

# Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin–neuraminidase protein

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Virulence of Newcastle disease virus (NDV) is mainly determined by the amino acid sequence surrounding the fusion (F) protein cleavage site, since host proteases that cleave the F protein of virulent strains are present in more tissues than those that cleave the F protein of non-virulent strains. Nevertheless, comparison of NDV strains that carry exactly the same F protein cleavage site shows that significant differences in virulence still exist. For instance, virulent field strain Herts/33 with the F cleavage site <sup>112</sup>RRQRRF<sup>117</sup> had an intracerebral pathogenicity index of 1·88 compared with 1·28 for strain NDFLtag, which has the same cleavage site. This implies that additional factors contribute to virulence. After generating an infectious clone of Herts/33 (FL-Herts), we were able to map the location of additional virulence factors by exchanging sequences between FL-Herts and NDFLtag. The results showed that, in addition to the F protein cleavage site, the haemagglutinin–neuraminidase (HN) protein also contributed to virulence. The effect of the HN protein on virulence was most prominent after intravenous inoculation. Interestingly, both the stem region and the globular head of the HN protein seem to be involved in determining virulence.

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## INTRODUCTION

Newcastle disease is a highly contagious and fatal viral disease affecting most species of birds. Because chickens are the most susceptible birds, the disease is frequently responsible for devastating losses in poultry (Alexander, 2000, 2001). For this reason, isolation of a virulent strain requires reporting to the 'Office International des Epizooties' (Alexander, 1997). This avian disease is caused by Newcastle disease virus (NDV), an avian paramyxovirus that has recently been assigned to the new genus *Avulavirus* within the family *Paramyxoviridae* (Mayo, 2002a, b).

NDV strains can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic). This classification is based on the results of the mean death time in chicken eggs (Beard & Hanson, 1984). The clinical signs of a highly virulent NDV infection in chickens can be extremely different depending on the strain of virus. Virulent strains that cause diarrhoea and frequently

haemorrhagic intestinal lesions are called viscerotropic velogenic. Strains that cause respiratory and neurotropic signs are called neurotropic velogenic (Alexander, 1997).

NDV is an enveloped virus with two membrane proteins: the haemagglutinin–neuraminidase (HN) protein involved in cell attachment and release, and the fusion (F) protein involved in mediating fusion of the viral envelope with cellular membranes. The F protein is synthesized as a precursor, F0, and is only fusogenic after cleavage into disulfide-linked F1 and F2 polypeptides. However, cleavage is not sufficient for the fusion process. The HN protein is also required for fusion to occur by cooperating with the F protein (Deng *et al.*, 1997). The consensus sequence of the F protein cleavage site of velogenic and mesogenic strains is <sup>112</sup>(R/K)RQ(R/K)RF<sup>117</sup>. The consensus sequence of the lentogenic F cleavage site is <sup>112</sup>(G/E)(K/R)Q(G/E)RL<sup>117</sup>. The different F protein cleavage sites are the substrates for different types of cellular proteases (Kawahara *et al.*, 1992; Sakaguchi *et al.*, 1991). The F protein of lentogenic viruses can be cleaved only by trypsin-like enzymes, as found in the respiratory and intestinal tracts, whereas the F protein of virulent viruses can be cleaved by a host

The GenBank/EMBL/DDBJ accession number of the sequence determined in this work is AY741404.

protease(s) found in a wide range of cells and tissues. Consequently, infection with a virulent strain results in fatal systemic infection (Garten *et al.*, 1980; Nagai *et al.*, 1976). For this reason, the F protein cleavage site has been postulated as the primary determinant of virulence. However, comparison of the amino acid sequence of the F protein cleavage site with the intracerebral pathogenicity indices (ICPI) for several NDV viruses has shown that significant differences in virulence can exist between strains with the same velogenic consensus sequence. For instance, virulent field strain Herts/33 with the cleavage site <sup>112</sup>RRQRRF<sup>117</sup> has an ICPI value of 1.88 whereas strains Beaudette C/45 and Komarov with the same sequence showed ICPI values between 1.4 and 1.5 (Collins *et al.*, 1993). In addition, NDV isolates recovered from many different bird species with the same velogenic F protein cleavage site were found to exhibit different virulence levels in chickens. These NDV isolates were either mesogenic strains that produced mild pathogenesis or velogenic strains causing severe pathogenesis and death (Brown *et al.*, 1999). Moreover, using reverse genetics techniques, we previously established an infectious clone of the non-virulent LaSota strain (NDFL) and demonstrated that modification of the cleavage site <sup>112</sup>GRQGRL<sup>117</sup> to <sup>112</sup>RRQRRF<sup>117</sup> (NDFLtag) increased the ICPI from 0.00 to 1.28 (Peeters *et al.*, 1999). Although NDFLtag contains exactly the same F protein cleavage site as Herts/33, the ICPI did not reach the level of 1.88, indicating that other factors contribute to virulence. The most likely candidate would be the HN protein, since it is involved in viral entry. Another candidate could be the V protein of NDV. The V protein is the result of an mRNA editing event in which a G residue is inserted within the P gene mRNA (Curran *et al.*, 1998). This accessory V protein has been shown to act as an interferon antagonist (Park *et al.*, 2003; Huang *et al.*, 2003) and is involved in pathogenesis and host-range restriction (Mebatsion *et al.*, 2001; Park *et al.*, 2003).

In this study, we generated an infectious clone of the highly virulent strain Herts/33 (FL-Herts). To map virulence factors, sequences from NDFL or NDFLtag were replaced with sequences from FL-Herts and the virulence of the rescued chimeric virus was determined. The results showed that, in addition to the F protein, the HN protein also contributes to virulence. Our results agree with the results of Huang *et al.* (2004), who exchanged the HN genes of the virulent recombinant NDV strain Beaudette and the non-virulent recombinant strain LaSota and showed that the HN protein affected virulence. Furthermore, our experiments showed that the effect of HN on virulence was most prominent after intravenous inoculation and that both the stem region and the globular head of the HN protein seemed to be involved in determining virulence.

## METHODS

**Cells and viruses.** QM5 cells (Antin & Ordahl, 1991) were grown in medium supplied by Gibco-BRL/LifeTechnologies. Media were supplemented with 5% fetal calf serum and antibiotics. The fowlpox

recombinant virus fpE-FLT7pol (Britton *et al.*, 1996) (hereafter called FPV-T7), which expresses T7 RNA polymerase, was grown on primary chicken embryo liver cells.

