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A Recombinant Newcastle Disease Virus with Low-Level V Protein Expression Is Immunogenic and Lacks Pathogenicity for Chicken Embryos

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Newcastle disease virus (NDV) edits its P-gene mRNA by inserting a nontemplated G residue(s) at a conserved editing site (3′-UUUUUCCC-template strand). In the wild-type virus, three amino-coterminal P-gene-derived proteins, P, V, and W, are produced at frequencies of approximately 68, 29, and 2%, respectively. By applying the reverse genetics technique, editing-defective mutants were generated in cell culture. Compared to the wild-type virus, mutants lacking either six nucleotides of the conserved editing site or the unique C-terminal part of the V protein produced as much as 5,000-fold fewer infectious progeny in vitro or 200,000-fold fewer in 6-day-old embryonated chicken eggs. In addition, both mutants were unable to propagate in 9- to 11-day-old embryonated specific-pathogen-free (SPF) chicken eggs. In contrast, a mutant (NDV-P1) with one nucleotide substitution (UU*C*UUCCC) grew in eggs, albeit with a 100-fold-lower infectious titer than the parent virus. The modification in the first two mutants described above led to complete abolition of V expression, whereas in NDV-P1 the editing frequency was reduced to less than 2%, and as a result, V was expressed at a 20-fold-lower level. NDV-P1 showed markedly attenuated pathogenicity for SPF chicken embryos, unlike currently available ND vaccine strains. These findings indicate that the V protein of NDV has a dual function, playing a direct role in virus replication as well as serving as a virulence factor. Administration of NDV-P1 to 18-day-old embryonated chicken eggs hardly affected hatchability. Hatched chickens developed high levels of NDV-specific antibodies and were fully protected against lethal challenge, demonstrating the potential use of editing-defective recombinant NDV as a safe embryo vaccine.

Newcastle disease virus (NDV) belongs to the genus *Rubulavirus* within the family *Paramyxoviridae*. Recent findings, however, have indicated that NDV is only distantly related to other members of the genus *Rubulavirus*, and it has been suggested that NDV should be assigned to a new genus within the subfamily *Paramyxovirinae* (6). NDV isolates are further categorized based on pathogenicity for chickens into velogenic, mesogenic, and lentogenic strains corresponding to high-, moderate-, and low-virulence strains, respectively. The molecular basis for this distinction lies mainly in the amino acid sequence of the protease cleavage site of the fusion (F) protein (14, 25). The precursor fusion glycoprotein F0 has to be cleaved into F1 and F2 for the progeny virus to be infectious and to be able to undergo multiple rounds of replication. Recently, experimental evidence for the presence of a direct correlation between the sequence of the cleavage site and NDV virulence was provided by changing the protease cleavage site of a lentogenic strain of NDV (GGRQGR↔ L) into the consensus cleavage site of a velogenic strain (GRRQRR↔ F). A dramatic increase in virulence of the genetically modified virus indicated that the key determinant for NDV virulence is the cleavage efficiency of the precursor protein (28). However, there is indirect evidence suggesting that cleavage efficiency is not the sole determinant governing NDV virulence (22, 28).

The negative-strand RNA virus genome of NDV contains six genes encoding six major structural proteins (3′-NP-P-M-F-HN-L-5′). A general feature of the *Paramyxovirinae*, however, is the presence of additional structural or nonstructural viral proteins resulting from the use of alternative reading frames and RNA editing of their P genes (19). Like other members of the *Paramyxovirinae*, NDV edits its P gene by inserting one or two G residues at the conserved editing locus (UUUUUCCC) and transcribes three P-gene-derived mRNA species. The mRNAs encode the open reading frame (ORF) of P (unedited), the V ORF (with a +1 frameshift), and the W ORF (with a +2 frameshift) (39). These proteins are amino coterminal and vary at their carboxy-terminal ends in length and amino acid composition. Of the three P-gene products, the P protein is known to be an essential component for viral RNA synthesis and, together with the L protein, was demonstrated to form an active transcriptive complex (15). However, not much is known about the two other P-gene products. The V protein is of particular interest since it is conserved in all three genera of the *Paramyxovirinae*, with the exception of human parainfluenza virus type 1 (HPIV-1), which lacks an intact V ORF (23). Moreover, the V protein is characterized by the presence of a highly conserved cysteine-rich carboxy-terminal domain, and there is evidence that this domain of simian virus (SV5) interacts with damage-specific DNA binding protein (21). The V

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proteins of NDV and SV5 were shown to bind zinc and were also demonstrated to be structural components of virions (26, 33, 38). On the other hand, the V proteins of Sendai virus (SeV) and measles virus (MV) are not structural components of virions and are not associated with the ribonucleoprotein complex (16, 20).

Further insight into the functions of the additional P-gene products of the *Paramyxoviridae* was obtained after the development of reverse genetics technology, which enabled genetic manipulation of the genomes of nonsegmented negative-strand RNA viruses (reviewed in references 5 and 31). Studies with SeV and MV showed that the V and/or W protein could be deleted without detrimental effects on replication of the virus in cell culture (7, 8, 17, 18, 35). Interestingly, however, the editing-defective SeV was found to replicate normally in vitro but was severely attenuated in pathogenicity for mice (8, 17, 18). The mechanism of the in vivo attenuation in certain members of the *Paramyxoviridae* may involve the interferon (IFN) system, in which accessory proteins, particularly V or C proteins (20), are responsible for blocking the activation of IFN-responsive genes (9, 10, 13).

