 0.01. The infected cells were maintained with DMEM containing 2% FBS at 37^0C in 5% CO_2. Supernatant samples were collected and replaced with an equal volume of fresh medium at 8 h intervals until 64 h post-infection. The titer of virus in the collected samples was determined by plaque assay. Briefly, monolayers of DF1 cells in six-well plates were infected with 0.1 ml of 10-fold diluted virus. One hour after infection, cells were covered with 1% methylcellulose and incubated at 37^0C in 5%

CO_2. Four days later, the cells were fixed with methanol and stained with crystal violet (Krishnamurthy *et al*., 2000).

4.4.6 Hemagglutination and Neuraminidase assay. Furthermore, the hemagglutination (HA) and neuraminidase (NA) activities of the parental and mutant viruses were compared by HA and NA assays following the standard protocols (Alexander, 1989; Huang *et al*., 2004).

Hemagglutination assay (HA) was performed with 1% (v/v) chicken erythrocytes (RBCs) according to the standard protocol (Alexander, 1989). Briefly, a serial two fold dilutions of 0.05 ml of virus suspension in PBS were made in a plastic V-bottom 96-well plate. Then 0.05 ml of 1% (v/v) chicken RBCs is dispensed to each well and mix well. The plate was incubated on ice for 30 min. HA titer was determined by as the highest serum dilution giving complete red cells agglutination.

A fluorescence based neuraminidase (NA) assay was done as described previously (Huang *et al*., 2004a). Briefly, a serial two fold dilutions of 0.05 ml of virus samples were prepared in enzyme buffer (32.5 ml of 0.1 2-N-Morpholinoethanesulfonic acid [MES], pH 6.5 and 4.0 ml of 0.1 M calcium chloride made up to a final volume of 100 ml with Mili-Q water)) in a 96 well plate. Ten ul of 12.5% DMSO was added to each samples and then transferred in duplicates to assay plate. The reaction was started by adding 10 ul of enzyme buffer and 30 ul of substrate mix per well at 37^0C for 15 min with shaking. Then the reaction was terminated by adding 150 ul of 0.014M sodium hydroxide in 83% (v/v) ethanol. NA activity of the recovered viruses was measured by quantifying the 4-methylumbelliferone released from the fluoregenic substrate with an excitation wavelength of 360nm and emission wavelength of 450nm.

4.4.7 Northern blot hybridization. DF1 cells in six-well plates were infected with recombinant NDVs at a MOI of 5. Twenty-four hours after infection, the total RNAs were isolated from virus-infected DF1 cells using RNeasy mini Kit (Qiagen). The RNAs were electrophoresed in denaturing 1.5% agarose gels containing 0.5 M formaldehyde, transferred onto nitrocellulose membranes, and then hybridized with ^{32}P-labeled double-stranded cDNA probes specific to NDV HN, F, L or NP mRNA. The bands corresponding to each mRNA were quantitated by Fuji phosphorimager. The 28S and 18S RNAs were used as loading controls.

4.4.8 Western blot analysis. To examine the expression of HN protein by the recombinant NDVs, Western blot assays were performed using a standard protocol (Huang *et al*., 2004b). Total cell lysates were collected from virus-infected DF1 cells after 24 hours of infection. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel, and then transferred to a nitrocellulose membrane. The membrane was incubated with a monoclonal antibody against the NDV HN protein (a gift from Dr. Ronald Iorio, University of Massachusetts Medical School) and a monospecific antibody against the M protein prepared in our laboratory. The membrane was washed, and subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-chicken immunoglobulin G antibody (Kirkegaard & Perry Laboratories, MD). Protein bands were visualized after incubation with ECL Western blotting detection reagent (GE Healthcare, UK). The bands corresponding to the NDV HN and M proteins were quantitated by ImageJ software. Western blot analyses were repeated three times using viral proteins obtained from different experiments.

4.4.9 Analysis of proteins present in purified recombinant NDVs. Proteins present in purified recombinant viruses were examined by 8% SDS-PAGE under reducing conditions (Shirtridge *et al*., 1980). Briefly, parental and mutant viruses were purified through a 30% sucrose cushion centrifuged in the SW28 rotor (Beckman coulter) for 2.5 hours at 20,000 rpm (Peter and Robinson, 1965). Purified viruses were dissolved in sterile PBS, and the amounts of total proteins were quantified by Micro BCA Protein Assay Reagent Kit (PIERCE). Equal amounts of proteins were separated by electrophoresis on an 8% SDS-PAGE gel. The viral protein bands were visualized after staining with Coomassie brilliant blue. These experiments were also repeated three times to confirm the results.

4.4.10 Evaluation of recombinant virus pathogenicity in chickens. The pathogenicity of the mutant viruses was determined by an internationally-accepted pathogenicity test, intracerebral pathogenicity index (ICPI) test in 1-day-old specific pathogen free (SPF) chicks (Yan and Samal, 2008). Furthermore, the pathogenicity of the parental and mutant viruses (rBC, rBC-5UTRΔ1-78, rBC-3UTRΔ1-162 and rBC-5UTRΔ1-78/3UTRΔ1-162) was evaluated in 2-week-old chickens by the natural route of infection. Briefly, groups of nine 2-week-old SPF chickens were inoculated with 10^6 PFU of virus per chicken via the oculonasal route. The birds were observed daily for clinical symptoms of disease until 7 days post-infection. To determine the replication ability of viruses, three birds from each group were euthanized at 3, 5 and 7 days post-infection. Tissue samples (brain, lungs, trachea and spleen) were collected, homogenized and titrated by plaque assay for virus content.

4.4.11 Analysis of the antibody response in chickens to mutant viruses. To examine the antibody response in infected chicken, twelve 4-week-old SPF chickens were infected with parental and mutant viruses (rBC, rBC-5UTRΔ1-78, rBC-3UTRΔ1-162 and rBC-5UTRΔ1-78/3UTRΔ1-162) using natural route of infection. After 14 days at infection, serum samples from each group (three chickens) were collected. ELISA, virus-neutralization and hemagglutination-inhibition (HI) tests were performed to determine the antibody level in each chicken. ELISA was performed using a commercial kit according to the manufacturer's recommendation (Infectious Newcastle Disease Antibody Test Kit, SYNBIOTICS Corporation, San Diego, CA). Virus-neutralization and HI tests were performed following the standard procedure (Beard, 1989).

4.4.12 Immunogold electron microscopy. To examine the amount of HN protein present on the surface of mutant viruses, immunogold electron microscopy was performed in a JEM-1200 EX II electron microscope using Aurion conventional gold reagents according to the manufacturer's recommendation (AURION, PA). The viruses were purified from infected allantoic fluid by sucrose cushion centrifugation and immunogold labeled with monoclonal antibodies against NDV HN protein.