NDV strain Herts/33 (CIDC-Lelystad) was grown in 9–11-day-old embryonated specific-pathogen-free (SPF) eggs. The recombinant strains NDFL and NDFLtag were derived from the NDV strain LaSota (ATCC VR-699) (Peeters *et al.*, 1999).

**Sequencing and cloning of full-length Herts/33 cDNA.** All standard cloning procedures were performed as described by Sambrook *et al.* (1989), unless otherwise noted. Viral RNA of Herts/33 was isolated using the Trizol procedure as described by the supplier (Invitrogen). To clone the entire Herts/33 genome, overlapping subgenomic cDNA fragments were generated by RT-PCR as described previously (de Leeuw & Peeters, 1999). The generated cDNA fragments were cloned into pGEM-T (Promega). The resulting plasmids were designated pH1, pH2, pH3, pH4 and pH5 and used for sequence analysis. The NDV-specific sequencing primers were derived from the NDV LaSota strain or from published Herts/33 sequences, or were established during this work.

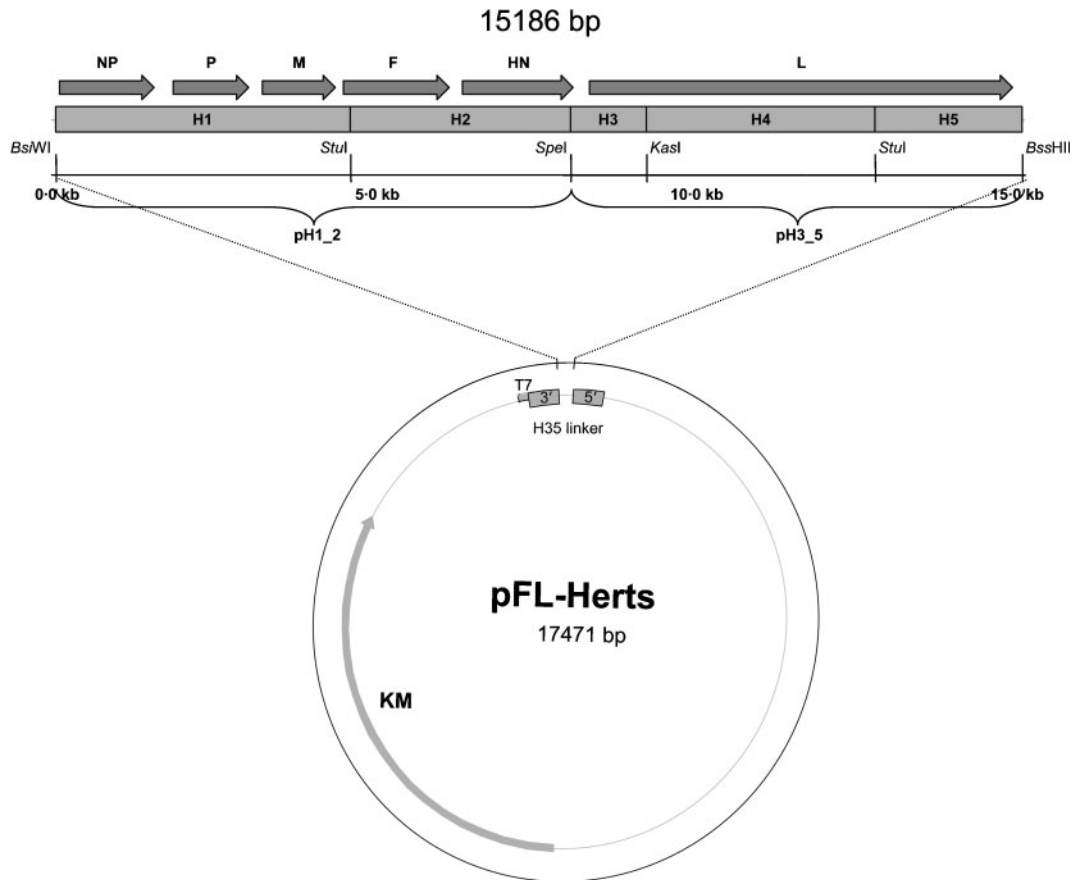
To construct a full-length cDNA clone of Herts/33, a large dsDNA H35 linker (5'-ACCAAACAGAGAATCCGTGAGTTACGATAAAA-GGCGAAGGAGCAATCGAAGTCGTACGGGTAGAAGACTAGTCAA-AGAAGCGCGCATAGTTGCTCTTGATTATACTCTCGTCATTCACC-AAATCTTTGTTTGGT-3') was obtained. The first 24 nt corresponded to the 3' end and the last 27 nt to the 5' end of NDV LaSota (de Leeuw & Peeters, 1999). The linker contained a *Bsi*WI site (bold), a *Spe*I site (underlined) and a *Bss*HII site (italic). The linker was cloned between the *Stu*I and *Sma*I sites of the transcription plasmid pOLTV5 (Peeters *et al.*, 1999). The resulting plasmid, with the H35 linker in the correct orientation, was designated pOLTV535NH. To clone the full-length Herts/33 cDNA in pOLTV535NH, the overlapping cDNA fragments H1 and H2, and H3, H4 and H5 were joined at shared restriction sites in pGEM-T. The resulting plasmids were designated pH1\_2 and pH3\_5, respectively. The *Spe*I–*Bss*HII fragment of pH3\_5 and the *Bsi*WI–*Spe*I fragment of pH1\_2 were sequentially cloned. The resulting plasmid was designated pFL-Herts (Fig. 1).

The sequences of the 3'- and 5'-terminal ends of the viral Herts/33 RNA were determined by rapid amplification of cDNA ends as described previously (de Leeuw & Peeters, 1999). The complete sequence of Herts/33 has been submitted to GenBank under accession no. AY741404.

**Cloning and expression of NP, P and L genes.** The NP, P and L genes of Herts/33 were cloned in the eukaryotic expression vector pCIneo (Promega) as described previously (Peeters *et al.*, 1999). The expression of functional NP, P and L proteins of Herts/33 was tested using the minigenome plasmid pOLTV553 (Peeters *et al.*, 2000).

**Exchanging sequences between the full-length cDNAs of pFL-Herts and pNDFL or pNDFLtag.** Conserved unique restriction sites, present in both full-length constructs, were used for the replacement of sequences of pNDFL with sequences from pFL-Herts. The *Apa*I–*Spe*I fragment of pFL-Herts, which contains the P'–HN' genes (nt 2290–8095) and the *Stu*I–*Spe*I fragment of pFL-Herts, which contains the F'–HN' genes (nt 4649–8095) were cloned in pNDFL. The resulting plasmids were designated pNDFL(P'–HN')<sup>Herts</sup> and pNDFL(F'–HN')<sup>Herts</sup>, respectively.