NDV is responsible for one of the most devastating diseases of poultry and has substantial economic impact in the poultry industry. Vaccination of chickens, particularly those raised for commercial consumption, is carried out throughout the world. The currently available live attenuated ND vaccines can be administered to hatched chickens only in drinking water, aerosols, or eye drops or by parenteral routes. These methods of applications have several disadvantages, the most important being labor costs. Embryo, or in ovo, vaccination has proved to be an effective and economical method of application for several commonly used vaccines, such as those for turkey herpesvirus and infectious bursal disease virus (36, 37). Moreover, in ovo vaccination was found to be advantageous due to the administration of a uniform dose of vaccine into each egg using automated machines. However, several live virus vaccines for poultry cannot be administered in ovo mainly because they cause high embryo mortality. For NDV, the use of a modified live vaccine for in ovo administration has been described previously (1). However, this involves the use of a chemical mutagenic agent, ethyl methanesulfonate, at each step of the vaccine preparation. Recombinant fowlpox vectors expressing NDV fusion protein and/or hemagglutinin-neuraminidase protein have been successfully constructed, and their safety and efficacy for in ovo vaccination have been studied in specific-pathogen-free (SPF) chickens (12). Although the recombinant vaccines were shown to be efficacious in SPF animals, no data were provided on the efficacy of such recombinant vaccines in commercial chickens with neutralizing maternal antibodies. Such passive antibodies, which are usually present at high levels in very young chickens from immunized parent flocks, can impair the effectiveness of live virus vaccines. Since conventional live ND vaccines confer full protection even in the presence of maternal antibodies, it is highly desirable that the currently available posthatching vaccines be further attenuated to make them suitable for embryo vaccination.

Recently, the recovery of infectious lentogenic NDV from full-length cDNA has been described (28, 32). We demonstrated that the recombinant virus was phenotypically identical to its parent virus, NDV Clone-30, which is currently used as a live posthatching vaccine (32). In the present study, this recombinant cDNA technology was used to introduce mutations into the conserved editing site of the P gene. A single U-to-C change within the U stretch substantially reduced the editing frequency and hence considerably lowered the level of additional proteins generated by RNA editing. The editing-defective virus was dramatically attenuated for chicken embryos. Here, we describe the effects of this and other mutations on viral replication and pathogenesis and discuss the potential use of such editing-defective viruses for the development of ND vaccines that can be used to immunize chicken embryos.

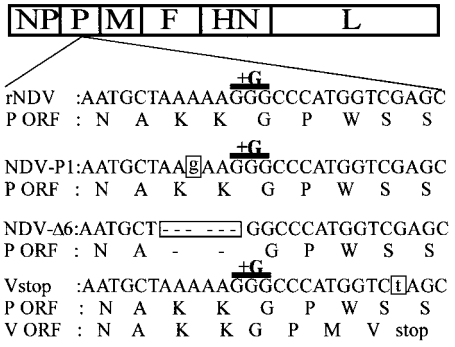

FIG. 1. Recombinant NDV constructs. A schematic representation of the NDV gene order in the negative-strand genomic RNA is shown. Sequences around the editing site (positions 2274 to 2300) are presented in a positive sense. The modifications resulting in interruption of the A stretch in NDV-P1, deletions of six nucleotides of the conserved editing site in NDV-Δ6, and the creation of a stop codon in the *trans*-V frame of NDV-Vstop (Vstop) are shown in boxes. +G indicates the position for insertion of nontemplated G residue(s).

MATERIALS AND METHODS

Viruses and cells. A recombinant NDV, rNDV, which was generated from a full-length cDNA copy of the lentogenic ND vaccine virus Clone-30 was described previously (32). A lentogenic posthatching ND vaccine, NDW, was obtained from a commercial source (Fort Dodge). The velogenic Herts strain 33/56 of NDV was used for challenge purposes. BSR-T7/5 cells stably expressing phage T7 RNA polymerase (4) were used to recover infectious NDV from cDNA.

Introduction of mutation into the full-length NDV cDNA. The plasmid pflNDV, expressing the full-length antigenome RNA of Clone-30 (32), was used to introduce mutations. Since NDV edits its P-gene mRNA by inserting nontemplated G residues (39), we modified the conserved editing site (UUUUUCCC) in the P gene of pflNDV. PCR was performed with the template pflNDV using forward primer 4 (5′-GCTCCTCGCGGCTCAGACTCG-3′, nucleotides 151 to 171) and reverse primers 1 (5′-CCATGGGCCCTTCTTAGCATTGGACG-3′, nucleotides 2269 to 2294) and 3 (5′-CCATGGGCCCGCATTGGACG-3′, nucleotides 2269 to 2294) to introduce one nucleotide change and a deletion of six nucleotides, respectively (Fig. 1). PCR products were then digested with *Aat*II and *Apa*I and cloned into the same sites of pflNDV. To selectively block expression of the unique C-terminal part of the V protein, a stop codon was introduced into the *trans*-V frame without affecting the P frame. PCR was performed using primer 20 (5′-CCCGGGAATCTTCTCTGGCGC-3′, nucleotides 3764 to 3784) and primer 29 (5′-AAGGGCCCATGGTCTAGCCCCCAAGAG-3′, nucleotides 2283 to 2309). The product was digested with *Apa*I and *Rsr*II and ligated into the same site of pflNDV. The nucleotide numbering is based on that of Römer-Oberdörfer et al. (32). The region newly introduced into each clone was sequenced to rule out PCR-introduced errors. The resultant full-length clones, with one nucleotide substitution at the editing site, a deletion of six nucleotides, or the insertion of a stop codon in the V ORF, were named NDV-P1, NDV-Δ6, and NDV-Vstop, respectively (Fig. 1).

