Journal of General Virology (2003), **84**, 475–484 DOI 10.1099/vir.0.18714-0

Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain

Olav S. de Leeuw,[1] Leo Hartog,[2] Guus Koch[2] and Ben P. H. Peeters[1]

[1]Department of Infectious Disease & Food Chain Quality, Institute for Animal Science and Health, PO Box 65, NL-8200 AB Lelystad, The Netherlands

[2]Central Institute for Animal Disease Control, PO Box 2004, NL-8203 AA Lelystad, The Netherlands

Correspondence
Ben Peeters
b.p.h.peeters@id.wag-ur.nl

Received 15 July 2002
Accepted 13 September 2002

Virulence of Newcastle disease virus (NDV) is mainly determined by the amino acid sequence of the fusion (F0) protein cleavage site. Full-length NDV cDNA clone pNDFL was used to generate infectious NDV with defined mutations in the F0 cleavage site (RRQRR ↓ **L**, **G**RQ**G**R ↓ F, RRQ**G**R ↓ F, R**G**QRR ↓ F and R**K**Q**K**R ↓ F). All the mutants were viable and the mutations were maintained after virus propagation in embryonated eggs. The mutants showed single-cell infections on chicken embryo fibroblasts, which suggested that they were non-virulent. However, virulence tests in 1-day-old chickens resulted in an intracerebral pathogenicity index (ICPI) between 0 and 1·3. Moreover, virulent virus was isolated from chickens that had died in the virulence tests. Subsequent sequence analysis showed that the mutants RRQRR ↓ **L,** RRQ**G**R ↓ F, R**G**QRR ↓ F and R**K**Q**K**R ↓ F gave rise to the appearance of revertants containing the virulent cleavage site RRQ(K/R)R ↓ F and an ICPI of 1·4 or higher. This indicated that reversion to virulence was caused by alteration of the amino acid sequence of the F0 cleavage site from a non-virulent to a virulent type. Furthermore, the ICPI of the revertants was higher than that of cDNA-derived strain NDFLtag, which has the same cleavage site, RRQRR ↓ F (ICPI=1·3). NDFLtag^{Pass}, which was isolated from dead chickens after intracerebral inoculation of NDFLtag, also showed an increase in the ICPI from 1·3 to 1·5. This study proves that reversion to virulence occurs within non-virulent NDV populations and that the virulence may increase after one passage in chicken brain.

INTRODUCTION

Newcastle disease is a serious disease in many species of birds, both domestic and wild. Because chickens are the most susceptible birds, the disease can cause serious economic problems in the poultry industry worldwide (Alexander, 2000, 2001). The virus that causes this avian disease is Newcastle disease virus (NDV) a member of the *Rubulavirus* genus of the subfamily *Paramyxovirinae* (family *Paramyxoviridae*, order *Mononegavirales*) (Murphy *et al.*, 1995). However, recent studies suggest that NDV should be assigned to a new genus (de Leeuw & Peeters, 1999; Seal *et al.*, 2000; Westover & Hughes, 2001).

NDV contains a non-segmented single-stranded RNA genome of negative polarity with a size of 15 186 nucleotides (Krishnamurthy & Samal, 1998; de Leeuw & Peeters, 1999; Phillips *et al.*, 1998; Römer-Oberdorfer *et al.*, 1999) and contains six genes encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase protein (HN) and large polymerase protein (L) (Lamb & Kolakofsky, 1996).

NDV is an enveloped virus with two membrane proteins, one of which is involved in cell attachment and release (HN protein) and the other in mediating fusion of the viral envelope with cellular membranes (F protein). The F protein is synthesized as a precursor, F0, and is only fusogenic after cleavage into disulfide-linked F1 and F2 polypeptides. This cleavage is accomplished by host-cell proteases (Nagai *et al.*, 1976; Morrison *et al.*, 1985; Ogasawara *et al.*, 1992). Thus, NDV becomes infective only when the precursor glycoprotein F0 is cleaved into F1 and F2. The ability to cleave F0 varies among different strains of NDV and is the main determinant of mortality and morbidity in infected chickens. Using mean death time (MDT) determined in chicken eggs, NDV strains can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic) (Beard & Hanson, 1984). Within the highly virulent NDV strains, two types of virus can be distinguished. These strains are termed neurotropic velogenic or

viscerotropic velogenic, based on the clinical signs induced (Alexander, 2000).

Phylogenetic studies at the molecular level of the NDV F0 cleavage site have determined the consensus amino acid sequence ^{112}R/K-R-Q-R/K-R-F^{117} for virulent and mesogenic strains and ^{112}G/E-K/R-Q-G/E-R-L^{117} for NDV strains of low virulence (Collins *et al.*, 1993). However, two exceptions to the consensus amino acid sequence for virulent strains have been observed for the so-called pigeon paramyxovirus type 1 (PPMV-1) isolates. These virulent isolates show fusion protein cleavage sites of ^{112}**G**RQKRF^{117} (Collins *et al.*, 1993) and ^{112}RR**K**KRF^{117} (Werner *et al.*, 1999).

The different F0 protein cleavage sites are the substrates for different kinds of cellular proteases (Kawahara *et al.*, 1992; Sakaguchi *et al.*, 1991). The F0 of lentogenic viruses can be cleaved only by trypsin-like enzymes, as found in the respiratory and intestinal tracts, whereas F0 of virulent viruses can be cleaved by a host protease (or proteases) 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). Until now, it has not been clear which specific host-cell protease is responsible for cleavage of the different F0 proteins. However, velogenic and mesogenic strains show a clear dibasic amino acid motif (K/R residues at amino acid positions 112/113 and 115/116) with a phenylalanine (F^{117}) at the N terminus of the F1 protein after cleavage (Glickman *et al.*, 1988; Morrison *et al.*, 1993; Toyoda *et al.*, 1987). This sequence motif is most comparable with the motif of the furin family of proteases (Fujii *et al.*, 1999; Sakaguchi *et al.*, 1994). In contrast to the velogenic and mesogenic strains, lentogenic strains show a monobasic motif and a leucine (L^{117}) at the N terminus of the F1 protein. This sequence motif is recognized by a factor X-like protease (vitamin K-dependent serine protease of the prothrombin family), which is present in the allantoic fluid and the amniotic fluid of the chicken egg (Gotoh *et al.*, 1990; Ogasawara *et al.*, 1992). In conclusion, there is a correlation between virulence or pathogenicity and a high content of basic amino acid residues at the F0 cleavage site.