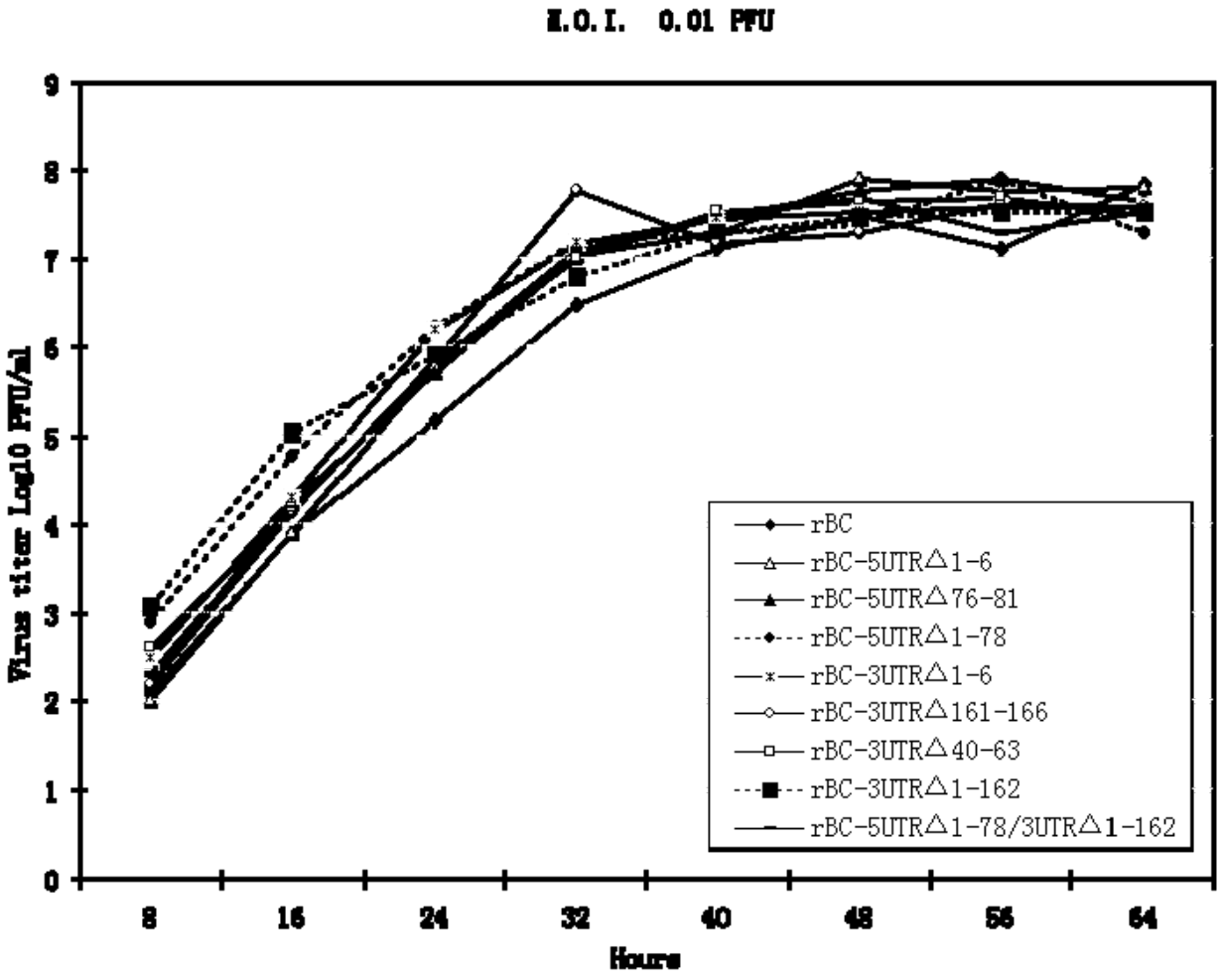
4.5 Results

4.5.1 Rescue of rNDVs with alterations in 5' and 3' UTRs of HN gene. In NDV strain BC, the HN mRNA has 81 nt at the 5' UTR and 166 nt at the 3' UTR. To determine the role of 5' and 3' UTRs of HN mRNA in NDV replication and pathogenicity, we made a total of nine different alterations in the UTRs of HN gene in a full-length BC antigenomic cDNA clone (Fig.4.1). In the 5' UTR, six nucleotides deletions were made downstream of HN GS end, six

nucleotides deletions were made upstream of HN AUG start codon, and 78 nt deletions were made in the 5' UTR from 1 to 78 nt of HN gene. In the 3' UTR, six nucleotides deletions were made downstream of HN TAG stop codon, six nucleotides deletions were made upstream of HN GE, and 24 nt or 162 nt deletions were made in the middle of 3'UTR of HN mRNA. One mutant was made containing both 78 nt deletions and 162 nt deletions in the 5' and 3' UTRs of HN gene, respectively. In addition, one mutant was constructed by substituting HN 5' and 3' UTRs with NP 5' and 3' UTRs, respectively. All modifications were made to follow the "Rule of Six" (Calain and Roux, 1993; Kolakofsky *et al*., 1998; Phillips *et al*., 1998; and Samal and Collins, 1996).

4.5.2 HN UTRs are not essential for NDV replication in vitro. Mutant viruses were recovered by reverse genetics technique as described previously (Krishnamurthy *et al*., 2000). The sequences of deletion/modification UTRs were confirmed by RT-PCR and sequencing. There were no noticeable differences in the recovery of recombinant viruses from different constructs. To examine the replication of rNDVs *in vitro*, multi-step growth kinetics was performed in DF-1 cells. There was no significant difference in growth kinetics between the various mutant viruses and the parental virus rBC (Fig.4.2). All mutant viruses and rBC grew to a similar titer after 32 h p.i. This result was consistent with the plaque morphology, which did not show any difference in size (Fig.4.3), indicating that both 5' and 3' UTRs of HN are not essential for NDV replication *in vitro*.

A.


M.O.I. 0.01 PFU
Virus titer Log10 PFU/ml
Hours
0
1
2
3
4
5
6
7
8
9
8
16
24
32
40
48
56
64
rBC
rBC-5UTR△1-6
rBC-5UTR△76-81
rBC-5UTR△1-78
rBC-3UTR△1-6
rBC-3UTR△161-166
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rBC-5UTR△1-78/3UTR△1-162

B.

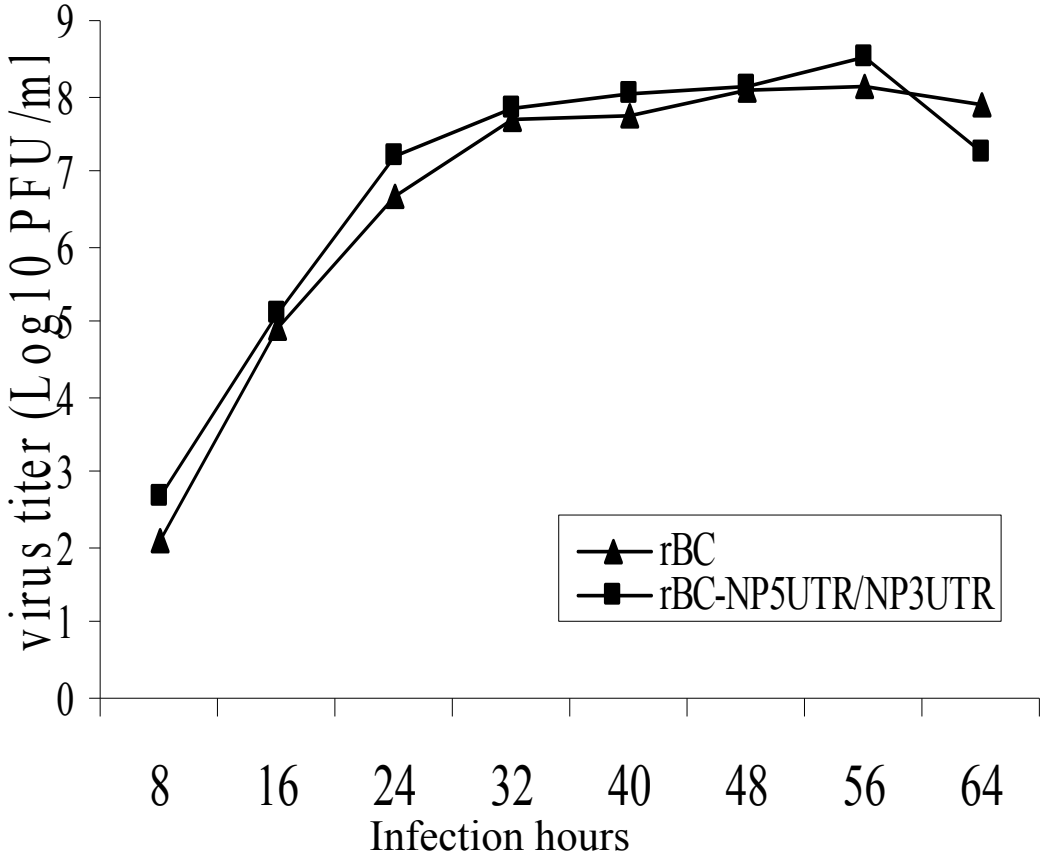


FIG.4.2 Kinetics of replication of mutant NDVs in DF1 cells.

DF1 cell monolayers were infected in duplicate with the indicated viruses at an M.O.I. of 0.01 PFU per cell for 1 h. The cells were washed with DMEM and then covered with DMEM containing 2% FBS at 37^0C. Aliquots of the supernatant medium were taken at 8 h intervals till 64 h post-infection, replaced with equal volumes of fresh medium, flash-frozen, and analyzed in a single plaque assay. (A) Kinetic of replication of parental virus, rBC, and UTR deletion viruses. (B) Kinetic of replication of parental virus, rBC, and UTR replacement virus, rBC-NP%UTR/NP3UTR.