In order to be able to grow and characterize the mutants in vitro without the addition of proteolytic enzymes, additional mutations were introduced at the F protein cleavage site. First, a 3.3-kb *Apa*I-*Acl*I fragment of pflNDV was cloned

into the *Sma*I site of the pUC18 vector. F protein cleavage site modification was performed using a site-directed mutagenesis kit (Amersham Pharmacia Biotech) with primer MP1 (5′-CTGTGACTACATCTGGAGGG**C**GGAGACAG**AA**GC GC**T**TTATAGGCGCCATT ATTGG-3′, nucleotides 4857 to 4911) according to the supplier's instructions. The modified plasmid was then digested using *Pml*I and *Not*I, and a fragment of approximately 1.2 kb was used to replace the corresponding fragment of pflNDV. The resultant full-length clone was then digested with *Pml*I and *Bsi*WI, and a fragment of approximately 5.1 kb containing the modified F cleavage site was used to replace the corresponding fragments of NDV-Δ6 and NDV-Vstop.

Recovery of recombinant viruses. Approximately 1.5×10^6 BSR-T7/5 cells stably expressing phage T7 RNA polymerase (4) were grown overnight in 3.2-cm-diameter dishes. Cells were transfected with plasmid mixtures containing 5 μg of pCite-NP, 2.5 μg of pCite-P, 2.5 μg of pCite-L, and 10 μg of one of the full-length clones using a mammalian transfection kit ($CaPO_4$ transfection protocol; Stratagene). Three to five days after transfection, supernatant was harvested and inoculated into the allantoic cavities of 9- to 11-day-old embryonated SPF chicken eggs. After 3 to 4 days of incubation, the presence of virus in the allantoic fluid was determined by a rapid plate hemagglutination (HA) test using chicken erythrocytes (3). Supernatants obtained from transfections involving full-length clones with modifications at the F cleavage site were serially passaged in BSR cells. Virus stocks were prepared from supernatants of infected BSR cells, and the infectious titers were determined by serial 10-fold dilutions and staining of infectious foci with an anti-F monoclonal antibody (MAb). The growth characteristics of the viruses were then analyzed in BSR and Vero cells as well as in 6- and 10-day-old embryonated SPF chicken eggs.

Reverse transcription-PCR and determination of P-gene mRNA editing frequency. BSR-T7/5 cells were infected with the recombinant viruses, and total RNA was prepared 24 to 36 h after infection using the RNeasy kit (Qiagen). Reverse transcription by avian myeloblastosis virus reverse transcriptase on 1 μg of total RNA was primed with NDV P-gene-specific oligonucleotide P13 (5′-C CACCCAGGCCACAGACGAAG-3′, nucleotides 2176 to 2196) or oligo(dT) primer to amplify only mRNAs. DNA amplification was then performed with primers P13 and P17 (5′-ATGAATTCAGCTGTTGGA-3′, nucleotides 2680 to 2696). The PCR products were analyzed on a 1% agarose gel and used directly for sequencing or were digested with *Eco*RV and *Sal*I and ligated into the same site of the pSKT7T vector. Cloned plasmids were sequenced from independent colonies and examined for the presence or absence of insertion of a nontemplated G residue(s) at the editing site.

Immunofluorescence analysis. For the analysis of viral protein expression, BSR-T7/5 cells were infected with the recombinant viruses and incubated for approximately 18 h. Infected cells were fixed for 1 h at room temperature with cold ethanol (96%). Cells were then incubated with antipeptide rabbit serum directed against the 16 C-terminal amino acids of V protein or MAbs reacting with NP or F protein. Cells were washed and stained with fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse antibody containing 0.05% Evans blue and examined by fluorescence microscopy.

Immunoblotting. For virus purification, 9- to 11-day-old embryonated SPF chicken eggs were infected and allantoic fluid was collected 3 to 4 days postinfection. Virus in the allantoic fluid was then purified and concentrated by centrifugation through a 20% sucrose cushion in a Beckman SW28 rotor at 21,000 rpm for 90 min. The pellet was resuspended and mixed with protein sample buffer to disrupt the virions. Viral proteins from purified virions were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), and incubated with antipeptide serum specific for the C-terminal 16 amino acids of the V protein of NDV Clone-30 or with a MAb specific for NDV NP protein. Membranes were then incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G. Proteins were visualized after incubation with peroxidase substrate (Vector).

Virus propagation in embryonated eggs. To determine virus titers and embryo mortality, serial 10-fold dilutions of the recombinant viruses were prepared, and 9- to 11-day-old embryonated SPF chicken eggs were inoculated in the allantoic cavity with the serial dilutions, in duplicate. A rapid-plate HA test (3) was carried out on one set of eggs after 4 days of incubation, and the titer, expressed as the 50% embryo-infectious dose (EID_{50}), was calculated using the method of Reed and Muench (29). The remaining eggs were observed daily for embryo mortality for at least 7 days, and the 50% embryo-lethal dose was then determined using the same method. To determine the susceptibility of chicken embryos to NDV-P1 infection at an early age of embryonation, chicken embryos at the ages of 7 and 8 days were infected with 2 $\log_{10}$ EID_{50} and observed for 1 week.

In ovo vaccination and challenge. Eighteen-day-old embryonated SPF or commercial chicken eggs were inoculated through a hole punched at the blunt end of the egg. Using a 23-gauge needle, 0.1 ml of the virus dilution or negative allantoic fluid was injected just below the air membrane. Eggs were further incubated until hatching. The percent hatchability was recorded, and chickens were observed daily for general health. At 14 days of age, chickens were weighed and blood samples were taken. Serum samples were assayed for NDV antibodies in the NDV hemagglutination inhibition test (3). At 14 days of age (~17 days postvaccination), all animals were challenged with intramuscularly administered virulent Herts strain of NDV. Chickens were observed daily for a period of 10 days for clinical signs of disease or mortality.