**Construction of pNDFLtag<sup>AF</sup>.** Using PCR mutagenesis, the unique restriction sites *Asc*I and *Fse*I were introduced into full-length cDNA of pNDFLtag (nt 4527 and 6347, respectively). To introduce the unique *Asc*I restriction site, PCR fragments were generated with pNDFLtag as template. The following pairs of primers were used: for PCR1(*Asc*I), primers p3587<sup>+</sup>



**Fig. 1.** Schematic representation of the vector pFL-Herts. pFL-Herts was assembled from subgenomic overlapping cDNA fragments H1 to H5. The cDNA fragments were joined at shared restriction sites and cloned in transcription plasmid pOLTV5 (Peeters *et al.*, 1999) in which the H35 linker was previously cloned between the *StuI* and *SmaI* sites (see Methods).

(5'-CGGAGATCTTGTGAGTTGG-3') and pAscI(F) (5'-CCCGTTGGCGCGCCCCAGGTGC-3'); for PCR2(AscI), pAscI(R) (5'-GCACCTGGGGCGCGCCAACCGGG-3') and p5390<sup>-</sup> (5'-GTAGAGTTACCTGTATACCC-3'). The two overlapping PCR fragments were joined in a second PCR using primers p3587<sup>+</sup> and p5390<sup>-</sup>. The resulting PCR fragment was digested with *PmlI* and *NofI* and cloned in pNDFLtag<sup>A</sup>. The resulting plasmid was designated pNDFLtag<sup>A</sup>. To introduce the unique *FseI* restriction site, PCR fragments were generated using the following pairs of primers: for PCR1(*FseI*), primers p4731<sup>+</sup> (5'-AAGCTCCTCCCGAATCTGCC-3') and pFseI(R) (5'-CGCAATTGAGGCGCGCCTCTCT-3'); for PCR2(*FseI*), pFseI(F) (5'-AGAGAGCCGCGCCTCAATTGCG-3') and p367 (5'-AGGGACCTCAACTAGCCAGTTC-3'). The two overlapping PCR fragments were joined in a second PCR using primers p4731<sup>+</sup> and p367. The resulting PCR fragment was digested with *NofI* and *SpeI* and cloned in pNDFLtag<sup>A</sup>. The resulting plasmid was designated pNDFLtag<sup>AF</sup>.

**Insertion of F<sup>Herts</sup>, HN<sup>Herts</sup> and the HN hybrid genes HN<sup>LH</sup> and HN<sup>HL</sup> in pNDFLtag<sup>AF</sup>.** The F gene of FL-Herts was PCR amplified using the primers FHf(AscI) (5'-TTGGCGCGCCCCAGGTGCAAGATGGGC-3') and FHr(XbaI) (5'-TCATCTGATCTAGAGTATTATTC-3'). The resulting PCR fragment was digested with *AscI* and *XbaI* (nt 6172) and ligated in the *AscI* and *XbaI* sites of pNDFLtag<sup>AF</sup>. The resulting plasmid was designated pNDFL(F)<sup>Herts</sup>. The HN gene of FL-Herts was PCR amplified using

the primers pFseI(HF) (5'-GGGGAGGCCCGCCCTCAATCGGG-3') and p8106<sup>-</sup> (5'-AATCTCAACTAGTAAAGGAACGATC-3'). The resulting PCR fragment was digested with *FseI* and *SpeI* and ligated in the *FseI* and *SpeI* sites of pNDFLtag<sup>AF</sup>. The resulting plasmid was designated pNDFL(HN)<sup>Herts</sup>. Hybrid HN genes (HN<sup>LH</sup> and HN<sup>HL</sup>) were constructed as described previously (Peeters *et al.*, 2001). The hybrid HN<sup>LH</sup> gene contained the stem region of NDFL HN (aa 1–143) and the globular head of FL-Herts HN (aa 144–561). For this hybrid gene, PCR fragments were generated with the following pairs of primers: for NDFL HN, primers pFseI(F) (5'-AGAGAGGCCCGCCCTCAATTGCG-3') and pHNLHR (5'-GACATCACT-AGCGTCATCTACAATAAGTTC-3'); for FL-Herts HN, primers pHNLHF (5'-GAACTATTGTAGATGACGCTAGTGATGTC-3') and p8106<sup>-</sup>. The two overlapping PCR fragments were joined in a second PCR using primers pFseI(F) and p8106<sup>-</sup>. For the reciprocal hybrid HN<sup>HL</sup> gene [the stem region of FL-Herts HN (aa 1–143) and the globular head of NDFL HN (aa 144–577)], PCR fragments were generated with the following pairs of primers: for FL-Herts HN, primers pFseI(HF) and pHNLHR; for NDFL HN, primers pHNLHF and p367. The two overlapping PCR fragments were joined in a second PCR using primers pFseI(HF) and p367. The PCR fragments LH and HL were digested with *FseI* and *SpeI* and ligated in *FseI* and *SpeI* sites of pNDFLtag<sup>AF</sup>. The resulting plasmids were designated pNDFLtag(HN)<sup>LH</sup> and pNDFLtag(HN)<sup>HL</sup>, respectively.

**Rescue of infectious virus.** To rescue viable virus from the various full-length (chimeric) constructs, QM5 cells were infected with FPV-T7 and co-transfected as described previously (Peeters *et al.*, 1999). One microgram of full-length cDNA, 1.6 µg pCIneoNP, 0.8 µg pCIneoP and 0.8 µg pCIneoL were used for each co-transfection. After 3 days, the culture supernatant was harvested and inoculated into the allantoic cavities of 9–11-day-old embryonated SPF eggs.