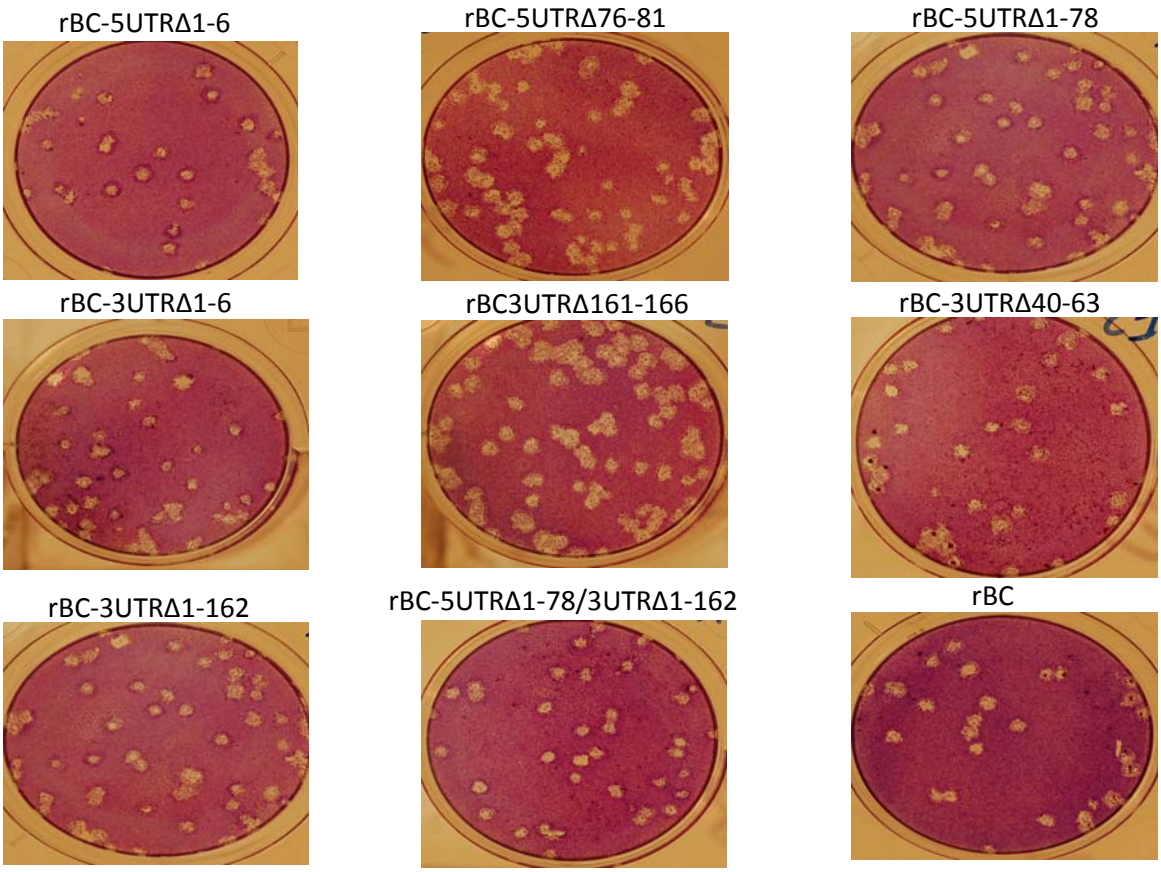
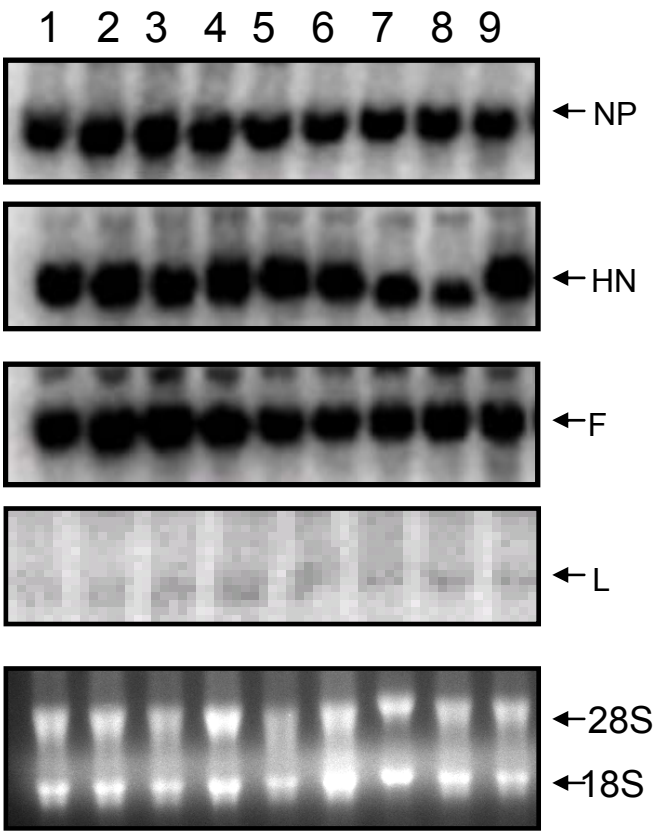


FIG.4.3 Plaque morphology of mutant NDVs containing modified HN UTRs.
Recovered viruses were titrated in duplicated in 6-well plates. Supernatant collected from virus-inoculated samples was serially diluted, and 0.2 ml of each diluents was added into confluent DF1 cells per well. After 1h of adsorption, cells were overlaid with DMEM containing 2% FBS and 1% methylcellulose, and incubated at 37^0C for 4 days. The cells were then fixed with methanol and stained with crystal violet.

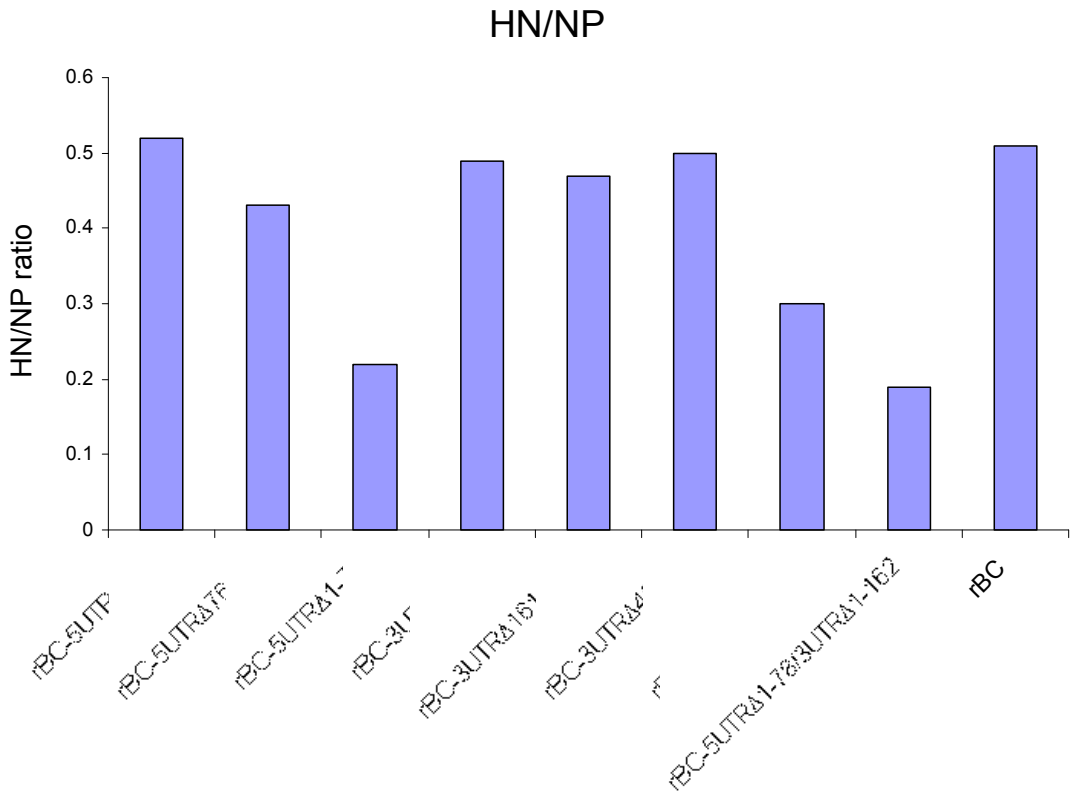
4.5.3 HN mRNA transcription was affected by deletion of HN UTRs. The level of HN mRNA transcription of different UTR deletion viruses was analyzed by Northern blot hybridization. The ^{32}P-labeled NDV gene-specific probes were used to hybridize with mRNA

species. The 28S and 18S RNAs were included as control. The ratio of HN to NP mRNA was quantified by measuring radioactivity using Fuji phosphorimager. Our results showed that the HN to NP mRNA ratios of 5' UTR complete deletion virus, rBC-5UTRΔ1-78, and the 5' and 3' UTR complete double- deletion virus, rBC-5UTRΔ1-78/3UTRΔ1-162, were approximately 20%; and the HN to NP mRNA ratio of 3' UTR complete deletion virus, rBC-3UTRΔ1-162, was approximately 30%; whereas, the HN to NP mRNA ratios of other HN UTR deletion viruses and the parental virus, rBC, were approximately 50% (Fig.4.4 B). These data indicated that complete deletion of 5' or 3' UTR affected HN mRNA transcription. It also indicated that 5'UTR may be more sensitive to HN mRNA transcription than 3'UTR, since 5' UTR showed more effect on HN mRNA transcription.

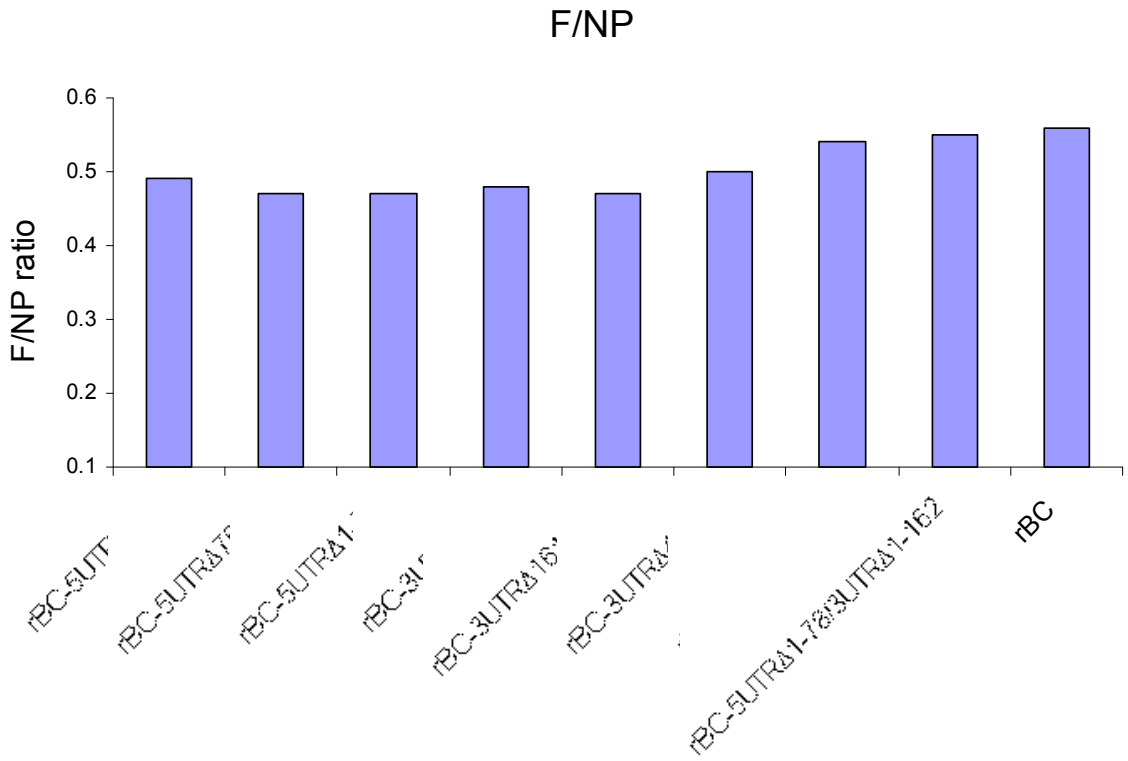
A.