RESULTS

Generation of mutant NDV from cDNA. In order to disrupt the conserved P-gene mRNA editing or selectively block expression of the unique C-terminal part of the V protein, the modifications shown in Fig. 1 were carried out on the full-length cDNA clone (pflNDV) of NDV Clone-30 (32). Each modified full-length cDNA clone, together with three support plasmids expressing NDV NP, P, and L proteins, was transfected into BSR-T7/5 cells. Transfection experiments were also performed with the unmodified full-length cDNA, pflNDV, to compare rescue efficiencies. After 3 to 5 days of incubation, supernatants were harvested and transfected cells were subjected to immunofluorescence staining using an anti-F MAb. At least 20 to 50 immunofluorescence-positive cells were detected in all of the transfection experiments involving pflNDV or modified full-length clones, showing that there were genome replication and expression of viral proteins in cell culture.

Embryonated SPF chicken eggs, which have long been known as the best substrates for propagation of lentogenic NDVs (14, 25), were then inoculated with transfection supernatants. After 3 to 4 days of incubation, allantoic fluid samples were harvested and subjected to an HA test. HA was detected in eggs inoculated with the supernatant from cells transfected with the pflNDV. However, two extra egg passages were required for NDV-P1 to be detected using the HA test, suggesting that this mutant grows slowly when inoculated into the allantoic cavity of 9- to 11-day-old embryonated SPF chicken eggs. Surprisingly, infectious virus was not detected in the allantoic fluid of embryonated eggs inoculated with supernatants obtained from NDV-Vstop and NDV-Δ6 transfections, even after four successive passages. In spite of three repeated rescue experiments, we were unable to detect infectious virus in the allantoic fluid after passage in 9- to 11-day-old embryonated eggs.

V protein of NDV is essential for efficient virus propagation. In order to determine whether these mutants grow in cell culture as efficiently as the wild-type virus, repeated passage in culture cells is necessary. However, due to the absence of efficient cleavage of the precursor F protein, lentogenic NDVs cannot be propagated in most tissue culture systems without the addition of proteases. In contrast, velogenic strains are able to undergo multiple rounds of replication in cell culture. To be able to grow the mutants in vitro, "virulent" versions of rNDV, NDV-Vstop, and NDV-Δ6 were constructed by modifying the F cleavage site. The alterations resulted in a change of the F cleavage site of Clone-30 (GGRQGR↔ L) to a cleavage site similar to that of a virulent strain (G**R**RQ**K**R↔ **F**). Using an anti-V-peptide serum specific for the C terminus of V protein, the absence of V expression in both mutants was confirmed, demonstrating that the introduced mutations were sufficient to completely abolish RNA editing. In order to compare the

TABLE 1. In vitro and in vivo propagation of V-deficient NDV mutants that posses an F protein cleavage site similar to that of a virulent NDV strain

Virus	Infectious titers[a] after propagation in:			
	BSR cells	Vero cells	6-day-old embryos	10-day-old embryos
NDV-Δ6	6×10^0	1×10^1	2×10^2	0
NDV-Vstop	6×10^0	1.4×10^2	4×10^2	0
Wild type	4×10^3	7×10^5	1×10^8	1.2×10^8

[a] For in vitro propagation, BSR and Vero cells were infected at a multiplicity of infection of 0.001, and the infectious titers in supernatants harvested after 4 days of infection were determined using end point dilutions in cell culture. For in ovo propagation SPF chicken eggs at 6 or 10 days of embryonation were inoculated with 1.7 $\log_{10}$ focus-forming units/egg and incubated for 4 days. Infectious titers in the allantoic fluid were then determined in cell culture after endpoint dilutions. Values are in focus-forming units per milliliter.

growth efficiency of the mutants with that of the wild-type virus, BSR and Vero cells were inoculated with the respective supernatants at a multiplicity of infection of 0.001. In addition, 6- and 10-day-old embryonated SPF chicken eggs were inoculated with 1.7 $\log_{10}$ focus-forming units/egg. Infected cell cultures and embryonated eggs were incubated for 4 days, and the titers of infectious viruses in cell culture supernatants or allantoic fluid were determined. In vitro, the titers of both mutants were 600- to 5,000-fold lower than the titers of the wild-type virus depending on the type of cells (Table 1). This remarkable growth impairment of both mutants in cell culture indicates that V protein plays a crucial role in NDV replication. In 6-day-old chicken embryos, both mutants yielded more than 200,000-fold-lower titers than the wild-type virus (Table 1) and did not cause any embryo mortality. Interestingly, the mutants were completely unable to propagate in 10-day-old embryonated eggs, even after serial passage, indicating that V is also a pathogenesis factor. The wild-type virus grew to identical titers in younger and older embryos and caused mortality of up to 100%.

NDV-P1 expresses a low level of V protein. The mutant which could be propagated in embryonated eggs, NDV-P1, was then serially passed two or three times in 9- to 11-day-old embryonated eggs. The infectious titers of this mutant after the fifth and sixth egg passages were 6.7 and 7.1 $\log_{10}$ EID_{50} per ml, respectively, which were at least 100-fold lower than the titer obtained for the parent virus after the third egg passage (9.2 $\log_{10}$ EID_{50} per ml). Experiments described here were carried out using the sixth passage of NDV-P1 except where it is stated that the fifth passage was employed. BSR-T7/5 cells were infected with NDV-P1 or the parent virus rNDV and subjected to immunofluorescence analysis. Using MAbs directed against NDV NP or F protein, the levels and patterns of NP and F protein fluorescence in cells infected with the mutant and the parent virus were indistinguishable (Fig. 2). In contrast, an anti-V peptide serum specific for the C terminus of V protein reacted with intense fluorescence only with cells infected with the rNDV. The same dilution of the serum revealed a specific but very weak fluorescence in NDV-P1-infected cells, suggesting a low level of V expression (Fig. 2).