The F0 protein is able to induce cell fusion only when the F0 protein is cleaved into F1 and F2. The HN protein is also needed in the promotion of fusion to co-operate with the F protein (Deng *et al.*, 1997). Syncytium formation by cells can be observed when the F0 protein and HN protein are co-expressed after co-transfection of plasmids containing the F and HN genes (Li *et al.*, 1998; Peeters *et al.*, 2001). This assay can be used to examine the effect of specific amino acid mutations on cleavability of the F0 protein.

Mutagenesis of the amino acids of a velogenic F0 cleavage site has recently been carried out to investigate which amino acid mutation influences syncytium formation (cell fusion) after co-transfection of cultured cells with F and HN genes (Fujii *et al.*, 1999; Li *et al.*, 1998). These data clearly showed that the dibasic structure as well as the phenylalanine at position 117 is necessary for the host-cell protease to cleave F0 and to form a biologically active complex with the HN protein. Using reverse genetics techniques, we recently established an infectious clone of NDV and we showed that modification of the lentogenic cleavage site ^{112}GRQGR↓L^{117} into the consensus sequence of virulent strain ^{112}RRQRR↓F^{117} increased the intracerebral pathogenicity index (ICPI) from 0 to 1·3 (Peeters *et al.*, 1999). These results confirmed that the presence of one or both arginines at positions 112 and 115 and/or the phenylalanine at residue 117 are necessary for efficient cleavage of the F0 protein and result in an increase in virulence.

With the already available data on the amino acid sequences of the F0 cleavage site, we wanted to examine different F0 cleavage site mutations at the whole-virus level. Li *et al.* (1998) showed that mutations of the F0 cleavage site such as K115G, F117L and R113G/K115G were fusion-negative. However, all mutant F0 proteins activated fusion after the addition of exogenous trypsin. Thus, it should be possible to generate viruses with different F0 cleavage sites that can grow in an environment with trypsin-like proteases (e.g. allantoic fluid and the amniotic fluid of the embryonated chicken egg). If it is possible to generate viruses with mutations of the F0 cleavage site, then the next step is to investigate the effect of those mutations on virulence and pathogenesis.

In this study, we have used our full-length NDV cDNA clone to generate infectious NDV with defined mutations in the F0 protease cleavage site. The following F0 cleavage site mutants were generated: RRQRR↓**L**, **G**RQ**G**R↓F, RRQ**G**R↓F, R**G**QRR↓F and R**K**Q**K**R↓F. Here, we show that all the F0 cleavage site mutants were viable and that the mutations were maintained after virus propagation in embryonated eggs. The F0 cleavage site mutants were non-virulent based on plaque assays in cell culture. However, after intracerebral inoculation in 1-day-old chickens, virulent revertants were isolated from infected animals.

METHODS

Cells and viruses. QM5 cells (Antin & Ordahl, 1991) were grown in medium supplied by Gibco BRL/LifeTechnologies (Cat. no. 041-91536; proprietary composition of Fort Dodge). Primary chicken embryo fibroblast (CEF) cells were grown in the Glasgow modification of Eagle's medium/Eagle's minimal essential medium (1:1; both from ICN). Both media were supplemented with 5% foetal calf serum (FCS) 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.

The strains NDFL and NDFLtag were obtained from the NDV strain La Sota (ATCC VR-699), as previously described (Peeters *et al.*, 1999).

Modification of the protease cleavage site of the F0 protein. All standard cloning procedures were performed as described in Sambrook *et al.* (1989), unless otherwise noted. Mutagenesis reactions on the sequence encoding the protease cleavage site of the F0

Table 1. Nucleotide and deduced amino acid sequences of the region encompassing the F0 cleavage site, and syncytium formation by QM5 cells after co-transfection with the F0 cleavage site mutants and pCIneo-HN^{LS}

The nucleotide changes that were introduced to modify the amino acid sequences of the cleavage site are underlined. The resulting amino acid changes are shown in bold. The arrow indicates the site of cleavage.

F0 cleavage site mutant	Sequence						Syncytium formation
F^{LS} (La Sota)	GGG	AGA	CAG	GGG	CGC	CTT	−
	G	R	Q	G	R	L	
F^{WT} (tag)	AGG	AGA	CAG	CGG	CGC	TTT	+
	R	R	Q	R	R	F	
F^{FM}	AGG	AGA	CAG	CGG	CGC	CTT	−
	R	R	Q	R	R	**L**	
F^{FM1}	GGG	AGA	CAG	GGG	CGC	TTT	−
	G	R	Q	**G**	R	F	
F^{FM2}	AGG	AGA	CAG	GGG	CGC	TTT	−
	R	R	Q	**G**	R	F	
F^{FM3}	AGG	GGA	CAG	CGG	CGC	TTT	−
	R	**G**	Q	R	R	F	
F^{FM4}	AGG	AAA	CAG	AAG	CGC	TTT	−
	R	**K**	Q	**K**	R	F	
Amino acid position	112	113	114	115	116 ↓	117	

protein were carried out using PCR as described previously (Peeters *et al.*, 1999). The same procedure was used for the new F0 cleavage mutants, which are listed in Table 1.