B.



C.



D.

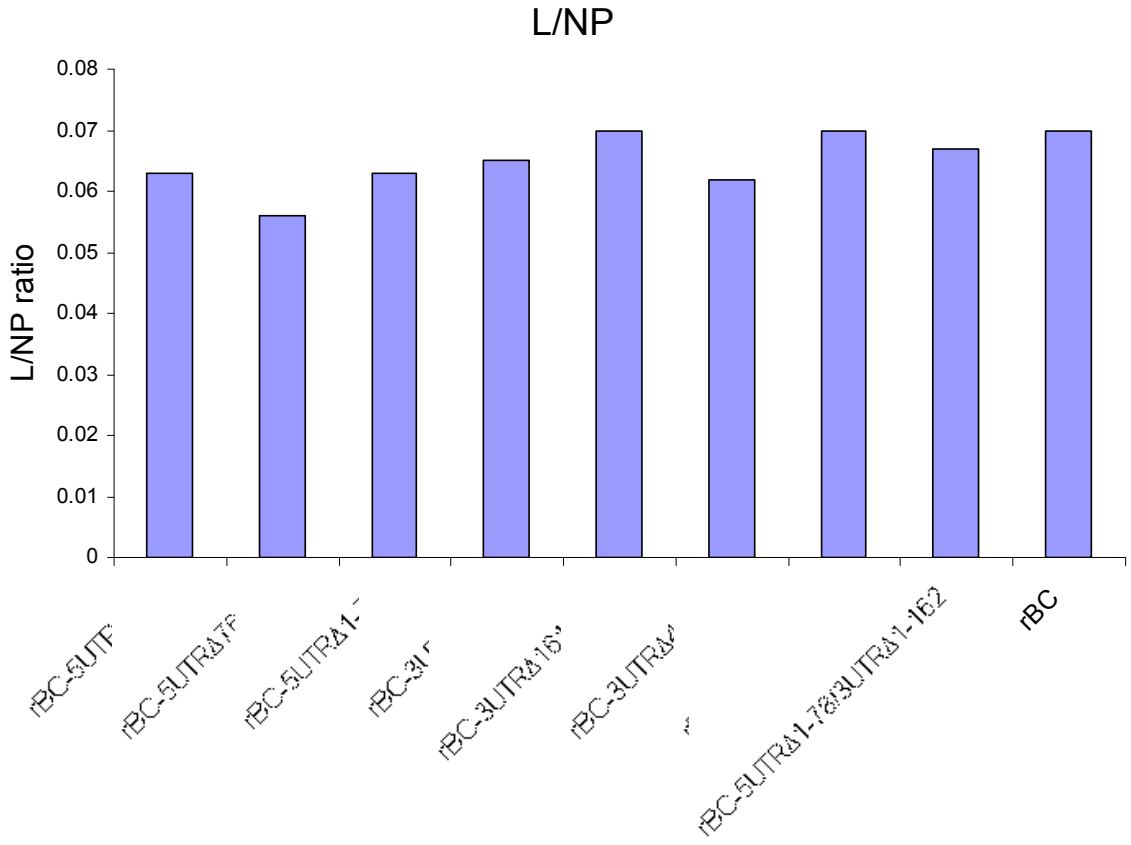


FIG.4.4 Northern blot analysis of RNAs synthesized by mutant NDVs with modified 5' and 3' HN UTR.

DF1 cells were infected with the indicated viruses (M.O.I. of 5 PFU), incubated for 24 h and harvested, and then total intracellular RNAs were extracted. The RNAs were separated by electrophoresis in formaldehyde agarose gel, transferred onto nitrocellulose membranes, and hybridized with ^{32}P-labeled double-stranded cDNA probes specific to the HN, NP, L, and F gene (A), respectively. Ratios of quantified HN to NP mRNA levels (B), F to NP mRNA levels

(C), and L to NP mRNA levels (D). The amounts of HN and NP mRNAs were normalized by the amount of 28 S RNA (A).

Lanes: 1, rBC-5UTRΔ1-6; 2, rBC-5UTRΔ76-81; 3, rBC-5UTRΔ1-78; 4, rBC-3UTRΔ1-6; 5, rBC-3UTRΔ161-166; 6, rBC-3UTRΔ40-63; 7, rBC-3UTRΔ1-162; 8, rBC-5UTRΔ1-78/3UTRΔ1-162; 9, rBC.

To ascertain the deletion of HN UTRs did not affect transcription of other virus genes, F and L mRNAs transcription were also examined following the same procedure. The ratio of F to NP mRNA did not show a detectable difference (Fig.4.4 C), indicating that UTRs of HN did not affect upstream gene transcription. We also examined the F-HN read-through transcription. The results were consistent with the HN mRNA transcription: viruses that had less HN mRNA transcription level also showed less F-HN read-through. This suggested that UTRs could also affect read-through transcription. Our results did not show any difference in the level of L mRNA (Fig.4.4 D), indicating that HN UTRs have no effect on transcription of downstream L gene.

4.5.4 Deletions of 5' and 3' UTRs of HN mRNA affect its translation and incorporation into virus particles. The HN protein production of HN UTR deletion NDV mutants in cell culture was examined by Western blot, using antibodies specific to NDV HN and M proteins. The ratios of HN to M protein level were quantified. Our results showed that two mutant viruses, rBC-5UTRΔ1-78 and rBC-5UTRΔ1-78/3UTRΔ1-162, showed significantly lower ratios of HN to M (5% for both viruses), compared to the parental virus, rBC (50%) (Fig.4.5). The ratios of HN to M proteins of other mutant viruses varied between 29% to 44%, compared to that of rBC. These results indicated that deletion of 5' and 3' UTRs of HN mRNA also affected the translation of HN mRNAs.

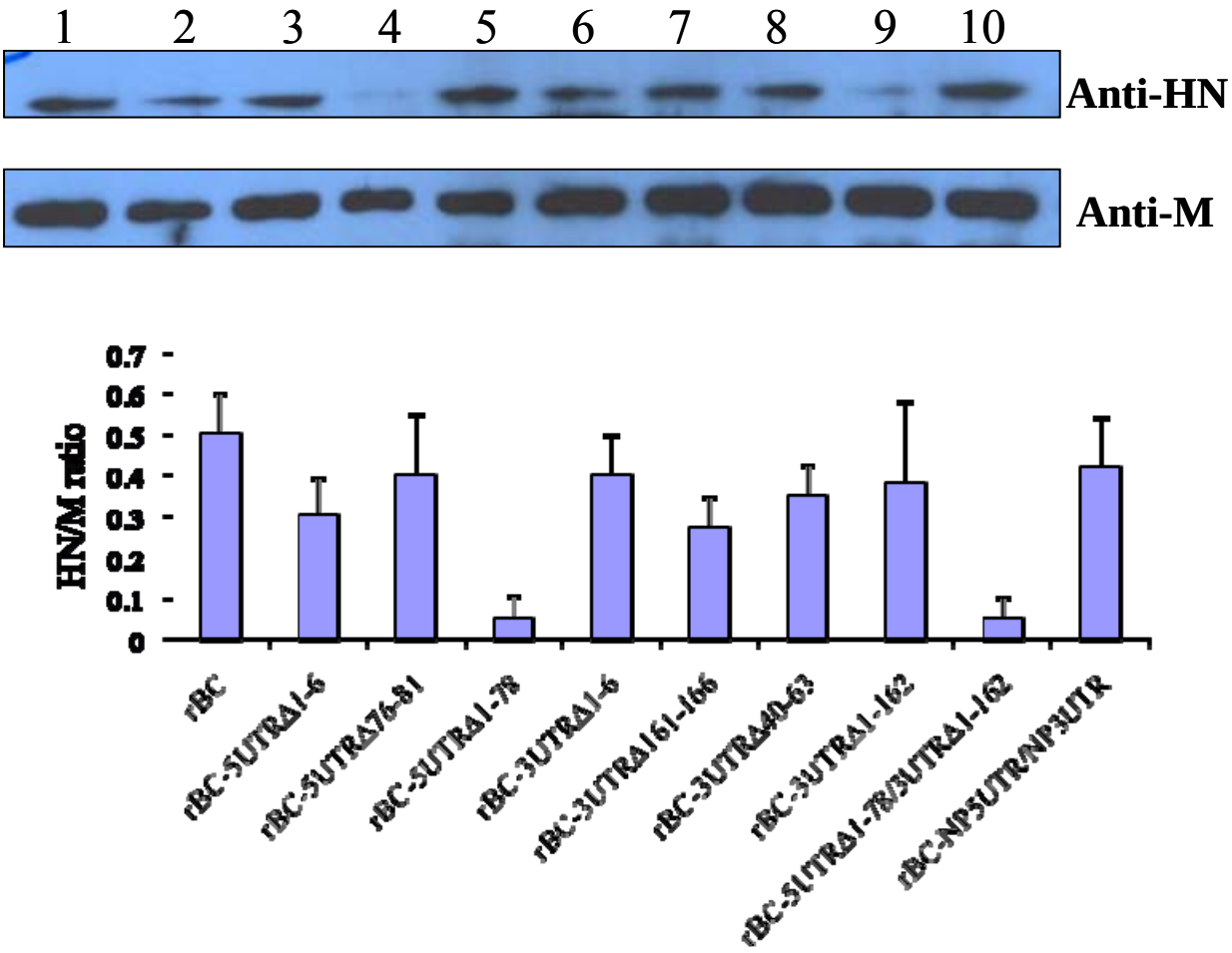


FIG.4.5 Protein synthesis in DF1 cells infected with recombinant viruses.