V protein is a structural component of NDV; therefore, it was of interest to determine whether the low level of V expression in infected cells would lead to low-level incorporation of V into virions. Thus, virions purified and concentrated through 20% sucrose were subjected to immunoblotting experiments. Using an NP-specific MAb, which is reactive with the NP protein of both viruses with equal sensitivity, it was possible to standardize the amount of protein loaded into the gel (Fig. 3). Although comparable amounts of rNDV and NDV-P1 proteins were subjected to the Western blot analysis, the amount of V protein of NDV-P1 was considerably smaller than that of rNDV, demonstrating low-level V protein incorporation into NDV-P1 virions. Analysis of diluted samples by Western blotting revealed that the V protein content of NDV-P1 virions was approximately 20-fold lower than that of rNDV.

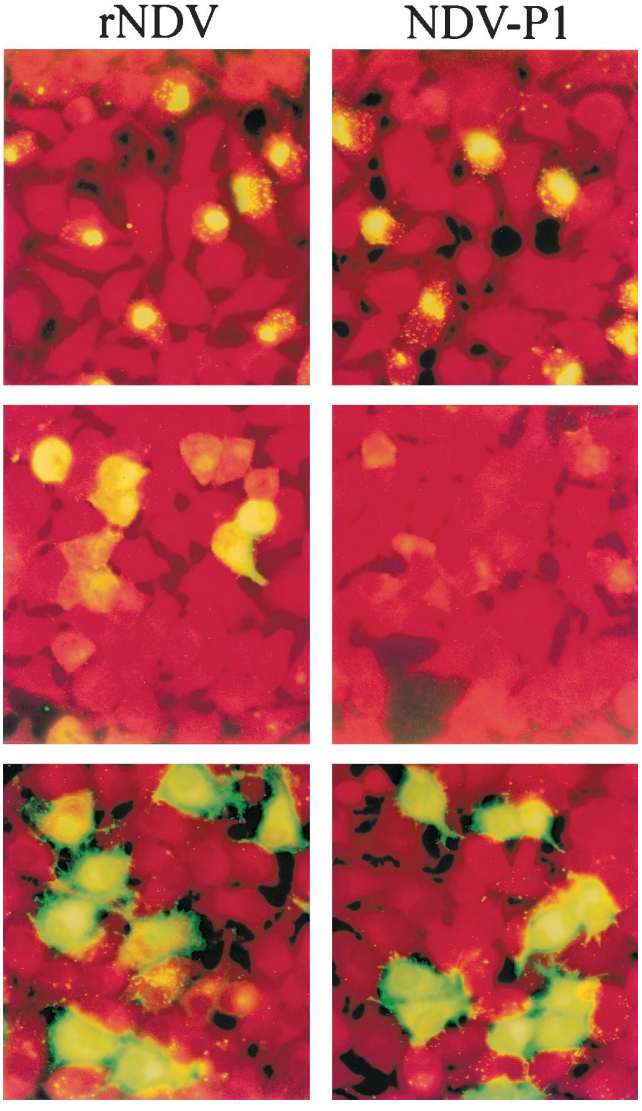

FIG. 2. Low-level V protein expression in NDV-P1-infected cells. BSR-T7/5 cells were infected with rNDV or NDV-P1 at a multiplicity of infection of approximately 0.01. Eighteen hours after infection, cells were processed for indirect immunofluorescence after incubation with MAbs specific for NP protein (top) or for F protein (bottom) or anti-V peptide serum (middle). Although the levels of NP and F protein expressions in cells infected with both viruses were indistinguishable, the level of V protein expression was considerably lower in cells infected with NDV-P1 than in those infected with rNDV.

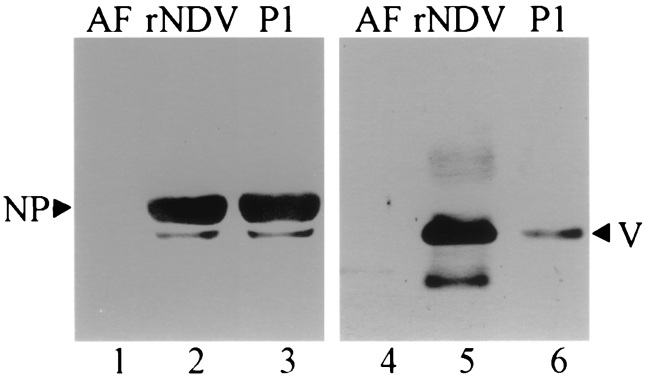

FIG. 3. NP and V proteins of sucrose-purified recombinant viruses. Virions in the allantoic fluid of infected embryonated eggs were purified by centrifugation through 20% sucrose. The volumes loaded for NDV-P1 were 4.5-fold greater than those for rNDV in order to normalize for NP protein content. Samples were loaded in duplicate, and blots were incubated with anti-NP MAb (lanes 1 through 3) or with anti-V peptide serum (lanes 4 through 6). AF, allantoic fluid from noninfected embryonated eggs; P1, NDV-P1.