**Cloning and expression of F<sup>Herts</sup>, HN<sup>Herts</sup> and hybrid HN genes in expression vector pCIneo.** The construction of the plasmids pCIneoF<sup>LS</sup> and pCIneoHN<sup>LS</sup> has been described previously (Peeters *et al.*, 2001). To clone the F<sup>Herts</sup> gene of pFL-Herts, primers HF5XHOF (5'-GAAGCTCGAGGATCCCGTTGGCAC-3') and HF3MLUR (5'-TTACACGCGTATTGCTATTGGGGATACC-3') were used for PCR and the resulting DNA fragment was digested with *XhoI* and *MluI* and cloned in pCIneo. Expression of the F<sup>Herts</sup> protein, in the presence of an HN protein, was verified in an immunoperoxidase monolayer assay (IPMA; Wensvoort *et al.*, 1986) using monoclonal antibody 8E12A8C3 (CIDC Lelystad). To clone the HN<sup>Herts</sup> gene of pFL-Herts, primers HN5XHOF (5'-TCAYAACTCGAGTTCTACCGCWTACC-3') and HN3NOTHR (5'-GGCCTCGCGCCGATTCATTTGATCC-3') were used for PCR and the resulting DNA fragment was digested with *XhoI* and *NotI* and cloned in pCIneo. To clone the hybrid HN<sup>LH</sup> gene of pNDFLtag(HN)<sup>LH</sup>, primers HN5XHOF and HN3NOTR (5'-AGCACGCGCCGATTCGTTGATTC-3') were used for PCR and the resulting DNA fragment was digested with *XhoI* and *NotI* and cloned in pCIneo. To clone the hybrid HN<sup>HL</sup> gene of pNDFLtag(HN)<sup>HL</sup>, primers HN5MLUF (5'-TCAYAAACGCGTTTCTACCGCWTACC-3') and HN3NOTR were used for PCR and the resulting DNA fragment was digested with *MluI* and *NotI* and cloned in pCIneo. The expression of the (hybrid) HN proteins was verified in an IPMA using monoclonal antibody 5H11 (CIDC Lelystad).

**Determination of fusion activity and haemadsorption.** DNA transfections using the FuGENE 6 procedure were carried out as described previously (de Leeuw *et al.*, 2003). Fusion activity of the NDV F<sup>LS</sup> and F<sup>Herts</sup> proteins was determined microscopically 24 and 48 h after co-transfection with the different HN genes by examining syncytium formation in QM5 cells. Before observation, the QM5 cells were washed with PBS and stained for 30 min with Giemsa (Merck; diluted 1:30 in water) and washed again with PBS.

Haemadsorption was carried out as described previously (Peeters *et al.*, 2001). QM5 cells were transfected with pCIneoHN<sup>LS</sup>, pCIneoHN<sup>Herts</sup> and the hybrid genes pCIneoHN<sup>LH</sup> and pCIneoHN<sup>HL</sup> (1 µg each). Forty-eight hours after transfection, the cells were incubated for 90 min with 1% chicken red blood cells at 4 °C. The monolayers were then carefully washed three times with cold PBS and the adhesion of erythrocytes to transfected cells was examined microscopically. pCIneo was used as a negative control.

**Haemagglutination (HA) assay and pathogenicity tests.** The HA assay and determination of the ICPI in 1-day-old chickens was performed as described previously (14 July, European Community, 1992). The intravenous pathogenicity index (IVPI) in 6-week-old chickens was performed as described previously (19 May, European Community, 1992).

## RESULTS

### Nucleotide sequence of Herts/33 and comparison with LaSota

Both strands of full-length Herts/33 cDNA were sequenced using five overlapping subgenomic cDNA fragments, H1 to

H5 (see Methods). The entire genome of NDV Herts/33 consisted of 15186 nt, which is identical to the genome sizes of previously published full-length sequences of other NDV strains (Krishnamurthy & Samal, 1998; de Leeuw & Peeters, 1999; Phillips *et al.*, 1998; Römer-Oberdörfer *et al.*, 1999). The nucleotide sequence and amino acid sequence of the lentogenic strain LaSota (de Leeuw & Peeters, 1999) were compared with those of the velogenic strain Herts/33. The percentage divergence of the nucleotides between the two genomes was 12.3%. The percentage divergence of the amino acids of the NP, P, M, F, HN and L proteins was 8.0, 10.6, 9.6, 8.3, 8.2 and 4.7%, respectively. The results of this comparison also showed that the sizes of the NDV proteins were the same except for the M protein (364 aa for LaSota; 380 aa for Herts/33) and the HN protein (577 aa for LaSota; 571 aa for Herts/33). These protein length differences resulted in different lengths of the untranslated regions (UTRs) between the M and F genes (162 nt for LaSota; 114 nt for Herts/33) and the HN and L genes (238 nt for LaSota; 256 nt for Herts/33). Furthermore, the transcription start and stop signals, the signals responsible for the sequential and discontinuous RNA synthesis of viral mRNAs (Lamb & Kolakofsky, 1996), were completely conserved except for the F gene stop signal, which showed one nucleotide difference (3'-TTAAGAAAAA-5' for LaSota F gene end signal; 3'-TTAAGAAAAAG-5' for Herts/33). The number of nucleotides in the intergenic regions between the start and stop signals were the same, but the sequences differed at several positions (data not shown). The RNA sequence of the 3'-terminal end of Herts/33 was 3'-UGGUUUGUCUCUAAAGACACUCCAU-5' and the RNA sequence of the 5'-terminal end was 5'-UGGUUUGUUUCUAAACCACUUACUGUC-3'. The underlined nucleotides were different compared with the LaSota 3'- and 5'-terminal ends. The complete sequence of NDV strain Herts/33 has been submitted to GenBank under accession no. AY741404.

### Rescue of infectious Herts/33 virus from full-length cDNA

The helper plasmids pCIneoNP<sup>Herts</sup>, pCIneoP<sup>Herts</sup> and pCIneoL<sup>Herts</sup> (see Methods) were used to rescue strain Herts/33 from full-length cDNA pFL-Herts. The rescued virus was designated FL-Herts and showed an ICPI of 1.63 (Table 1), which was higher than that of NDFLtag (ICPI = 1.28) but lower than the published value of Herts/33 field strain (ICPI = 1.88). The IVPI value for FL-Herts was 2.29 (Table 2), which was slightly lower than the published IVPI value of 2.64 for the Herts/33 field strain (Collins *et al.*, 1993).