(A) Western blot of HN and M proteins from parental and HN UTR mutant virus-infected cells. DF1 cells were infected at 5 PFU/cell and total proteins were collected at 24 h postinfection. The top panel shows HN protein, and the bottom panel shows M protein. (B) Ratios of quantified HN protein to M protein levels from wild type rBC and the HN UTR mutant viruses. The mean standard deviations in triplicate samples are shown. *P<0.005. Lanes: 1, rBC;

2, rBC-5UTRΔ1-6; 3, rBC-5UTRΔ76-81; 4, rBC-5UTRΔ1-78; 5, rBC-3UTRΔ1-6;

6, rBC-3UTRΔ161-166; 7, rBC-3UTRΔ40-63; 8, rBC-3UTRΔ1-162;

9, rBC-5UTRΔ1-78/3UTRΔ1-162; 10, rBC-NP5UTR/NP3UTR.

We then wanted to examine whether lower levels of production of HN proteins affect incorporation of HN into the NDV particles. The parental and mutant viruses harvested from allantoic fluids were purified through a sucrose cushion. The proteins present in the purified viruses were separated on an 8% SDS-PAGE gel. The amount of each virus protein was quantified after Coomassie brilliant blue staining. The 5' UTR complete deletion virus, rBC-5UTRΔ1-78, and the 5' and 3' UTRs double-deletion virus, rBC-5UTRΔ1-78/3UTRΔ1-162, showed lower ratios of HN to M proteins (Fig.4.6). However, the ratios of HN to M proteins of other deletion mutant viruses were similar to that of the parental virus. The amount of HN protein present on the surface of virus particle was further examined by electron microscopy using immunogold labeling with HN antibody. Our results confirmed that on complete 5' UTR deletion and 5' and 3' UTRs double-deletion viruses, HN protein was present in much less quantity on the surfaces of virus particles compared to that of the parental virus, rBC (Fig.4.7). These results were consistent with our Western blot analysis, indicating that complete HN 5' UTR deletions drastically affected HN protein expression level, which, in turn, resulted in less incorporation of the HN protein into the virus particles.

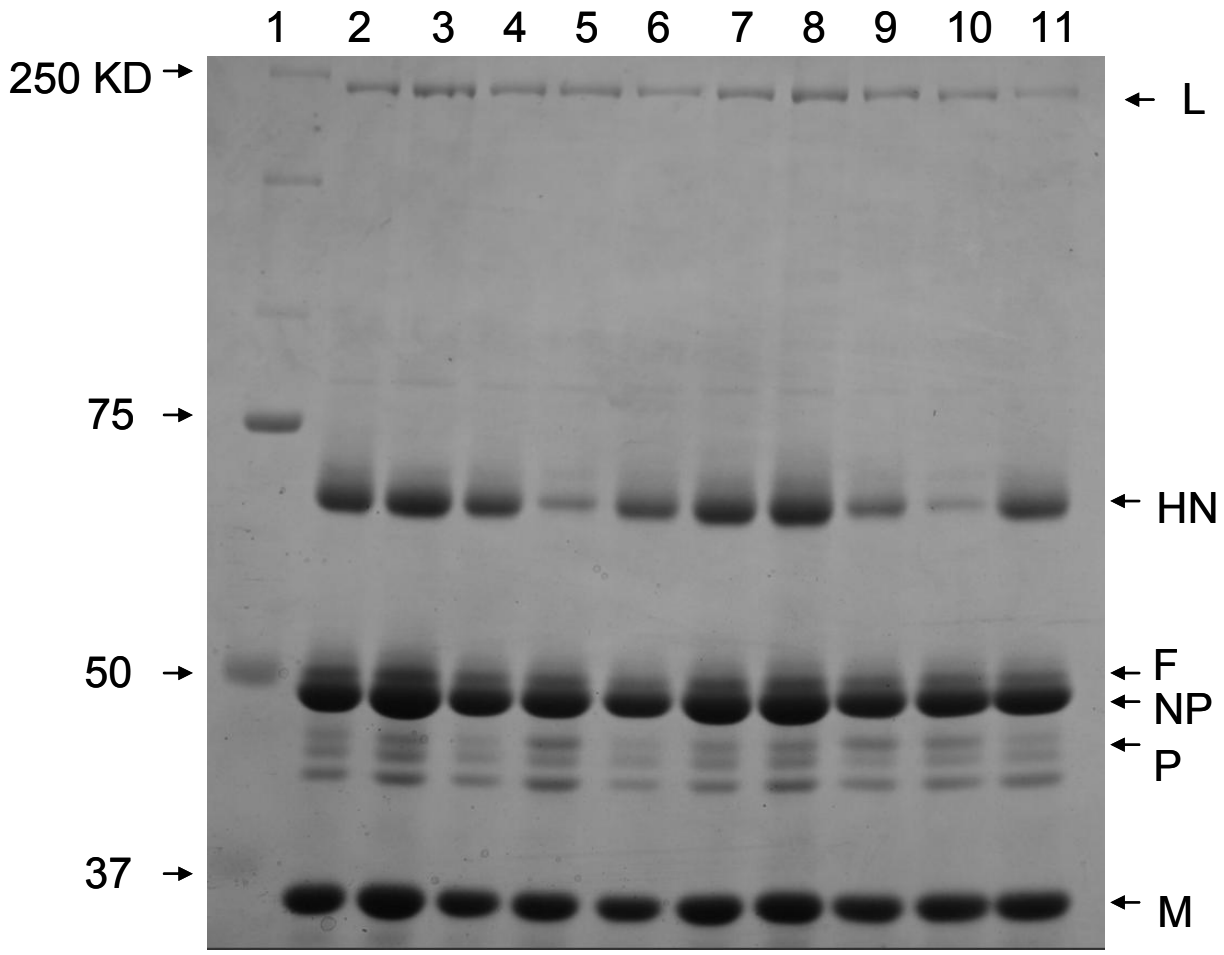


FIG.4.6 Incorporation of viral proteins into virus particles.

Ultra centrifuge-purified viruses from infected allantoic fluids were separated by electrophoresis on an 8% SDS-PAGE gel. The gel was then stained with Coomassie brilliant blue. The molecular weight and individual viral proteins are indicated.

Lanes: 1, protein standard; 2, rBC; 3, rBC-5UTRΔ1-6; 4, rBC-5UTRΔ76-81; 5, rBC-5UTRΔ1-78; 6, rBC-3UTRΔ1-6; 7, rBC-3UTRΔ161-166; 8, rBC-3UTRΔ40-63; 9, rBC-3UTRΔ1-162; 10, rBC-5UTRΔ1-78/3UTRΔ1-162; 11, rBC-NP5UTR/NP3UTR.

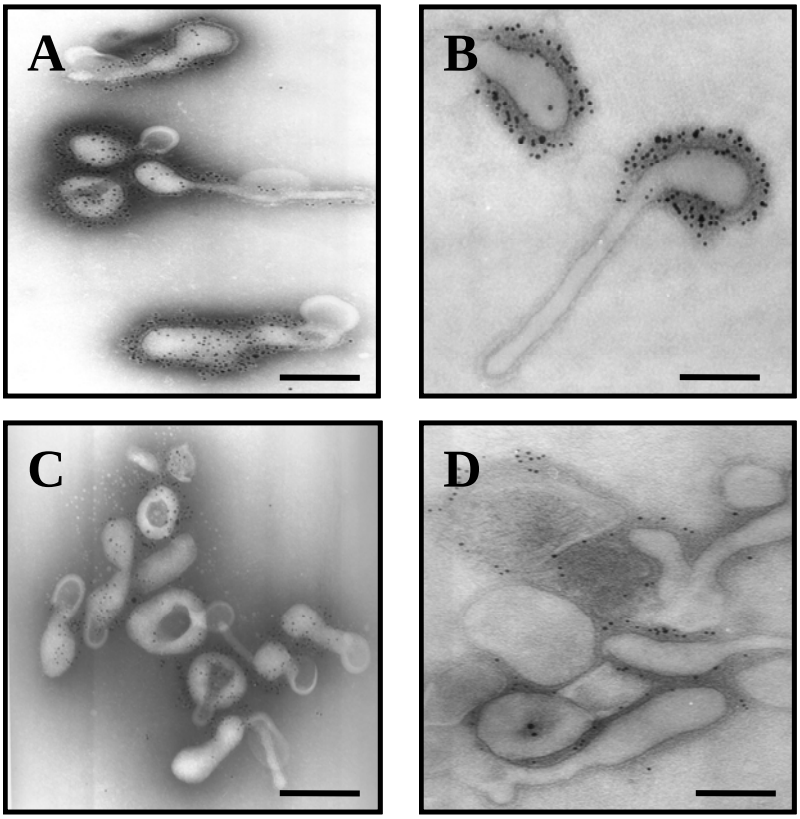


FIG.4.7 Immunogold electron micrographs of parental and complete 5’ and 3’ UN UTRs deletion viruses.