The first PCR fragment was generated using the forward primer NDV5Fxho (5′-GAAGCTCGAGGATCCCGGTTGGCAC-3′) and one of the following reverse primers: Ffmr (5′-AAGGCG-CCGCTGTCTCCTCCCTCCAGATGTAGTCAC-3′); Ffm1r (5′-AAAGCGCCCCTGTCTCCCCCCTCCAGATGTAGTCAC-3′); Ffm2r (5′-AAAGCGCCCCTGTCTCCTCCCTCCAGATGTAGTCAC-3′); Ffm3r (5′-AAAGCGCCGCTGTCCCCTCCCTCCAGATGTAGTCAC-3′); Ffm4r (5′-AAAGCGCTTCTGTTTCCTCCCTCCAGATGTAGTCAC-3′); or Ffm5r (5′-AAAGCGCTTCTGTCTCCTCCCTCCAGATGTAGTCAC-3′). The second PCR fragment was generated using one of the following forward primers: Ffmf (5′-GGAGGAGACAGCGGC-GCCTTATAGGCGCCATTATTGG-3′); Ffm1f (5′-GGGGGA-GACAGGGGCGCTTTATAGGCGCCATTATTGG-3′); Ffm2f (5′-GGAGGAGACAGGGGCGCTTTATAGGCGCCATTATTGG-3′); Ffm3f (5′-GGAGGGGACAGCGGCGCTTTATAGGCGCCATTATTGG-3′); Ffm4f (5′-GGAGGAAACAGAAGCGCTTTATAGGCGCCATTATTGG-3′); or Ffm5f (5′-GGAGGAGACAGAAGCGCTTTATAGGCGCCATTATTGG-3′) and the reverse primer NDV3Fmlu (5′-TTACACGCGTATT-GCTATTGGGGATACC-3′). The two overlapping PCR fragments were joined in a second PCR by using primers NDV5Fxho and NDV3Fmlu. The resulting fragments, which contained the entire open reading frame of the F gene, were cloned in pCIneo (Promega) between the *Xho*I and *Mlu*I sites, yielding pCIneo-F^{fm}, pCIneo-F^{fm1}, pCIneo-F^{fm2}, pCIneo-F^{fm3} and pCIneo-F^{fm4}. In order to introduce the F0 mutations in the full-length NDV cDNA, the *Stu*I–*Not*I fragment (nt 4646–4952) of pNDFL (Peeters *et al.*, 1999) was replaced by the *Stu*I–*Not*I fragments of the pCIneo constructs F^{FM}–F^{FM4}. The resulting plasmids were designated pNDFL-F^{fm}, -F^{fm1}, -F^{fm2}, -F^{fm3}, -F^{fm4} and F^{fm5}, respectively.

Co-transfections. DNA transfections using Fugene6 were carried out essentially as recommended by the manufacturer (Roche). QM5 cells were grown in six-well culture dishes and after 24 h the cells were infected at an m.o.i. of 1 with FPV-T7 for 1 h at 37 °C. The cells were washed with PBS and co-transfected with 1 μg of the various pCIneo-F constucts and 1 μg pCIneo-HN in QM5 medium containing 5 % allantoic fluid, 5 % FCS and antibiotics. After 48 h, expression of the F0 protein was verified in an immunoperoxidase monolayer assay (Wensvoort *et al.*, 1986) using mAb 8E12A8C3 (ID-Lelystad).

Fusion activity of NDV F0 cleavage site mutants was determined microscopically 48 h post-transfection by examining syncytium formation of QM5 cells. Before observation, the QM5 cells were washed with PBS, stained for 30 min with Giemsa (Merck; 1 : 30 dilution in water) and washed again with PBS.

Rescue of infectious virus. To rescue viable virus from the various full-length pNDFL-F constucts, QM5 cells were infected with FPV-T7 and co-transfected as describe above. One μg pNDFL-F, 0·8 μg pCIneo-NP, 0·4 μg pCIneo-P and 0·4 μg pCIneo-L were used for each co-transfection. After 3 days, the culture supernatant was harvested and inoculated into the allantoic cavities of 8–11-day-old embryonated specific pathogen-free (SPF) eggs (Peeters *et al.*, 1999).

Haemagglutination (HA) assay and pathogenicity tests. The HA assay and determination of the intracerebral pathogenicity index (ICPI) in 1-day-old chickens was performed as described in the European Community Council Directive 92/66/EEC (14 July 1992).

Re-isolation of virulent virus from dead chickens. Brains, livers and lungs were collected from three dead chickens and homogenized with a mortar and sterile sand in tryptose/phosphate broth (Invitrogen) with antibiotics. After incubation at room temperature for 2 h, the organ suspensions of three chickens were pooled and centrifuged for 10 min at 2500 r.p.m. The supernatant was used for further virus propagation.

RT-PCR and sequence analysis. To sequence the region of the F gene that encodes the protease cleavage site, an RT-PCR fragment was obtained from the viral genome using primer 3UIT (5′-ACCAAACAGAGAATCCGTGAGTTA-3′) for reverse transcription and primers NDV5Fxho and NDV3Fmlu for the PCR. The resulting PCR fragment was purified using a High Pure PCR

purification kit (Roche) and sequenced with primer p4731+ (5′-AAGCTCCTCCCGAATCTGCC-3′) using a PRISM kit (Perkin-Elmer) and an Applied Biosystems AB310 automatic sequencer.

RESULTS

Syncytium formation induced by mutant F0 proteins after co-expression with the HN protein

Table 1 shows the nucleotide and amino acid sequences of the NDV F0 cleavage site mutants that we generated. These mutations have never been observed in any known F0 protein sequence from field isolates.

Mutagenesis of the fusion cleavage site of F0 was performed by PCR using plasmid pCIneo-F^{WT} (RRQRR ↓ F) as the template (see Methods). In F^{WT}, the following cleavage site mutations were introduced: F117L (F^{FM}), R115G (F^{FM2}), R113G (F^{FM3}), R112G+R115G (F^{FM1}) and R113K+R115K (F^{FM4}). These five mutant F0 genes were cloned in plasmid pCIneo.