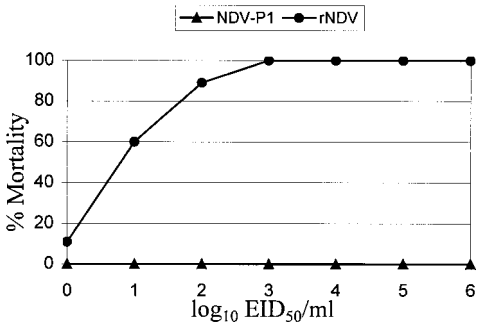

FIG. 5. Pathogenicity of rNDV and NDV-P1 in SPF chicken embryos. Eleven-day-old embryonated SPF chicken eggs were inoculated with the parent rNDV (passage 3) or the mutant NDV-P1 (passage 5) and incubated for 7 days or until the embryos had died. NDV-P1 caused no embryo mortality for 7 days at all indicated doses (0.2 ml/egg), whereas rNDV was lethal at a dose as low as 1 EID_{50}/ml (approximately 10% mortality). Embryos inoculated with rNDV started to die as early as 3 days postinoculation at higher doses.

As the V protein of NDV can be produced only by the RNA editing process, we determined the sequence around the editing locus from a total of 72 independent colonies of plasmids derived from NDV-P1. As expected, we found a plasmid containing an insertion of one nontemplated G residue leading to V-ORF (1.4%), in spite of the presence of a modification at the editing site (Fig. 4). For comparison, 41 independent colonies were sequenced for rNDV; 28 out of 41 (68.3%) of the sequenced plasmids encoded the unedited version of P protein, and 12 out of 41 (29.3%) encoded the V protein with an insertion of one nontemplated G residue. Only one plasmid out of 41 (2.4%) possessed an insertion of two nontemplated G residues and hence encoded W protein. The frequency of RNA editing of the wild-type virus is very similar to the results obtained by Steward et al. (39), except that plasmids encoding W protein were approximately threefold less abundant in this study. Compared with the wild-type virus, the NDV-P1 virus edits its P-gene mRNA at an approximately 20-fold lower frequency and hence synthesizes V protein at a correspondingly low level. Taken together, these results showed that the substitution that interrupts the U stretch at the editing locus did not completely block P-gene mRNA editing but dramatically reduced the RNA editing frequency.

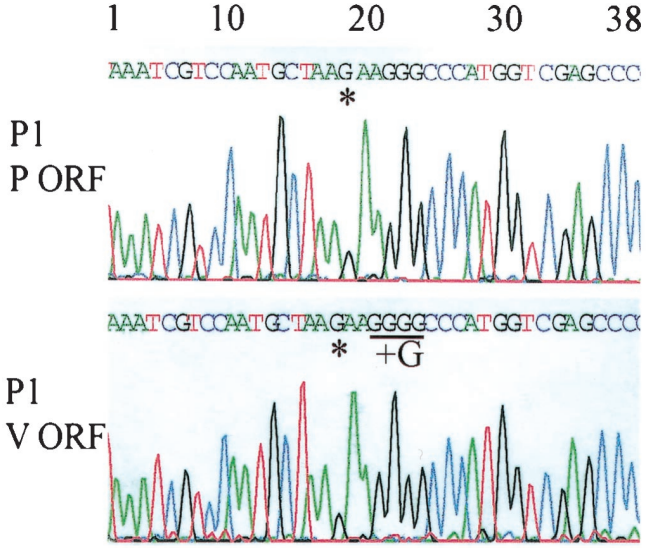

FIG. 4. P-gene mRNA editing in NDV-P1-infected cells. mRNA sequences in the regions of the editing site with the unedited P ORF or with insertion of one G residue (+G) coding for V ORF are shown. NDV-P1 (P1) edits its P-gene mRNA in spite of the interruption of the five A residues by A-to-G substitution (*).

NDV-P1 is attenuated for chicken embryos. NDV isolates vary in their virulence for chicken embryos as well as for chickens. The degree of virulence of a given NDV isolate can be measured by assessing the pathogenicity of the virus for 1-day-old chickens after intracerebral inoculation (2). Using this method, the intracerebral pathogenicity index of the rNDV was found to be identical to that of the wild-type parent, Clone-30 (32). Another method is to evaluate the time required for the virus to cause embryo mortality after allantoic inoculation. To determine the embryo mortality caused by NDV-P1, 10-fold serial dilutions of passage 5 of NDV-P1 were inoculated into 11-day-old embryonated SPF chicken eggs (0.2 ml/egg), which were then incubated for 1 week. Interestingly, no specific embryo mortality was detected during the 7-day incubation period, showing that NDV-P1 was not lethal for embryos even with a dose as high as 6 $\log_{10}$ EID_{50}/ml (Fig. 5). Chicken embryos inoculated with the parent virus, rNDV, started to die as early as 3 days postinoculation, at doses higher than 4 $\log_{10}$ EID_{50}/ml. The difference between the 50% infectious dose and 50% lethal dose of rNDV was only 0.3 $\log_{10}$. In contrast, this difference was as high as 6.7 $\log_{10}$ for NDV-P1, showing that it was attenuated at least 10^6-fold more than its parent virus. To further analyze the pathogenicity of NDV-P1 for younger embryos, 7- and 8-day-old embryonated SPF chicken eggs were inoculated at a dose of 2 $\log_{10}$ EID_{50}/egg and observed for 1 week for embryo mortality. Interestingly, NDV-P1 was capable of causing embryo mortality reaching 62 and 23% for 7- and 8-day-old embryos, respectively. NDV-P1

TABLE 2. Hatchability and body weight of chickens after in ovo vaccination

Virus	Dose[a]	Hatchability (%)[b]	Wt[c]
NDV-P1	3.5	22 (73)	133
NDV-P1	4.3	28 (93)	135
NDV-P1	5.4	21 (70)	115
rNDV	5.0	7 (23)	ND
NDW	5.1	7 (23)	85
Control	0	29 (96)	141

[a] Log_{10} EID_{50} per egg calculated after back titration of the samples.
[b] Number of chickens hatched from 30 eggs.
[c] Mean body weight in grams at 2 weeks of age. ND, not determined.

reached a 10-fold-higher titer in these younger embryos than the virus grown in 9- to 11-day-old embryonated eggs. NDV-P1 was not lethal for SPF chicken embryos after 8 days of embryonation, indicating an age-dependent resistance of chickens to disease caused by NDV-P1.