A: rBC; B: rBC-3UTRΔ1-162; C: rBC-5UTRΔ1-78; D: rBC-5UTRΔ1-78/3UTR Δ1-162. Bar=100nm.

In order to determine whether lower incorporation of HN protein in rBC-5UTRΔ1-78 and rBC-5UTRΔ1-78/3UTRΔ1-166 viruses affected the HA activity of the viruses, freshly-harvested allantoic fluids of parental and mutant viruses were examined simultaneously by HA and plaque assays. The PFU to HA ratios of the parental and mutant viruses were determined. Our results showed that the complete 5' UTR deletion and complete 5' and 3' UTR double-deletion viruses had 10- fold lower HA activity compared to their parental virus; whereas, the HA activities of other mutant viruses did not show any significant difference compared to their parental virus. NA assay also showed that complete 5' UTR deletion and complete 5' and 3' UTR double-deletion viruses possessed lower NA activities compared to their parental virus (Fig.4.8).

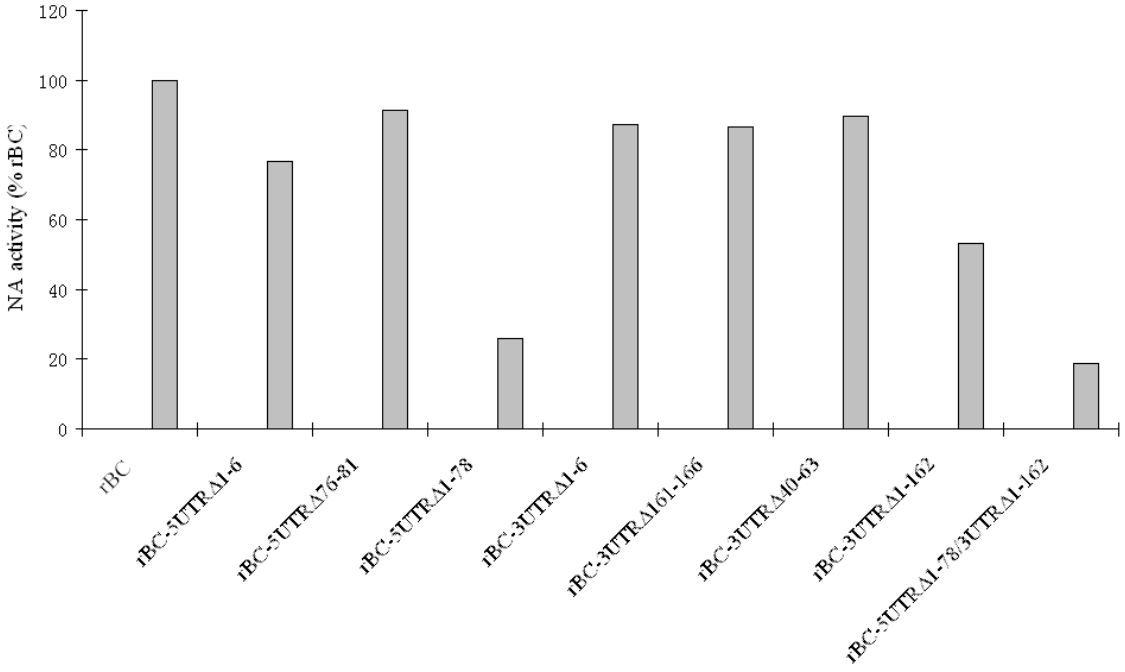


FIG.4.8 The neuraminidase (NA) activities of the parental and HN UTR deletion viruses.

The percent NA activity of mutant viruses are compared to the rBC, whose activity was considered to be 100%. The NA activity of purified parental and HN UTR deletion viruses was measured by a fluorometric assay.

4.5.5 The 5' UTR of HN affects the pathogenicity of NDV. Our results showed that deletion of either 5' or 3' UTR of HN lowered the ICPI values of mutant viruses compared to their parental rBC virus (Table 4.2). However, the ICPI values were lowest in the cases of complete 5'UTR deletion virus rBC-5UTRΔ1-78 and complete 5' and 3' UTR double-deletion virus rBC-5UTRΔ1-78/3UTRΔ1-162. These results indicated that deletions of UTRs of HN gene ultimately reduce the pathogenicity of NDV.

TABLE 4.2 Pathogenicity studies of recombinant viruses in 1-day-old chickens by ICPI test

Virus	ICPI[a]
rBC	1.49
rBC-5UTRΔ 1-6	1.43
rBC-5UTRΔ 76-81	1.24
rBC-5UTRΔ 1-78	0.86
rBC-3UTRΔ 1-6	1.35
rBC-3UTRΔ 161-166	1.33
rBC-3UTRΔ 40-63	1.16
rBC-3UTRΔ 1-162	1.26
rBC-5UTRΔ 1-78/3UTRΔ 1-162	1.08
rBC-NP5UTR/NP3UTR	0.98

[a]The ICPI values for velogenic strains approach the maximum score of 2.00, whereas, lentogenic strains give values close to 0.

We further evaluated the replication abilities of the mutant viruses in 2-week-old chickens by inoculating 10^6 PFU of virus per bird through the natural route of infection. Three mutant viruses, which included the two complete 5' or 3' UTR deletion viruses (rBC-5UTRΔ1-78 and rBC-3UTRΔ1-162) and the complete 5' and 3' UTR double-deletion virus (rBC-5UTRΔ1-78 /3UTRΔ1-162), along with the parental virus, rBC, were tested in this study. Nine birds in each group were inoculated by intranasal and intraocular routes of infection. Three birds each were sacrificed on 3, 5 and 7 dpi. Tissue samples (brain, lungs, trachea and spleen) collected from each bird were titrated by plaque assay for virus content. Our results showed that viruses could not be detected in any of the tissue samples collected from the birds collected at 7 dpi, suggesting that viruses were probably cleared by the host immune system within 7 days after infection. Virus was detected in all of four tissues of all three birds infected with the parental rBC virus at 3 and 5 dpi (Table 4.3). All three birds infected with complete 3' UTR deletion virus also showed virus replication in most tissue samples at 3 and 5 dpi (Table 4. 3); whereas, only one of the birds infected with complete 5' UTR deletion virus showed virus replication in trachea and spleen at 5 dpi. Similarly, only one of the birds infected with complete 5' and 3' UTR double-deletion virus showed virus replication in the trachea and spleen at 3 dpi.(Table 4.3). Our results also showed that the birds infected with complete 5' UTR deletion virus and complete 5' and 3' UTR double-deletion virus were either mildly sick or healthy throughout the course of the study; whereas, the birds infected with the parental virus and the complete 3' UTR deletion virus exhibited symptoms of sickness and paralysis. These results suggested that complete deletion of 5' UTR of HN highly debilitated the replication ability of NDV in chickens, which, in turn, resulted in lower pathogenicity compared to that of the parental virus.

TABLE 4.3. Replication of recombinant viruses in 2-week-old chickens

Virus [a]	Day 3				Day 5			
	Brain (PFU/g)	Lungs (PFU/g)	Trachea (PFU/g)	Spleen (PFU/g)	Brain (PFU/g)	Lungs (PFU/g)	Trachea (PFU/g)	Spleen (PFU/g)
rBC (1)	$1.5x10^3$	$1.6x10^2$	$1.0x10^4$	$2.5x10^2$	$1.0x10^3$	$4.4x10^2$	$1.0x10^4$	$1.0x10^3$
rBC (2)	$2.1x10^3$	$7.0x10^2$	$2.4x10^3$	$7.5x10^2$	$1.2x10^3$	$1.0x10^3$	$2.0x10^3$	$7.3x10^2$
rBC (3)	$1.6x10^3$	$2.7x10^3$	$2.4x10^3$	$7.0x10^2$	$1.0x10^3$	$2.0x10^3$	$1.0x10^4$	$2.3x10^2$
rBC-5UTRΔ1-78 (1)	ND[b]	ND	ND	ND	ND	ND	$1.0x10^4$	$8.0x10^2$
rBC-5UTRΔ1-78 (2)	ND	ND	ND	ND	ND	ND	ND	ND
rBC-5UTRΔ1-78 (3)	ND	ND	ND	ND	ND	ND	ND	ND
rBC-3UTRΔ1-162 (1)	$3.0x10^2$	ND	$7.5x10^2$	ND	ND	$2.0x10^3$	$3.0x10^4$	$7.0x10^1$
rBC-3UTRΔ1-162 (2)	ND	ND	$1.2x10^2$	$2.0x10^2$	$3.0x10^2$	$1.0x10^3$	$1.0x10^4$	$4.3x10^3$
rBC-3UTRΔ1-162 (3)	$1.2x10^3$	$1.5x10^2$	$9.0x10^3$	ND	$1.2x10^3$	$6.0x10^3$	$9.0x10^3$	$4.4x10^2$
rBC-5UTRΔ1-78/3UTRΔ1-162 (1)	ND	ND	ND	ND	ND	ND	ND	ND
rBC-5UTRΔ1-78/3UTRΔ1-162 (2)	ND	$1.5x10^2$	$6.3x10^2$	ND	ND	ND	ND	ND
rBC-5UTRΔ1-78/3UTRΔ1-162 (3)	ND	ND	ND	ND	ND	ND	ND	ND
rBC-NP5UTR/NP3UTR (1)	$1.6x10^3$	ND	$1.1x10^4$	ND	$1.0x10^3$	ND	ND	ND
rBC-NP5UTR/NP3UTR (2)	$8.0x10^2$	$5.0x10^2$	$1.5x10^3$	$2.8x10^3$	$6.0x10^2$	$3.0x10^2$	$1.8x10^3$	ND
rBC-NP5UTR/NP3UTR (3)	$3.3x10^2$	$3.3x10^2$	$3.5x10^3$	ND	$5.0x10^2$	ND	ND	ND