Expression of the different F0 proteins was controlled by transfection into QM5 cells followed by immunological staining of the fixed monolayer with mAb 8E12A8C3 directed against the F protein. Immunological staining of the expressed (mutant) F0 proteins did not show any significant differences in the number of positive cells (data not shown).

To determine whether the F0 mutants were able to induce syncytium formation, QM5 cells were co-transfected with the fusion cleavage site mutants and pCIneo-HN^{LS} (La Sota). None of the F0 cleavage site mutants showed syncytium formation after 48 h on QM5 cells (Table 1). Only the positive control pCIneo-F^{WT} showed syncytium formation.

Rescue of infectious virus containing the F0 cleavage site mutations

The pCIneo plasmids with the five different F0 cleavage site mutations were used to introduce these mutations into full-length NDV cDNA in plasmid pNDFL (see Methods). The resulting plasmids were named pNDFL-F^{FM}, pNDFL-F^{FM1}, pNDFL-F^{FM2}, pNDFL-F^{FM3} and pNDFL-F^{FM4}.

These plasmids were used to generate infectious NDV with the different F0 cleavage site mutations (see Methods). The supernatants of transfected monolayers were used to inoculate the allantoic cavities of 8–11-day-old embryonated SPF eggs. The allantoic fluid was harvested 4 days after infection and analysed in a HA assay. Allantoic fluid with a positive HA titre was used for the isolation of the viral RNA genome followed by sequence analysis of an RT-PCR fragment that covered the F0 cleavage site. The results showed that all NDFL F0 cleavage site mutants could be recovered from cloned full-length cDNA. The mutants grew to similar titres compared with the parent strain, and the sequences of the F0 cleavage sites were still intact after two passages in embryonated eggs (data not shown).

The F0 cleavage site mutants were used to examine plaque formation on CEF cells. None of the mutants produced plaques 3 days post-inoculation except for the positive controls NDFLtag (RRQRR ↓ F) (Peeters *et al.*, 1999) and NDFL-F^{FM5} (RRQKR ↓ F) (for details of F^{FM5}, see below) (Fig. 1; data for F^{FM1} are not shown). All other mutations gave rise to single-cell infections, indicating that the F0 protein was not cleaved.

Determination of the effect of the F0 cleavage site mutations on virulence

Next we tested the virulence of the newly generated strains by determining the ICPI in 1-day-old chickens. Table 2(a) shows that the ICPI values of the NDV fusion cleavage site mutants varied from 0·0 for NDFL-F^{FM1} to 1·1 for NDFL-F^{FM3}. With the exception of NDFL- F^{FM1}, all the NDV fusion cleavage site mutants showed a delay in morbidity and mortality compared with virulent strain NDFLtag during the observation period of 8 days of the ICPI experiment. For mutants NDFL-F^{FM}, NDFL-F^{FM2} and NDFL-F^{FM4}, mortality increased further after day 8 (data not shown).

Organ material from dead chickens from the different ICPI experiments was used for virus re-isolation. After one egg passage, the nucleotide sequence of the fusion cleavage site was determined. The sequence data showed that all cleavage sites had reverted to the velogenic type RRQRR ↓ F with the exception of the double mutant of NDFL-F^{FM4}. In this strain, only the lysine at position 113 had reverted to an arginine, which resulted in the velogenic cleavage site (RRQ**K**R ↓ F) (Table 2b). These results indicated that all of the cleavage sites reverted to the consensus amino acid sequence of virulent strains after one passage in the chicken brain.

To determine the phenotype of the revertants in cell culture, CEF cells were infected with the revertants and examined for plaque formation. Plaque formation was observed for all the revertants after 3 days (Fig. 1), which indicated that the revertant F0 proteins had been cleaved as for the F0 protein of NDFLtag.

Increase in virulence of NDV after one passage in chicken brains

The revertant NDV strains, which were called NDFL-F^{FMR}, NDFL-F^{FM2R}, NDFL-F^{FM3R} and NDFL-F^{FM4R}, were again used to determine the ICPI. The resulting ICPI values varied from 1·4 to 1·7 (Table 2b), well above the value of the parent strain NDFLtag, which has the same cleavage site (RRQRR ↓ F) but which has an ICPI value of 1·3. The revertant NDV strains were propagated in embryonated eggs and again used for sequence analysis of the F0 cleavage site. These sequence analyses showed no further alteration of the F0 cleavage sites.