In ovo vaccination of SPF chicken embryos with NDV-P1. NDV-P1 did not cause embryo mortality when applied to 9- to 11-day-old embryos; therefore, an in ovo (embryo) vaccination experiment was carried out to determine the safety of NDV-P1 in older embryos. We chose to perform this experiment in 18-day-old embryonated SPF chicken eggs because commercially available embryo vaccines are routinely administered at this age of embryonation. Hatchability was found to be up to 93% for NDV-P1-vaccinated chickens, compared to 96% for the control group (Table 2). The lowest hatchability (23%) was seen in eggs inoculated with either the parent rNDV or NDW, a live attenuated posthatching vaccine. At 2 weeks of age, the mean body weights of chickens hatched from NDV-P1-inoculated eggs ranged from 115 to 135 g, compared to 85 g for the animals that had received a comparable dose of NDW (Table 2).

NDV-P1 protects SPF chickens against a lethal challenge. To determine whether protective antibodies were induced in chickens hatched from eggs vaccinated in ovo, blood samples were collected at 2 weeks of age and animals were challenged with a velogenic Herts strain of NDV. Chickens vaccinated as embryos with NDV-P1 developed high antibody levels in a dose-dependent manner (Table 3). Interestingly, the level of protection against lethal challenge reached more than 95% in a dose-dependent manner. All control chickens died within 3 days of challenge. These data show that NDV-P1 can confer full protection when administered to 18-day-old SPF chicken embryos.

TABLE 3. NDV-P1 applied at day 18 of embryonation protects SPF chickens against lethal NDV challenge

Virus	Dose (log_{10} EID_{50}/egg)	Mean HI[a]	Survival (%)[b]
NDV-P1	3.5	4.0 ± 1.1	19/20 (95)
NDV-P1	4.3	4.8 ± 1.0	20/20 (100)
NDV-P1	5.4	5.4 ± 1.2	19/19 (100)
Control	0	0.7 ± 0.5	0/20 (0)

[a] HI, log_2 hemagglutination-inhibition titer at 2 weeks of age.
[b] Chickens were challenged with the Herts strain of NDV (5.5 log_{10} EID_{50}/chicken) intramuscularly.

TABLE 4. Safety and efficacy of NDV-P1 in commercial chickens vaccinated in ovo at 18 days of embryonation

Virus	Dose[a]	Hatchability (%)[b]	HI[c]	Body wt[d]	Survival (%)[e]
NDV-P1	3.7	29 (96)	1.4 ± 1.0	439	7/20 (35)
NDV-P1	4.5	29 (96)	1.5 ± 0.9	413	15/20 (75)
NDV-P1	5.7	27 (90)	1.8 ± 1.1	438	17/20 (85)
Control		29 (96)	1.2 ± 0.9	440	4/20 (20)

[a] Log_{10} EID_{50} per egg, calculated after back titration of the samples.
[b] Number of chickens hatched from 30 eggs.
[c] HI, hemagglutination-inhibition titer (log_2) against NDV at 2 weeks of age.
[d] Mean body weight in grams at 2 weeks of age.
[e] Chickens were challenged with the Herts strain of NDV at a dose of 5.5 log_{10} ELD_{50}/chicken intramuscularly.

NDV-P1 in commercial chicken embryos. In the study involving SPF chickens in which NDV specific antibodies are absent, a dose as low as 3.5 log_{10} EID_{50} protected 95% of the animals. In contrast, embryos from commercial chickens acquire passive immunity by the transfer of maternal immunoglobulins from serum to egg yolk. Such passive antibodies, which can be present at high levels (4 to 7 log_2 hemagglutination inhibition units) in very young chickens from immunized parent flocks, might impair the effectiveness of live virus vaccines by neutralizing the vaccine virus. To examine the safety of NDV-P1 and its ability to confer protection in the presence of maternally derived antibody, in ovo vaccination of commercial chicken embryos was performed. Hatchability of embryonated commercial chicken eggs was not affected by in ovo administration of NDV-P1 (Table 4). Moreover, the body weights of all groups of chickens vaccinated with NDV-P1 were comparable to those of the unvaccinated control group, demonstrating the safety of NDV-P1 when administered in ovo to 18-day-old embryonated commercial chicken eggs. The level of antibody response and protection of chickens vaccinated as embryos with NDV-P1 depended on the dose administered (Table 4). In the group that had received the highest dose, 85% of the chickens were protected against challenge, demonstrating the ability of NDV-P1 to break through maternal antibody and confer protection.