[a] Two-week-old chickens in groups of 9 were infected with 10^6 PFU of each virus per chicken by oculonasal route. On days 3, 5, and 7, three chickens per group were sacrificed. Brain, lungs, trachea and spleen were collected and virus titers were determined by plaque assay. No virus was detected in all the tissues samples from each group on day 7.

[b] Not detected

TABLE 4.4. Serum antibody response of recombinant viruses in 4-week-old chickens

Virus[a]	**ELISA[b] (s/p ratio)**	**NT[c] ($Log_{10}ID_{50}$)**	**HI[d]**	**Virus[a]**	**ELISA[b] (s/p ratio)**	**NT[c] ($Log_{10}ID_{50}$)**	**HI[d]**
rBC(1)	1.17	2.85	256	rBC-3UTRΔ 1-162(1)	1.17	>3.0	256
rBC(2)	1.18	>3.0	724	rBC-3UTRΔ 1-162(2)	1.16	>3.0	256
rBC(3)	1.08	2.85	362	rBC-3UTRΔ 1-162(3)	1.01	>3.0	256
rBC-5UTRΔ 1-78(1)	0.57	2.25	128	rBC-5UTRΔ 1-78/3UTRΔ 1-162(1)	0.67	2.10	128
rBC-5UTRΔ 1-78(2)	0.81	2.25	128	rBC-5UTRΔ 1-78/3UTRΔ 1-162(2)	0.66	2.50	128
rBC-5UTRΔ 1-78(3)	1.01	2.10	128	rBC-5UTRΔ 1-78/3UTRΔ 1-162(3)	0.70	2.10	128

[a] Four-week-old chickens in groups of 3 were infected with 10^6 PFU of each virus per chicken by oculonasal route. Serum samples were collected from the infected chickens after 14 days of infection. [b] S/P ratio: (sample-negative)/(positive-negative)

[c] virus-neutralization test [d]Hemagglutination-inhibition test

We also evaluated the ability of the above mutant viruses to elicit antibody response in 4-week-old chickens since these mutant viruses produced much less HN proteins. Groups of three chickens were infected with 10^6 PFU of virus per bird through the natural routes of infection. Fourteen days after infection, serum samples were collected from each chicken and tested for antibody levels by ELISA and virus neutralization tests. Our results showed that chickens infected with complete 5' UTR deletion and complete 5' and 3' UTR double-deletion viruses elicited significantly less neutralizing antibody response than that of complete 3' UTR deletion and parental rBC viruses (Table 4.4). These results indicated that the lower replication ability of complete 5' UTR and complete 5' and 3' UTR double-deletion viruses was probably responsible for the induction of lower levels of neutralizing antibody.

4.5.6 Replacement of 5' and 3' UTRs of HN with the corresponding UTRs of NP. To determine whether the function of UTRs of HN could be fulfilled by the UTRs of another NDV gene, we replaced the UTRs of HN with the corresponding UTRs of NP. The recombinant mutant virus rBC-NP5UTR/NP3UTR, whose HN gene contained UTRs of NP in place of its own, was rescued and confirmed by RT-PCR and sequencing. Our results showed that the growth kinetics of rBC-NP5UTR/NP3UTR virus was similar to that of the parental virus (Fig.4.1b). Western blot analysis showed that the HN protein was produced at the same level as the parental rBC virus (Fig.4.5). Analysis of the proteins present in the purified virus also showed that the same amount of HN protein as the parental virus was incorporated into virus particles (Fig.4.6). The pathogenicity studies showed that the virulence of the virus rBC-NP5UTR/NP3UTR was similar to that of the parental virus, and the virus replicated in all organs

at 2-week-old chickens as the parental virus at 3dpi (Table 4.3). These results suggested that the UTRs of HN could be replaced with the UTRs of NP without affecting the functions of HN.

4.6 Discussion

In a paramyxovirus, the sequence composition and length of UTRs vary among the different mRNAs. For example, in NDV strain Beaudette C, the 5' and 3' UTRs of NP mRNA are 56 nt and 200 nt, respectively; whereas, those of HN mRNA are 81 nt and 166 nt, respectively. Furthermore, the sequence and length of UTRs for the same mRNA are also sometimes not conserved among NDV strains. For example, between NDV strain 99-0868lo (Gene Bank Accession: AY935496) and strain Beaudette C, the HN 5' UTRs are same length, but vary in sequence; whereas, the HN 3' UTRs vary in both sequence and length. Currently, functions of 5' and 3' UTRs of mRNA in a paramyxovirus life cycle are not well understood.

In this study, we used reverse genetics to investigate the functions of 5' and 3' UTRs of HN mRNA of NDV. Since we did not know which regions of the HN UTRs were essential for virus replication, several partial and complete deletions of the 5' and 3' UTRs were constructed. We were able to rescue all mutant viruses carrying deletions in the HN 5' or 3' UTR, and one more mutant virus in which both HN 5' and 3' UTRs were deleted. All the mutant viruses exhibited similar multi-step growth curves and plaque sizes in cell culture. These results suggested that HN UTRs are nonessential for virus growth *in vitro*; therefore, it was possible to recover recombinant viruses with deletions in the HN UTRs.

Quantification of the relative ratio of HN/NP mRNA accumulated in parental NDV-infected cells and the cells infected with NDV mutants indicated that deletion of HN 5' and 3' UTRs affected the levels of HN mRNA accumulation. There was significant reduction in

accumulations of HN mRNA levels in the case of complete 5' UTR deletion, complete 3' UTR deletion and complete 5' and 3' UTR double-deletion viruses. Quantification of the relative ratio of F/NP mRNA levels indicated that deletion of HN UTRs did not affect accumulations of other viral mRNAs. In influenza A virus, mutation of nonconserved nucleotides in the 3' and 5' UTRs of the NA segment was found to decrease levels of both NA antigenome RNA and mRNA segments (Zheng *et al*., 1996).

Several processes contribute to the overall accumulation of viral mRNAs in infected cells, and impairment in any of the processes could lead to a decrease in mRNA level: (i) Reduced binding of viral polymerase complex or host factors are involved in transcription of viral mRNA. It is possible that the UTRs contain sites essential for binding viral polymerase complex or host factors. Loss of these sites due to deletion of UTRs may decrease efficiency of viral polymerase or host factors binding to the genome. In fact, computation analysis of HN UTR sequences showed activator/repressor binding sites in the 3' UTR of HN mRNA and viral myb-like transcription regulators in the 5' UTR of HN mRNA. (ii) Inefficient transcription terminator can cause decreased initiation of the downstream gene (Bousse *et al*., 2002; Harmon and Wertz, 2002; Moudy *et al*., 2003). It is possible that HN UTRs are involved in termination of F mRNA transcription, and deletion of these UTRs leads to decreased transcription of HN mRNA. However, our Northern blot assays did not show any increase in the level F-HN bicistronic mRNA, indicating that HN UTRs did not affect termination of F mRNA synthesis. (iii) One possibility we can not discount is that the reduced accumulation of HN mRNA is due to its decreased stability, rather than its decreased synthesis.

Comparison of the relative ratio of HN/M protein present in parental NDV-infected cells and the cells infected with HN UTR deletion mutant NDVs showed that complete deletion of HN

5’ UTR and complete deletion of HN 5’ and 3’UTRs drastically affected the synthesis of HN protein. Since complete deletion of HN 3’UTR did not significantly affect the level of HN present in infected cells, the drastic reduction of HN protein observed in the cells infected with virus containing complete HN 5’ and 3’ UTRs was most likely due to the complete deletion of HN 5’ UTR. Our results suggested that in the virus-infected cells the HN 5’UTR promotes the HN protein translation, since the ratio of the HN protein production to the amount of the HN mRNA was significantly higher in the absence of the 5’ UTRs. Our results confirm previous studies on the functions of the 5’ UTR of F mRNAs of MeV, canine distemper virus and rinderpest virus performed using plasmid system (Evans *et al*., 1990; Hasel *et al*., 1987) and in an avian sarcoma mRNA (Katz *et al*., 1986), which showed deletion of 5’ UTR can have an inhibitory effect on translation. Katz *et al*. (1986) suggested that a secondary structure predicted to form in the 5’ UTR may allow ribosome to avoid scanning through the entire region, and perturbation at such a structure may then inhibit translation efficiency. It may be possible that the 5’ UTR of NDV HN mRNA form a secondary structure necessary for efficient translation, which is absent in 5’ UTR deleted viruses.