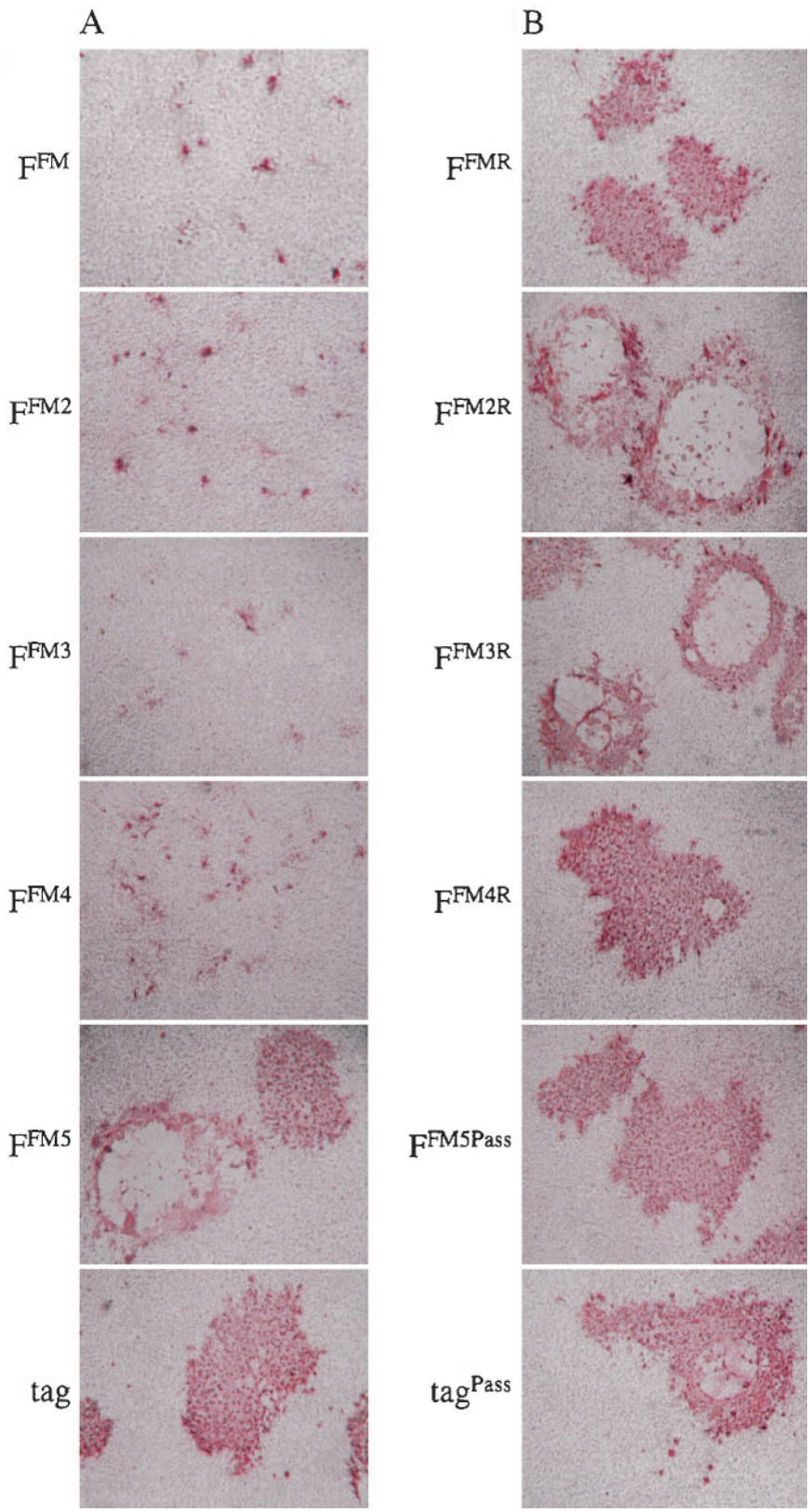

Fig. 1. Plaques produced in a CEF cell monolayer by NDV F0 cleavage site mutants before (A) and after (B) a passage in chicken brains. CEF cells were infected with virus and incubated for 3 days under a 1 % methylcellulose overlay containing 5 % FCS and antibiotics. Plaques were visualized by immunological staining (Wensvoort *et al.*, 1986) using a mAb against the NDV F protein. Magnification $\times 25$.

The complete sequence of the F0 gene of strain NDFL-F^{FM4R} was determined and compared with the sequence of the F0 gene of the parent strain NDFL-F^{FM4} and of the cDNA of pNDFL-F^{FM4}. This analysis showed that no other mutations were present in the F gene except for the one in the cleavage site. This means that the increase in virulence of the revertant NDV strains is not caused by another mutation in the F0 gene but probably by a mutation(s) elsewhere in the NDV genome.

To confirm that the virulence indeed increases after one passage in chicken brains, we determined the ICPI of isolate $NDFLtag^{Pass}$, which was recovered after one passage of strain NDFLtag in chicken brains. The ICPI value increased from 1·3 (NDFLtag) to 1·5 ($NDFLtag^{Pass}$) (Table 2a, b). Nucleotide sequence analysis showed that the F0 cleavage site of strain $NDFLtag^{Pass}$ was identical to that of NDFLtag. Additional proof for increase in virulence was obtained by generating NDFL-F^{FM5}, which has the same cleavage site as NDFL-F^{FM4R} (RRQKR ↓ F). The ICPI value of NDFL-F^{FM5} was found to be 1·3, which is equal to that of NDFLtag. This means that the lysine (K) instead of the arginine (R) at position 115 does not have any effect on the virulence. However, after one passage of NDFL-F^{FM5} in chicken brain, the ICPI of the re-isolate (NDFL-$F^{FM5Pass}$) also showed an increase in the ICPI from 1·3 to 1·5. In this case also, nucleotide sequence analysis showed that the F0 cleavage site of strain NDFL-$F^{FM5Pass}$ was similar to that of NDFL-F^{FM5} (Table 2a, b). Although the virulence of NDV increased after a passage in chicken brains, significant differences in plaque size, for example between NDFLtag and $NDFLtag^{Pass}$, on CEF cells could not be observed.

DISCUSSION

In this study we generated infectious NDV from cloned cDNA with mutations in the protease cleavage site of the F0 protein (Table 1). These protease cleavage sites are distinct from the consensus sequence of the cleavage site of velogenic/mesogenic field isolates, which are characterized by dibasic amino acids flanking the glutamine (Q^{114}) and a phenylalanine (F^{117}) as the first amino acid of the F1 protein

Table 2. ICPI of the NDV F0 cleavage site mutants

(a) After one passage in embryonated eggs

NDV F0 cleavage site mutants	Cleavage site*	HA titre†	P.f.u.‡	Clinical view	Days 1	2	3	4	5	6	7	8	Score§
NDFL	GRQGRL	7	8·1	Normal	10	10	10	10	10	10	10	10	
				Sick									
				Dead									
													0
NDFLtag	RRQRRF	5	6·5	Normal	10	10		1					
				Sick			10	5	1				
				Dead				4	9	10	10	10	
													1·3
NDFL-F^{FM}	RRQRRL	6	7·5	Normal	10	10	10	10	7	2		1	
				Sick					3	7	9	5	
				Dead						1	1	4	
													0·5
NDFL-F^{FM1}	GRQGRF	7	7·5	Normal	10	10	10	10	10	10	10	10	
				Sick									
				Dead									
													0
NDFL-F^{FM2}	RRQGRF	6	7·7	Normal	10	10	10	9	8	7	6	5	
				Sick				1	2	1	2	3	
				Dead						2	2	2	
													0·3
NDFL-F^{FM3}	RGQRRF	7	7·3	Normal	10	10	10	2					
				Sick				5	4				
				Dead				3	6	10	10	10	
													1·1
NDFL-F^{FM4}	RKQKRF	7	7·2	Normal	10	10	10	9	7	4	2	1	
				Sick				1	3	5	5	4	
				Dead						1	3	5	
													0·5

Table 2. (*cont.*)

(b) Re-isolated from dead birds in the first ICPI test

Viruses were passaged once in embryonated eggs before the second ICPI test was determined.