### Mapping virulence factor(s) of NDV by replacing genome sequences of NDFL or NDFLtag with corresponding genome sequences from FL-Herts

To map virulence factor(s) of NDV, sequences of pNDFL or pNDFLtag were replaced with sequences from pFL-Herts

**Table 1.** ICPI of the chimeric viruses

Virus	HA titre (log <sub>2</sub> )	Clinical view	Days post-inoculation								ICPI score†	
			1	2	3	4	5	6	7	8		
NDFL	7	Normal Sick Dead	10	10	10	10	10	10	10	10	10	0
NDFLtag	5	Normal Sick Dead	10	10		1						1.28
NDFLtag <sup>AF</sup>	6	Normal Sick Dead	10	10	1	9						1.36
FL-Herts	6	Normal Sick Dead	10	1			10	10	10	10	10	1.63
NDFL(P'-HN') <sup>Herts</sup>	6	Normal Sick Dead	10	2	8	3						1.56
NDFL(F'-HN') <sup>Herts</sup>	7	Normal Sick Dead	10		7	10	10	10	10	10	10	1.56
NDFLtag(HN) <sup>Herts</sup>	10	Normal Sick Dead	10	9	1	6	1					1.40
NDFL(F) <sup>Herts</sup>	5	Normal Sick Dead	10	10	1	9	3	1				1.31
NDFLtag(HN) <sup>LH</sup>	8	Normal Sick Dead	10	9	6	1	7	10	10	10	10	1.28
NDFLtag(HN) <sup>HL</sup>	7	Normal Sick Dead	10	10	1	7	4	2				1.31
						2	6	8	10	10	10	

†Calculated as (total numbers of sick chickens) + (total number of dead chickens × 2)/80 observations.

using shared restriction sites according to the strategy shown in Fig. 2(a). The recovered chimeric viruses NDFL(P'-HN')<sup>Herts</sup> and NDFL(F'-HN')<sup>Herts</sup> grew to similar titres as the parent strain NDFL (data not shown). Both viruses showed an ICPI value of 1.56, which was comparable with the ICPI value of the reference FL-Herts virus (Table 1). This means that virulence is determined by sequences between the *StuI* site (nt 4649) and the *SpeI* site (nt 8094) of the FL-Herts sequence.

pNDFL(F)<sup>Herts</sup> and pNDFLtag(HN)<sup>Herts</sup> were constructed according to the strategy shown in Fig. 2(b). To this end, pNDFLtag<sup>AF</sup> with two new unique restriction sites, *AscI* and *FseI*, was constructed (see Methods). The ICPI of the rescued virus NDFLtag<sup>AF</sup> showed a value that was slightly higher than that of NDFLtag (Table 1).

The rescued virus NDFL(F)<sup>Herts</sup> contained the F protein of FL-Herts except for the last 10 aa, which were derived from

the F protein of NDFL, and showed an ICPI value of 1.31, which was comparable with the ICPI value of NDFLtag (Table 1). Interestingly, NDFL(F)<sup>Herts</sup> showed an IVPI value of 0.41, which was much lower than that of FL-Herts and even lower than that of NDFLtag (Table 2). NDFLtag(HN)<sup>Herts</sup>, which contained the HN gene of FL-Herts, showed an ICPI value of 1.40 and an IVPI value of 1.83 (Tables 1 and 2). These results showed that the HN protein is involved in virulence. However, NDFLtag(HN)<sup>Herts</sup> did not reach the virulence level of the reference FL-Herts strain.

The HN protein is a type II membrane glycoprotein and contains a long stem region supporting the globular head (Lamb & Kolakofsky, 1996). The hybrid HN genes HN<sup>LH</sup> and HN<sup>HL</sup> were generated to investigate whether both the stem region and the globular head contribute to virulence. HN<sup>LH</sup> consists of the first 143 aa of NDFL and aa 144–561 of FL-Herts. Hybrid HN<sup>HL</sup> consists of the first 143 aa of

**Table 2.** IVPI of the chimeric viruses

Virus	HA titre (log <sub>2</sub> )	Clinical view	Days post-inoculation										IVPI score†
			1	2	3	4	5	6	7	8	9	10	
NDFLtag	5	Normal	10	10	10	7	5	5	4	3	3	3	0·76
		Sick				3	2	2	2	3			
		Very sick					3	3	3	3	4	4	
		Dead							1	1	3	3	
FL-Herts	6	Normal	8										2·29
		Sick	2	10	2								
		Very sick			8	6	3	2					
		Dead				4	7	8	10	10	10	10	
NDFL(F'-HN') <sup>Herts</sup>	8	Normal	10	1									2·58
		Sick		1									
		Very sick		7									
		Dead		1	10	10	10	10	10	10	10	10	
NDFLtag(HN) <sup>Herts</sup>	10	Normal	10	4	1			1	1				1·83
		Sick		6	3	1	1	0	0	1	1		
		Very sick			6	8	6	6	4	3	3	4	
		Dead			1	3	3	5	6	6	6	6	
NDFL(F) <sup>Herts</sup>	5	Normal	10	10	10	9	8	7	7	6	4	4	0·41
		Sick				1	1	2	1	2	4	4	
		Very sick					1	1	1	1			
		Dead							1	1	2	2	
NDFLtag(HN) <sup>LH</sup>	8	Normal	10	10	10	4	2						1·52
		Sick				5	1	2					
		Very sick				1	3	4	4	4	4	4	
		Dead				0	4	4	6	6	6	6	
NDFLtag(HN) <sup>HL</sup>	7	Normal	10	10	9	2							1·82
		Sick			1	1	1						
		Very sick				6	6	3	2	2			
		Dead				1	3	7	8	8	10	10	

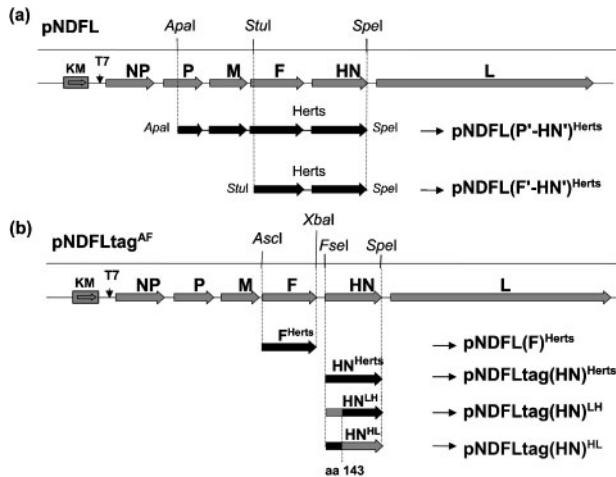
†Calculated as (total numbers of sick chickens)+(total numbers of very sick chickens × 2)+(total number of dead chickens × 3)/100 observations.