The amount of HN protein present in purified parental and each mutant virus was examined. Interestingly, complete HN 5’UTR deletion and complete HN 5’ and 3’ UTR double-deletion viruses showed significantly lower amounts of HN protein in virus particles, following the same level of HN protein present in cells infected with the respective viruses (Figs.4.6 & 4.7). However, other 5’ and 3’ UTR deletion viruses that showed lower levels of HN protein synthesis in infected cells possessed the same amount of HN protein as their parental virus in purified particles. One possible explanation for this observation could be that in these viruses, although the amount of HN protein synthesis was relatively low compared to that of the parental

virus, the amount of HN was sufficient for required incorporation into viral particles. But, in complete HN 5'UTR deletion and complete 5' and 3' UTR double-deletion viruses, the amount of HN protein synthesis was drastically low, which was below the amount required for full incorporation into virus particles. It was noteworthy that decreased incorporation of the HN protein did not increase incorporation of the F protein into virus particles, suggesting that F and HN proteins have specific anchor sites inside the virus particles and these sites are not exchangeable.

HN protein of NDV possesses both the hemagglutinin (HA) and neuraminidase (NA) activities associated with the virus (Crennell *et al*., 2000; Sheehan and Iorio, 1992). We examined the HA and NA activities of the parental and deletion mutant viruses. Our results showed that the complete HN 5'UTR and complete HN 5' and 3' UTR double-deletion viruses which had lower HN protein in the virus particles also had low HA and NA activities, confirming that HN protein is responsible for both HA and NA activities, not other viral proteins.

Pathogenicity studies showed that HN UTR deletion NDVs were viable *in vivo*, but were less virulent than parental NDV. The ICPI test results showed that all HN UTR deleted viruses were attenuated in 1-day-old chicks. The complete HN 5' UTR and complete 5' and 3' UTR double-deletion viruses showed greater levels of attenuation compared to other HN UTR deletion viruses. In order to determine the mechanisms responsible for the reduction in virulence of these viruses, 2-week-old chickens were inoculated with the mutant viruses by the oculonasal route. Interestingly, our research showed that the complete 5' UTR and complete 5' and 3' UTR double-deletion viruses, which have low amounts of HN, replicated inefficiently in most of the organs tested, compared to their parental virus (Table 4.3). These results suggest that the low amount of HN in complete HN 5'UTR deletion and complete 5' and 3' UTR double-deletion

viruses may not affect the growth of these viruses *in vitro*, but the low amount of HN affects replication of these viruses in most organs *in vivo*. Thus, the correct amount of HN on the NDV particle is essential for replication and pathogenicity of the viruses in its natural host, chickens. Furthermore, we examined the antibody response against these mutant viruses in chickens. Four-week-old chickens were inoculated with the mutant viruses by the oculonasal route, and the antibody levels in infected chickens were determined by ELISA, HI and virus neutralization tests (Table 4.4). Our results showed that all these deletion mutant viruses (rBC-3UTRΔ1-162, rBC-5UTRΔ1-78, and rBC-5UTRΔ1-78/3UTRΔ1-162), along with the parental virus, rBC, induced antibody in all the inoculated chickens, indicating that all mutant viruses were replication-competent in 4-week-old chickens. However, the mutants rBC-5UTRΔ1-78 and rBC-5UTRΔ1-78/3UTRΔ1-162 induced lower levels of antibody, compared to their parental virus, rBC. It is possible that the inefficient replication of these mutant viruses in chickens was probably responsible for the low level of antibody production. The HI antibody titers of these two viruses were also lower than that of their parental viruses, suggesting that the lower amounts of HN protein present in the virus particles was most likely responsible for the HI titer.

In order to determine whether the functions of UTRs of HN gene could be substituted by the UTRs of other NDV genes, we constructed a recombinant virus, rBC-NP5UTR/NP3UTR, in which both 5' and 3' UTRs of HN were replaced with the corresponding UTRs of NP gene. Our results showed that the rBC-NP5UTR/NP3UTR virus replicated at the same level as the parental virus *in vitro*. The UTR-replaced virus produced similar level of HN protein in infected cells as that of the parental virus (Fig.4.5). Also, a similar amount of HN protein was incorporated into the UTR-replaced virus as in the parental virus (Fig.4.6). Furthermore, pathogenicity studies in 2-week-old chickens showed that the UTR-replaced virus replicated similarly in almost all

organs as the parental virus (Table 4.3). These results suggest that UTRs can be exchanged between NDV mRNAs without affecting the replication of virus *in vitro* and *in vivo*. It will be interesting to determine whether the UTRs from a heterologous virus mRNA can also be used to substitute the functions of UTRs of NDV mRNAs.

The data presented in this paper provide insight into the functions of NDV UTRs in particular and paramyxoviruses in general. Our results showed that the HN UTRs may not be critical for replication *in vitro*, but are essential for replication and pathogenicity of NDV *in vivo*, and thereby, demonstrate that UTR deletion is a possible strategy for designing live attenuated recombinant NDV vaccines. Development of better live attenuated NDV vaccines will be highly beneficial to poultry industries throughout the world.

Chapter 5

5.1 Title

Conclusion and Prospects

5.2 Conclusion and Prospects

The family *Paramyxoviridae* includes some important human pathogens, such as mumps virus, measles virus and Nipah virus; and some important animal pathogens, such as Rinderpest virus, Canine distemper virus, and NDV. NDV is one of the most important animal viruses that cause severe economic losses to the poultry industry worldwide. NDV strains vary widely in their pathogenicity. Very little is known about the molecular mechanism responsible for the variation pathogenicity of NDV.

In this study, I applied reverse genetics system that we previously established in our laboratory (Krishnamurthy *et al*., 2000) to evaluate the role of the noncoding regions in NDV transcription, replication, and pathogenesis. I show that the pathogenicity of NDV mesogenic strain Beaudette C (BC) can be modulated by changing the length of the noncoding regions.

Recombinant viruses containing extended or shorten of intergenic sequences (IGS) in F-HN and/or HN-L altered the level of downstream gene transcription, which in turn, changed the ratios of the viral transcripts and affected the virulence (Yan and Samal, 2008). I also demonstrated that NDV can tolerate an IGS length of at least 365 nt and was stable after five passages in chicken embryos (chapter 3). To my knowledge, this is one of the largest IGS in *Mononagavirales*. My study provides an alternative way to reduce virulence of NDV by extending the length of IGS regions, since studies on the attenuation of virulence usually focus on

insertion of complete open reading frame into virus genome (Sakai *et al*., 1999; Bukreyev *et al*., 2005). However, a couple of interesting questions remain to be answered: First, what is the longest foreign sequence that can be inserted into NDV IGS without changing its stability? Second, can the length of the first three IGSs affect the virulence of NDV?

Besides the length of IGS, the length of untranslated regions (UTR) of HN mRNA could also alter the pathogenicity of the mesogenic strain BC. As shown in chapter 4, complete deletion of HN UTR reduced the transcription and translation levels, leading to attenuation of virulence. However, in the current study, I could not exclude the possibility that the reduced HN mRNA accumulation is due to reduced transcription or imparity of the mRNA stability in the mutant viruses. The study of mRNA stability of the recombinant HN UTR viruses will provide some insights into the transcriptional mechanisms of NDV. I also demonstrated that the HN UTRs could be replaced by the corresponding UTRs of the NP gene without affecting the function of the HN gene (Chapter 4). The more detailed study of replacing UTRs with the heterologous virus mRNA UTRs may pave the way towards studying the transcription mechanism of negative-sense RNA viruses. For a single-stranded, positive-sense RNA virus, its genome contains a single open reading frame encoding a polyprotein which is flanked by 5' and 3' UTRs (Tajima *et al*., 2007; Hunziker *et al*., 2007). The 5' UTR usually contains an internal ribosome entry site (IRES) that direct cap-independent translation of the viral RNA (Rijnbrand *et al*., 1995; Wang *et al*., 1993), and the 3' UTR contains essential domains for viral infectivity *in vivo* (Yanagi *et al*., 1999; Yi *et al*., 2003). Based on my results on replacement of HN UTRs with corresponding NP UTRs, it would be interesting to examine whether the UTRs could be exchanged between negative-sense and positive-sense RNA viruses, and how this would affect the transcription of these viruses.

Although vaccines are currently available to provide certain level of protection against NDV in poultry, occasional outbreaks of virulent NDV strains have been reported. Moreover, depending on the type of NDV vaccine strains used, the environmental conditions, and immune system of individual birds, disease signs may occur. This study not only provides basic information of NDV pathogenicity but also provides new methods to attenuate the pathogenicity of NDV strains by changing the length of noncoding regions, which could be applied for development of novel NDV vaccines.

NDV can be used as an ideal model to study the molecular mechanisms of pathogenesis of other paramyxoviruses, since the virulence of NDV strains vary from completely avirulent to highly virulent. Moreover, the pathogenesis study of NDV can be directly carried out in its natural host, the chicken. Therefore, the detailed study of molecular mechanisms of NDV pathogenesis will be a great benefit for basic molecular study of paramyxoviruses.

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