NDV F0 cleavage site mutants	Cleavage site‖	HA titre	P.f.u.	Clinical view	Days 1	2	3	4	5	6	7	8	Score
NDFLtag^{Pass}*	RRQRRF	9	6·0	Normal	10	4							
				Sick		6	5						
				Dead			5	10	10	10	10	10	
													1·5
NDFL-F^{FMR}	RRQRR**F**	6	7·1	Normal	9	8							
				Sick	1	2	9						
				Dead	0	0	1	10	10	10	10	10	
													1·4
NDFL-F^{FM2R}	RRQ**R**RF	6	6·0	Normal	9								
				Sick	1	2							
				Dead		8	10	10	10	10	10	10	
													1·7
NDFL-F^{FM3R}	R**R**QRRF	7	6·8	Normal	10	9							
				Sick		1	7						
				Dead			3	10	10	10	10	10	
													1·4
NDFL-F^{FM4R}	R**R**QKRF	5	6·3	Normal	10								
				Sick		10	2						
				Dead			8	10	10	10	10	10	
													1·6

*The modified amino acids in the cleavage sites are underlined.

†Haemagglutination titres (log_2).

‡Titres (log_{10}) expressed as p.f.u. ml^{-1}.

‖The resulting amino acid changes after the ICPI test are shown in bold.

§ICPI score=[(total numbers of sick chickens $\times 1$)+(total number of dead chickens $\times 2$)]/80 observations.

^{112}R/K-R-Q-R/K-R-F^{117}. To investigate which amino acids are important for cleavage of the F0 protein by host-cell protease(s), the mutants F^{FM1} (**G**RQ**G**R↓F), F^{FM2} (RRQ**G**R↓F) and F^{FM3} (R**G**QRR↓F) were generated. In these mutants, one or two basic amino acid residues were replaced by a non-basic amino acid. In mutant F^{FM4} (R**K**Q**K**R↓F), the pair of basic amino acid residues was not changed, but the type of the amino acid residue was changed from an arginine into a lysine at amino acid positions 113 and 115. Mutation F^{FM} (RRQRR↓**L**) was generated to investigate the effect of changing the amino acid at position 117 from a phenylalanine into a leucine.

Compared with the parent strain, all the newly generated NDV F0 mutants grew to similar titres in the allantoic cavity of embryonated eggs. Sequence analysis of the cleavage sites of the F0 mutants showed that no change occurred after two egg passages. This indicated that the allantoic fluid of the chicken egg contains a trypsine-like protease that is able to cleave the mutant F0 proteins shown in Table 1. However, transient expression of the mutant F0 proteins together with the HN protein did not result in syncytium formation of QM5 cells. This suggests that the mutant cleavage sites behaved like the lentogenic cleavage site of F^{LS} (La Sota). Only F^{WT} protein induced syncytium formation, indicating that it was cleaved by the host-cell protease (Table 1). The same lentogenic behaviour of the F0 cleavage site mutants was observed at the whole-virus level after examining plaque formation on CEF cells (Fig. 1A).

To analyse the effect of the mutant cleavage sites on virulence, the ICPI was determined (Table 2a). Interestingly, even though the ICPI values were all below the value of the parent strain NDFLtag (ICPI=1·3), they were much higher than expected based on the lentogenic behaviour of the F0 cleavage site mutants. These high ICPI values were caused by a dramatic increase in morbidity and mortality after day 5 for most of the NDV F0 cleavage site mutants (Table 2a). This increase in morbidity and mortality was the result of the presence of revertants with an F0 cleavage site RRQ(K/R)R↓F. These revertants are the result of replication errors and were probably already present after virus propagation in eggs. Apparently the cells in chicken brain constitute a highly selective environment for NDV viruses with the velogenic F0 cleavage site RRQ(K/R)R↓F. Initially, the ICPI values indicated that the cleavage site mutants were

either lentogenic or mesogenic. But in fact these values were influenced by the presence of virulent revertants. Therefore, precautions must be taken when the ICPI of strains containing cleavage site mutations is determined. It is sensible to sequence the F0 cleavage site of the re-isolate from dead chickens after the ICPI test to be sure that the amino acid sequence of the cleavage site is identical before and after the ICPI test.

The ICPIs of the velogenic revertants were all above the ICPI of the parent NDFLtag (ICPI=1·3). However, the fusion cleavage site of NDFLtag is the same as the fusion cleavage site of NDFL-F^{FM2R} (ICPI=1·7) and NDFL-F^{FM3R} (ICPI=1·5). Sequence analysis of the entire F0 gene of NDFL-F^{FM4}, NDFL-F^{FM4R} and pNDFL-F^{FM4} did not show any mutations other than the mutations of the cleavage site. This suggests that the revertants have somehow adapted to the host cells after one passage in the chicken brain. This might be due to another mutation somewhere in the NDV genome that results in an increase of the ICPI. NDFLtag^{Pass} and NDFL-$F^{FM5Pass}$ indeed showed an increase in ICPI compared with NDFLtag and NDFL-F^{FM5}, respectively, whereas the cleavage sites remained unaltered. Thus, the pathogenicity of NDV is not only related to the F0 cleavage site. A similar increase in virulence has been observed by Collins *et al.* (1994) for a variant of NDV, PPMV-1. Similarly, in this case the F0 cleavage site of PPMV-1 isolates did not change after passage in chickens.