FL-Herts and aa 143–577 of NDFL. Both genes were cloned in pNDFLtag<sup>AF</sup> (Fig. 2b). The rescued viruses NDFLtag(HN)<sup>LH</sup> and NDFLtag(HN)<sup>HL</sup> showed ICPI values that were comparable to those of NDFLtag and were lower than those of FL-Herts (Table 1). However, the IVPI values of NDFLtag(HN)<sup>LH</sup> and NDFLtag(HN)<sup>HL</sup> were similar to the values of NDFLtag(HN)<sup>Herts</sup> and much higher than that of NDFLtag (Table 2). These results showed that both the stem region (aa 1–143) and the globular head (aa 144–561) of the HN protein of FL-Herts are involved in determining virulence. Based on the IVPI values, the contribution to virulence of the stem region of the HN protein was comparable with the contribution of the entire HN protein.

#### Effect of the HN proteins on fusion activity of NDV F<sup>LS</sup> (LaSota) and F<sup>Herts</sup>

The HN protein is involved in attachment and release and in the promotion of fusion by cooperating with the F

protein. To determine the fusion activity of the F protein in combination with the different HN proteins, the fusion activity of NDV F<sup>LS</sup> and F<sup>Herts</sup> proteins was determined microscopically 24 and 48 h after transfection by examining syncytium formation in QM5 cells. An interesting observation was that F<sup>Herts</sup> showed much more syncytium formation than F<sup>Tag</sup>. Syncytium formation induced by F<sup>Tag</sup> was observed at only a few locations in the QM5 monolayer more than 48 h after co-transfection with any of the HN genes (data not shown). In contrast, by 24 h after co-transfection with F<sup>Herts</sup>, syncytia of QM5 cells were clearly visible. The proteins HN<sup>LS</sup> and HN<sup>LH</sup>, after co-transfection with F<sup>Herts</sup>, showed more syncytium formation than HN<sup>Herts</sup> and HN<sup>HL</sup>. In order of rank at 24 h post-transfection, HN<sup>LH</sup> showed the most syncytia directly followed by HN<sup>LS</sup> and then HN<sup>Herts</sup>. HN<sup>HL</sup> did not show syncytium formation after 24 h, but did after 48 h (Fig. 3a and b). This result showed that the stem region of LaSota could increase the fusion activity of the F<sup>Herts</sup> protein.



**Fig. 2.** (a) Strategy for cloning the *Apal*–*SpeI* (nt 2290–8095) and *StuI*–*SpeI* (nt 4649–8095) cDNA fragments of FL-Herts in pNDFL. This cloning strategy resulted in the plasmids pNDFL(P'-HN')<sup>Herts</sup> and pNDFL(F'-HN')<sup>Herts</sup>, respectively. (b) Cloning strategy for the F<sup>Herts</sup>, HN<sup>Herts</sup> and hybrid genes HN<sup>LH</sup> and HN<sup>HL</sup> in pNDFLtag<sup>AF</sup>. pNDFLtag<sup>AF</sup> contains two unique restriction sites in the genome sequence of pNDFL cDNA. The F gene was cloned between the restriction sites *AscI* (nt 4527) and *XbaI* (nt 6172). The HN genes were cloned between the restriction sites *FseI* (nt 6347) and *SpeI* (nt 8095). The cDNA fragments of FL-Herts are represented by black bars. aa 143 is the junction between the stem region and the globular head of the HN protein.

Attachment of the HN protein to the cellular receptor is the first step in the fusion process. To determine differences in attachment of the HN proteins that could explain differences in the fusion activity of the F protein, a haemadsorption assay was performed, which showed no changes in the ability of the HN proteins to bind chicken red blood cells (Fig. 3c).

The transfection efficiencies of F<sup>Tag</sup>, F<sup>Herts</sup> and the HN genes were verified in an IPMA using monoclonal antibodies 8E12A8C3 (F protein) and 5H11 (HN protein). After 24 h, immunological staining of the expressed F and HN genes did not show any significant differences in the number of positive cells (data not shown). Interestingly, the F<sup>Herts</sup> protein could only be detected with antibody 8E12A8C3 when the HN protein (either HN<sup>LS</sup>, HN<sup>Herts</sup>, HN<sup>HL</sup> or HN<sup>LH</sup>) was present (data not shown).