From our results, it is clear that the amino acid sequence ^{112}R-R-Q-K/R-R-F^{117} is required for efficient cleavage of the velogenic/mesogenic F0 protein by a furin-like protease in chicken tissues. This amino acid sequence is identical to the consensus amino acid sequence ^{112}R/K-R-Q-R/K-R-F^{117} of virulent NDV strains. Interestingly, a basic lysine (K) at amino acid position 113 is not accepted by this protease. In agreement with this observation, a K at position 113 has never been observed in field isolates. Thus, whereas the dibasic combination K-R at amino acid positions 112 and 113 does exist, the combination R-K does not. The dibasic structure at amino acid positions 112 and 113 was not required for some velogenic strains that were isolated from pigeons (PPMV-1) and had the fusion cleavage site **G**RQ(K/R)R ↓ F (Collins *et al.*, 1994). Surprisingly, the F0 cleavage mutant NDFL-F^{FM1} (GRQGR ↓ F) did not revert to GRQ(**K/R**)R ↓ F, even though just a single nucleotide mutation is needed to change the amino acid at position 115 from a G (codon= GGG) to an R (codon=**C**GG). Fujii *et al.* (1999) demonstrated with F0 cleavage site mutants expressed by vaccinia virus vectors in CEF cells that the R residue at position 112 was only required for maximal cleavage by the host-cell protease. This also suggested that the revertant of NDFL-F^{FM1} could exist. The fact that the revertant did not appear after intracerebral inoculation could indicate that the cleavage site GRQ(K/R)R ↓ F of NDFL-F^{FM1}, in contrast to the pigeon isolate with the same cleavage site, is not virulent.

It is clear that our NDV F0 cleavage site mutants give rise to revertants with a velogenic cleavage site by point mutations. After just one passage in chicken brains, we were able to isolate such revertants. This evolution of the NDV F0 cleavage site is reminiscent of the emergence of virulent strains, which were responsible for the outbreaks of Newcastle disease in Ireland in 1990 (Alexander *et al.*, 1992) and in Australia in the years 1998–2000 (Gould *et al.*, 2001; Westbury, 2001). Analysis of these virulent NDV isolates showed that they were closely related to a lentogenic strain (antigenically and genetically) that was considered to be the precursor of the virulent virus. The Australian NDV isolate Peats Ridge with the cleavage site RRQGR ↓ L probably required two mutations to evolve via RRQRR ↓ L (Somersby), RRQKR ↓ L (MP-2000) or RRQGR ↓ F (PR-32) to the velogenic site RRQ(K/R)R ↓ F. Toyoda *et al.* (1989) have already concluded that different strains of NDV have evolved through various degrees of accumulation of point mutations rather than gene exchange by recombination.

We have shown in this study that NDV can evolve from a non-virulent type to a virulent type by point mutations. At this moment, there is no evidence that this frequently happens in the field. One reason for this is that the chance of accumulating three mutations from GRQGR ↓ L to (K/**R**)RQ(**R/K**)R ↓ **F** is probably very low. This is confirmed by NDFL-F^{FM1} (GRQGR ↓ F), which needs just two mutations but did not revert to a virulent strain. A second reason is the fact that mutations to virulence have only been observed in a relatively small non-vaccinated chicken population (in a country like Australia) and never in an immune population. This might be explained by the low transmission of mutants in vaccinated populations. Furthermore, it is not clear where mutations to virulence occur in the field. Do the mutations take place in feral birds and are then passed to poultry or do they occur once the virus has been introduced in poultry? In this study, the NDV F0 cleavage site mutants were propagated in embryonated eggs followed by a passage in chicken brains where the virulent revertants appeared. This is far from the natural infection route of NDV and therefore it is of interest to know whether NDV F0 cleavage site mutants give rise to revertants when the natural infection route, i.e. the respiratory tract, is used.

Nevertheless, attention must be paid when a non-virulent NDV strain is isolated from the field that has just one or two amino acid differences in the F0 cleavage site compared with a virulent strain. Such NDV strains can evolve to a virulent strain, which can cause devastating disease in poultry.

REFERENCES

Alexander, D. J. (2000). Newcastle disease and other avian paramyxoviruses. *Sci Tech Rev* **19**, 443–462.

Alexander, D. J. (2001). Newcastle disease. *Br Poult Sci* **42**, 5–22.

Alexander, D. J., Campbell, G., Manvell, R. J., Collins, M. S., Parsons, G. & McNulty, M. S. (1992). Characterisation of an antigenically unusual virus responsible for two outbreaks of

Newcastle disease in the Republic of Ireland in 1990. *Vet Res* **130**, 65–68.

Antin, P. B. & Ordahl, C. P. (1991). Isolation and characterization of an avian myogenic cell line. *Dev Biol* **143**, 111–121.

Beard, C. W. & Hanson, R. P. (1984). Newcastle disease. In *Disease of Poultry*, pp. 452–470. Edited by M. S. Hofstad. Iowa: Iowa State University Press.

Britton, P., Green, P., Kottier, S., Mawditt, K. L., Penzes, Z., Cavanagh, D. & Skinner, M. A. (1996). Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. *J Gen Virol* **77**, 963–970.

Collins, M. S., Bashiruddin, J. B. & Alexander, D. J. (1993). Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch Virol* **128**, 363–370.

Collins, M. S., Strong, I. & Alexander, D. J. (1994). Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed ''pigeon PMV-1 viruses''. *Arch Virol* **134**, 403–411.

de Leeuw, O. & Peeters, B. (1999). Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. *J Gen Virol* **80**, 131–136.

Deng, R., Mirza, A. M., Mahon, P. J. & Iorio, R. M. (1997). Functional chimeric HN glycoproteins derived from Newcastle disease virus and human parainfluenza virus-3. *Arch Virol* **13**, 115–130.

Fujii, Y., Sakaguchi, T., Kiyotani, K. & Yoshida, T. (1999). Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle disease virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. *Microbiol Immunol* **43**, 133–140.

Garten, W., Berk, W., Nagai, Y., Rott, R. & Klenk, H. D. (1980). Mutational changes of the protease susceptibility of glycoprotein F of Newcastle disease virus: effects on pathogenicity. *J Gen Virol* **50**, 135–147.

Glickman, R. L., Syddall, R. J., Iorio, R. M., Sheehan, J. P. & Bratt, M. A. (1988). Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. *J Virol* **62**, 354–360.

Gotoh, B., Ogasawara, T., Toyoda, T., Inocencio, N. M., Hamaguchi, M. & Nagai, Y. (1990). An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. *EMBO J* **9**, 4189–4195.

Gould, A. R., Kattenbelt, J. A., Selleck, P., Hansson, E., Della-Porta, A. & Westbury, H. A. (2001). Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998–2000. *Virus Res* **77**, 51–60.

Kawahara, N., Yang, X. Z., Sakaguchi, T., Kiyotani, K., Nagai, Y. & Yoshida, T. (1992). Distribution and substrate specificity of intracellular proteolytic processing enzyme(s) for paramyxovirus fusion glycoproteins. *J Gen Virol* **73**, 583–590.

Krishnamurthy, S. & Samal, S. K. (1998). Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *J Gen Virol* **79**, 2419–2424.

Lamb, R. A. & Kolakofsky, D. (1996). *Paramyxoviridae*: the viruses and their replication. In *Fundamental Virology*, pp. 1177–1204. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lipincott–Raven.

Li, Z., Sergel, T., Razvi, E. & Morrison, T. (1998). Effect of cleavage mutants on syncytium formation directed by the wild-type fusion protein of Newcastle disease virus. *J Virol* **72**, 3789–3795.

Morrison, T., Ward, L. J. & Semerjian, A. (1985). Intracellular processing of the Newcastle disease virus fusion glycoprotein. *J Virol* **53**, 851–857.

Morrison, T., McQuain, C., Sergel, T., McGinnes, L. & Reitter, J. (1993). The role of the amino terminus of F1 of the Newcastle disease virus fusion protein in cleavage and fusion. *Virology* **193**, 997–1000.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A. & Summers, M. D. (editors) (1995). *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*. Vienna & New York: Springer-Verlag.

Nagai, Y., Klenk, H. D. & Rott, R. (1976). Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* **72**, 494–508.

Ogasawara, T., Gotoh, B., Suzuki, H., Asaka, J., Shimokata, K., Rott, R. & Nagai, Y. (1992). Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. *EMBO J* **11**, 467–472.

Peeters, B. P., de Leeuw, O. S., Koch, G. & Gielkens, A. L. (1999). Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *J Virol* **73**, 5001–5009.

Peeters, B. P., de Leeuw, O. S., Verstegen, I., Koch, G. & Gielkens, A. L. (2001). Generation of a recombinant chimeric Newcastle disease virus vaccine that allows serological differentiation between vaccinated and infected animals. *Vaccine* **19**, 1616–1627.

Phillips, R. J., Samson, A. C. & Emmerson, P. T. (1998). Nucleotide sequence of the 5′-terminus of Newcastle disease virus and assembly of the complete genomic sequence: agreement with the ''rule of six''. *Arch Virol* **143**, 1993–2002.

Römer-Oberdorfer, A., Mundt, E., Mebatsion, T., Buchholz, U. J. & Mettenleiter, T. C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *J Gen Virol* **80**, 2987–2995.

Sakaguchi, T., Matsuda, Y., Kiyokage, R., Kawahara, N., Kiyotani, K., Katunuma, N., Nagai, Y. & Yoshida, T. (1991). Identification of endoprotease activity in the trans Golgi membranes of rat liver cells that specifically processes in vitro the fusion glycoprotein precursor of virulent Newcastle disease virus. *Virology* **184**, 504–512.

Sakaguchi, T., Fujii, Y., Kiyotani, K. & Yoshida, T. (1994). Correlation of proteolytic cleavage of F protein precursors in paramyxoviruses with expression of the fur, PACE4 and PC6 genes in mammalian cells. *J Gen Virol* **75**, 2821–2827.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Seal, B. S., King, D. J. & Meinersmann, R. J. (2000). Molecular evolution of the Newcastle disease virus matrix protein gene and phylogenetic relationships among the paramyxoviridae. *Virus Res* **66**, 1–11.

Toyoda, T., Sakaguchi, T., Imai, K., Inocencio, N. M., Gotoh, B., Hamaguchi, M. & Nagai, Y. (1987). Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. *Virology* **158**, 242–247.

Toyoda, T., Sakaguchi, T., Hirota, H., Gotoh, B., Kuma, K., Miyata, T. & Nagai, Y. (1989). Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. *Virology* **169**, 273–282.

Wensvoort, G., Terpstra, C., Boonstra, J., Bloemraad, M. & Van Zaane, D. (1986). Production of monoclonal antibodies against

swine fever virus and their use in laboratory diagnosis. *Vet Microbiol* **12**, 101–108.

Werner, O., Römer-Oberdörfer, A., Köllner, B., Manvell, R. J. & Alexander, D. J. (1999). Characterization of avian paramyxovirus type 1 strains isolated in Germany during 1992 to 1996. *Avian Pathol* **28**, 79–88.

Westbury, H. (2001). Newcastle disease virus: an evolving pathogen? *Avian Pathol* **30**, 5–11.

Westover, K. M. & Hughes, A. L. (2001). Molecular evolution of viral fusion and matrix protein genes and phylogenetic relationships among the Paramyxoviridae. *Mol Phylogenet Evol* **21**, 128–134.