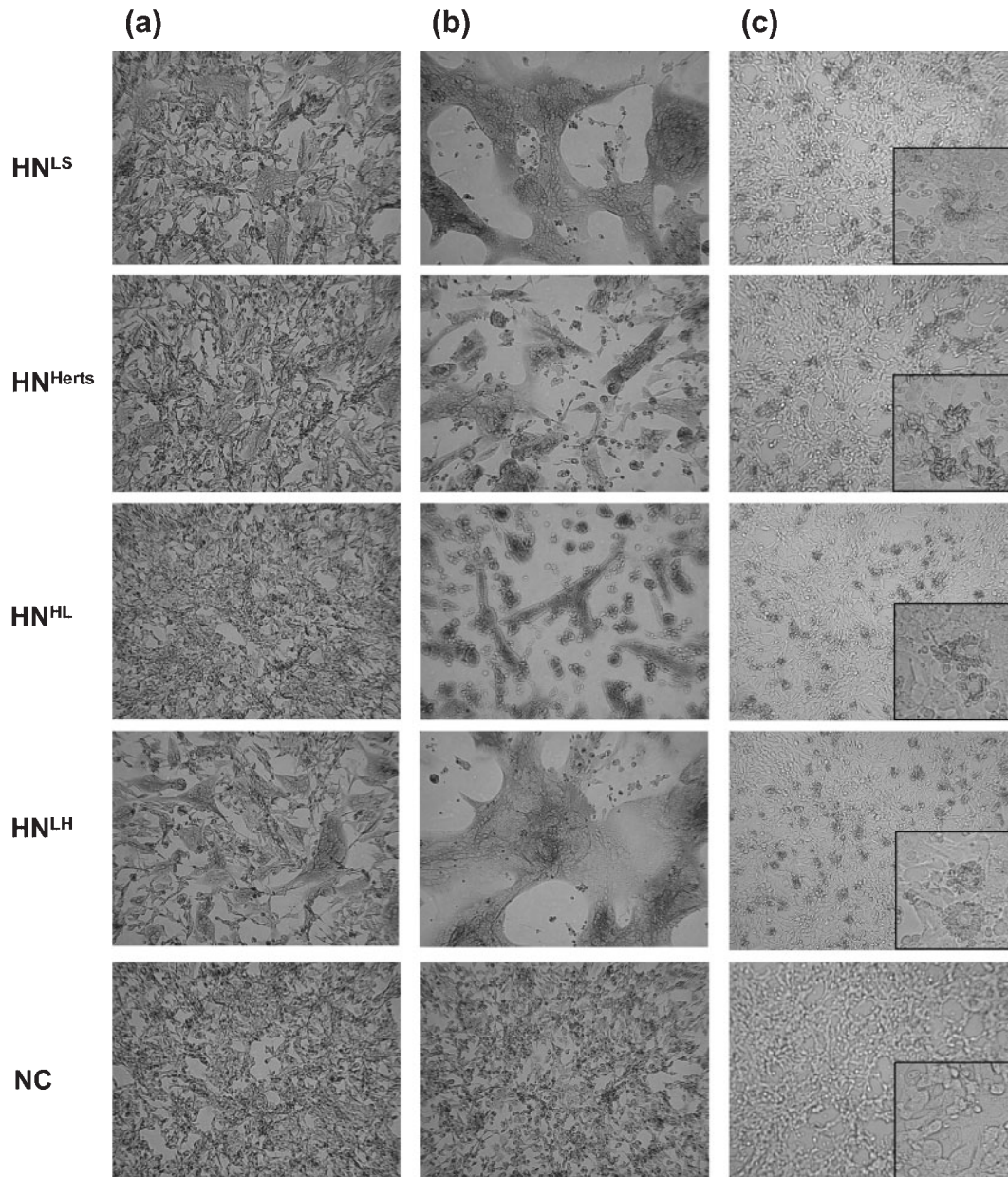
## DISCUSSION

Data from recent publications have implied that the pathogenesis of NDV is determined not only by its F protein cleavage site but also by other factors in its genome (Peeters *et al.*, 1999; Brown *et al.*, 1999; Römer-Oberdörfer *et al.*, 2003). To map additional virulence factor(s) of NDV, we made chimeric viruses consisting of sequences of a highly

virulent and a non-virulent strain. To do this, we generated an infectious cDNA clone of the virulent strain Herts/33 (FL-Herts; Fig. 1). Previously, we had generated the infectious clones NDFL (lentogenic) and NDFLtag (mesogenic), which were derived from NDV strain LaSota (Peeters *et al.*, 1999). The pathogenicity of FL-Herts was determined using both ICPI and IVPI tests. FL-Herts showed an ICPI value of 1.63 and an IVPI value of 2.29 (Tables 1 and 2). Both values were lower than values published in the literature (ICPI = 1.88 and IVPI = 2.64; Collins *et al.*, 1993) but were within the range of velogenic values. The lower values could be ascribed to the heterogeneity of the Herts/33 population. FL-Herts was generated from a cDNA clone, which may not represent the consensus of the Herts/33 population. In addition, the 3' and 5' ends of FL-Herts were derived from LaSota. The three nucleotide differences at the 3' end or the two nucleotide differences of the 5' end could perhaps decrease the virulence of FL-Herts compared with the parental Herts/33 strain.

To map roughly the virulence factors of FL-Herts, we first constructed the chimeric viruses NDFL(P'-HN') and NDFL(F'-HN'). These both showed an ICPI value of 1.56, comparable to the reference FL-Herts strain (Table 1). These ICPI results suggested that the virulence of FL-Herts was almost completely determined by the sequence located between nt 4649 (*StuI*) and nt 8094 (*SpeI*). This sequence contains the F and HN genes plus the non-coding region between them. Thus, the NP, P, M and L genes and their intergenic non-coding regions did not appear to contribute to the difference in virulence between LaSota and Herts. However, based on the IVPI results of FL-Herts (IVPI = 2.29) and NDFL(F'-HN') (IVPI = 2.58), it could not be excluded that NP, P, M, L or even the V protein are involved in the virulence of NDV. It is important to note that the chimeric virus NDFL(F'-HN') did not contain the complete FL-Herts F and HN genes. The first 35 aa of the F protein and the last 16 aa of the HN protein were derived from NDFL. This means that the first 35 aa of the F protein are not involved in virulence and that the last amino acids from aa 561 to 571 of the HN protein (571 aa is the length of the HN protein of FL-Herts) can be substituted with amino acids from NDFL HN. Even enlargement of the protein with 6 aa from NDFL HN (aa 572–577) did not influence the virulence. Similar results were obtained by Römer-Oberdörfer *et al.* (2003), who showed that velogenic recombinant strains with different HN lengths (616, 577 and 571 aa) showed similar ICPI values.

We subsequently determined the contribution of the F<sup>Herts</sup> and HN<sup>Herts</sup> proteins. The ICPI value of NDFL(F)<sup>Herts</sup> clearly showed that the F protein cleavage site was the only virulence determinant on the F protein of FL-Herts. The level of virulence was comparable with NDFLtag, which contained the same cleavage site (Table 1). In contrast, replacement of the HN protein by HN<sup>Herts</sup> resulted in a remarkable increase in virulence. This effect of HN<sup>Herts</sup> on virulence was most prominent after intravenous inoculation



**Fig. 3.** Syncytium formation in co-transfection experiments with pCIneoF<sup>Herts</sup> and pCIneoHN<sup>LS</sup>, pCIneoHN<sup>Herts</sup> and the hybrid genes pCIneoHN<sup>LH</sup> and pCIneoHN<sup>HL</sup>. (a, b) After 24 (a) and 48 (b) h, the monolayers were washed with PBS and stained with Giemsa. The negative control (NC) was pCIneoF<sup>Herts</sup>. Magnification  $\times 63$ . (c) Haemadsorption was examined 48 h after transfection with pCIneoHN<sup>LS</sup>, pCIneoHN<sup>Herts</sup> and the hybrid genes pCIneoHN<sup>LH</sup> and pCIneoHN<sup>HL</sup>. After incubation for 90 min with chicken red blood cells at 4 °C, cells were washed three times and adhesion of erythrocytes to transfected cells was examined microscopically. The negative control (NC) was pCIneo. Magnification  $\times 63$  and  $\times 200$  (inserts). The amount of plasmid DNA used for each transfection was 1  $\mu$ g.

(IVPI), which suggested that the HN protein is involved in the tropism of NDV. This result is in agreement with the data of Huang *et al.* (2004), who showed that the amino acid differences between the NDV HN proteins of the Beaudette and LaSota strains determined tropism and virulence. However, the virulence of NDFLtag(HN)<sup>Herts</sup>

(ICPI = 1.40; IVPI = 1.83) did not reach the virulence levels of NDFL(F'-HN')<sup>Herts</sup> (ICPI = 1.56; IVPI = 2.58) and the reference FL-Herts strain (ICPI = 1.63; IVPI = 2.29). This lower virulence of NDFLtag(HN)<sup>Herts</sup> could be explained by suboptimal interaction between the LaSota F protein and the Herts HN protein. This could also be the reason why



NDFL(F)<sup>Herts</sup> (IVPI=0.41) showed a lower IVPI value compared with NDFLtag (IVPI=0.76) (Table 2).

It has been shown that the stem region of HN is responsible for a type-specific functional interaction with the F protein. However, it is unclear whether this interaction triggers fusion or whether the release of the HN protein mediates conformational changes in the F protein leading to fusion (Deng *et al.*, 1997; Morrison, 2003). Based on the results of syncytium formation of QM5 cells, fusion activity of F<sup>Tag</sup> was not visible 48 h after co-transfection with any of the HN genes (HN<sup>LS</sup>, HN<sup>Herts</sup>, HN<sup>LH</sup> or HN<sup>HL</sup>). Thus, the lower virulence of NDFLtag(HN)<sup>Herts</sup> could not be explained by a difference in fusion activity of F<sup>Tag</sup> in combination with the different HN proteins. However, F<sup>Herts</sup> clearly showed syncytium formation of QM5 cells with the different HN proteins (Fig. 3). Thus, the amino acid sequence of the F<sup>Herts</sup> protein, which is different from F<sup>Tag</sup>, must determine this efficient syncytium formation. Although the transfection efficiency of F<sup>Tag</sup> and F<sup>Herts</sup> were comparable, detection of F<sup>Herts</sup> with monoclonal antibody 8E12A8C3 was only possible in the presence of HN (data not shown), which suggested conformational differences between F<sup>Herts</sup> and F<sup>Tag</sup>. These differences could be responsible for their different fusion activities. Furthermore, the fusion activity of F<sup>Herts</sup> was higher in combination with HN<sup>LS</sup> compared with HN<sup>Herts</sup>. This higher fusion activity of F<sup>Herts</sup> with HN<sup>LS</sup> was not due to differences in haemadsorption between HN<sup>LS</sup> and HN<sup>Herts</sup> (Fig. 3). In view of the virulence of NDFL(F)<sup>Herts</sup> (ICPI=1.31; IVPI=0.41) and NDFL(F'-HN')<sup>Herts</sup> (ICPI=1.56; IVPI=2.58), the latter suggests that increased fusion activity does not result in higher virulence. This is in agreement with the results of von Messling *et al.* (2001) who showed that canine distemper viruses that produced more syncytia were also less virulent. However, co-expression of F<sup>Herts</sup> and HN<sup>Herts</sup> also clearly showed syncytia (Fig. 3) although the virulence of FL-Herts (ICPI=1.63; IVPI=2.29) was very high. This suggests that fusion activity is not directly associated with the virulence of NDV.

An alternative explanation for the lower virulence of NDFLtag(HN)<sup>Herts</sup> compared with NDFL(F'-HN')<sup>Herts</sup> and FL-Herts could be that NDFLtag(HN)<sup>Herts</sup> contains the UTR between F and HN of NDFLtag. In this UTR, the F gene transcription stop signal of NDFL (3'-TTAAG-AAAAA-5') showed 1 nt difference compared with FL-Herts (3'-TTAAGAAAAAG-5'). This nucleotide sequence difference of the F gene end signal or other nucleotide sequence differences between the F-HN UTR of NDFL and FL-Herts could result in different amounts of transcripts of the F and HN genes and thus of the F and HN proteins, which could influence the functional interaction between the F and HN proteins on the viral envelope, thereby affecting the virulence of NDV. This is in agreement with the results of Rassa & Parks (1999), who showed that the sequence diversity at a simian virus 5 (SV5) gene junction may differentially affect SV5 gene expression.

The globular head of the HN protein mediates attachment

of the virus to sialic acid-containing receptors on the surface of the target cell and contains neuraminidase activity for the release of the virions from infected cells (Lamb & Kolakofsky, 1996). These important molecular determinants could be involved in the virulence of NDV. However, the IVPI of NDFLtag(HN)<sup>LH</sup> and NDFLtag(HN)<sup>HL</sup> showed that both the stem region and the globular head of FL-Herts HN were involved in virulence. Interestingly, the stem region of FL-Herts HN in the virus NDFLtag(HN)<sup>HL</sup> was sufficient to show IVPI values similar to NDFLtag(HN)<sup>Herts</sup>. Deng *et al.* (1999) reported that amino acid sequences from different HN proteins in the F-specific domain in the protein stem could change the interaction between the HN and F protein. Alignment of the amino acid sequences of the HN proteins of FL-Herts and NDFL (data not shown) showed that the stem region is less conserved than the globular head [percentage divergence of the stem region (aa 1-143)=13.3% and globular head (aa 144-561)=6.5%]. A possible explanation for the virulence of NDFLtag(HN)<sup>HL</sup> is that the interaction between the stem region of the FL-Herts HN protein with the specific domain of the F protein (probably the HR2 domain; Gravel & Morrison, 2003) could induce a conformational change in the NDFL HN globular head, which improves the binding of the NDFL HN globular head to host-cell receptors and/or could affect the neuraminidase activity. In NDFL(HN)<sup>LH</sup>, the conformation of the globular head of FL-Herts HN is probably changed by the stem region of NDFL HN in such a way that receptor binding and/or neuraminidase activity are not optimal, resulting in a slightly lower IVPI value. Recently the crystal structure of the globular head (aa 124-570) of NDV HN has been published (Crenell *et al.*, 2000). The crystal structure indeed showed that the globular head of HN appears to be a dynamic molecule that switches from one conformational state to another, resulting in a change of an active site, which is responsible for both receptor binding and neuraminidase activity.

In summary, the F protein cleavage site primarily determines the virulence of NDV. When the F protein contains the cleavage site of a velogenic type, it can be cleaved by host proteases that are available in a wide range of different cells. The ability of NDV to reach these cells probably depends on the activities of the HN protein, which is involved in both attachment and release and which activates the F protein to promote fusion (Morrison, 2003). In this report, we showed that both the stem region and the globular head of the FL-Herts HN protein were involved in the virulence of NDV. Thus, both the stem region and globular head of HN are probably necessary to exert its biological functions. Future work will focus on the location of the specific amino acids of the HN protein that are responsible for the mechanism by which it determines NDV virulence and on the determination of tropism in chickens of the different recombinant NDV viruses using the natural infection route, i.e. the respiratory tract.

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