DISCUSSION

The V protein of the *Paramyxoviridae* is one of the most conserved P-gene-derived accessory proteins and is characterized by a cysteine-rich C-terminal region. Based on in vitro and in vivo results, the V protein and other P-gene-derived accessory proteins of members of the *Paramyxoviridae* were categorized as nonessential gene products. In this study, NDV mutants lacking V protein showed severe growth impairment in vitro and in 6-day-old embryonated chicken eggs. In contrast, no virus growth could be detected in 9- to 11-day-old embryonated eggs, indicating that V protein plays a dual role in virus replication and pathogenesis. Apart from the mutants completely lacking V protein, we succeeded in recovering attenuated NDV by introducing specific mutations at the conserved editing locus, which resulted in down regulation of V protein expression instead of complete abrogation.

It has long been documented that lentogenic NDVs are unable to produce infectious viruses in most tissue culture

systems without the addition of proteases. This is mainly due to the absence of efficient cleavage of the precursor fusion protein F0 to FI and F2 (25). Chicken embryos, in contrast to cell cultures, support the propagation of lentogenic NDVs to high titers and are obviously the best choices for the propagation of newly generated recombinant viruses. Thus, transfection supernatants were passed into 9- to 11-day-old embryonated SPF chicken eggs. However, apart from the rNDV, the only viable recombinant virus that was recovered after passage in embryonated eggs was NDV-P1. The mutant NDV-P1, in spite of the one nucleotide substitution at the editing site, was found to edit its P-gene mRNA, albeit at a 20-fold-lower frequency. NDV-P1 was able to propagate autonomously in 9- to 11-day-old embryonated eggs and reached a peak titer of 7.1 $\log_{10}$ EID_{50}/ml after six egg passages. Interestingly, a 10-fold-higher titer was obtained when this mutant was grown in 7- or 8-day-old embryos. In contrast, NDV-Vstop and NDV-Δ6 mutants were unable to grow in 9- to 11-day-old embryonated eggs despite repeated rescue and passage experiments. In order to be able to propagate the mutants in cell culture, we constructed virulent versions of the mutants and the wild-type virus by modifying the F protein cleavage site. Compared with the virulent wild-type virus, the mutant viruses required one or two extra cell culture passages to produce cytopathic effects in approximately 80% of infected BSR cells, suggesting that abolition of V expression may lead to prolonged replication. The mutant viruses showed severe impairment in replication in both BSR and Vero cells and grew to titers which were as much as 5,000-fold lower than the titer of the virulent wild-type virus, demonstrating the requirement of V protein for efficient virus replication in vitro. The difference in growth between the wild type and the mutants was very dramatic in embryonated eggs. Although the virulent wild-type virus grew to identical titers both in young and older embryos (approximately 10^8 focus-forming units/ml), the mutants grew to more than 200,000-fold-lower titers in 6-day-old embryonated eggs. In 9- to 11-day-old embryonated eggs, which are commonly used for NDV propagation, no virus growth could be detected. This indicates that V plays an important role in NDV pathogenesis in addition to its involvement in virus replication.

The mutant NDV-Vstop was constructed in order to distinguish the role played by V from that of W. The severely impaired in vitro and in vivo growth of NDV-Vstop, therefore, provides evidence that the cysteine-rich C terminus of V protein was mainly responsible for this incompetence. A mutant of SeV lacking the cysteine-rich C-terminal portion of V protein was also attenuated in vivo but replicated well in vitro, suggesting that this portion of the V protein is particularly responsible for in vivo attenuation (18). However, our results demonstrate that V protein is not only a pathogenesis factor in vivo but also an important regulatory protein in virus replication. Interestingly, this cysteine-rich C-terminal portion of V protein is expressed by all members of the *Paramyxoviridae* except HPIV-1 and HPIV-3 (11, 23), suggesting an important function associated with V protein. Whether the C-terminal portion of NDV V protein interacts with other viral or host cell proteins to modulate NDV replication and pathogenesis remains to be established.

In general, lentogenic NDVs are propagated by inoculating them into 9- to 11-day-old embryonated chicken eggs and harvesting allantoic fluid containing infectious virus 2 to 4 days after inoculation. Prolonging the incubation period to 7 days causes embryo mortality of up to 100%. During prolonged incubation, the infectious dose and the lethal dose do not differ much. In contrast to the situation with the parent virus, the use of high doses of NDV-P1 and prolonged incubation were not lethal to embryos, demonstrating that NDV-P1 is dramatically attenuated for chicken embryos (Fig. 5). The difference between the infectious and lethal doses of NDV-P1 was as high as 6.7 $\log_{10}$, compared to 0.3 $\log_{10}$ for the parent virus, showing that NDV-P1 is attenuated more than 10^6-fold (Fig. 5). Interestingly, NDV-P1 was able to cause embryo mortality when administered to embryos younger than 9 days old. The mortality reached 62% at day 7 of embryonation and decreased to 23% at day 8. NDV-P1 also reached a 10-fold-higher titer in these younger embryos than the virus grown in 9- to 11-day-old embryos. This age-dependent resistance of chicken embryos to NDV-P1 and V-deficient mutants suggests a possible role for the innate or adaptive immune response in completely preventing growth of V-deficient mutants and pathogenicity of NDV-P1 after 8 days of embryonation. It has long been known that IFN-mediated resistance of chicken embryos to viral infections increases with age (24). The phenotype of these V-defective mutants strongly suggests that they have an impaired ability to antagonize the host's innate response, in addition to having a severe replication impairment. The specific role of NDV V protein in virus replication and its involvement in counteracting innate immune responses is currently under investigation.

Recombinant SeV and MV that are defective for RNA editing and are, therefore, unable to express V protein were shown to be attenuated in vivo, although in vitro replication was not impaired (8, 17, 18, 40). Recent publications suggest that SeV and SV5 block activation of IFN-responsive genes by interacting with a cellular target, STAT1 (9, 10, 13). For SeV, the C protein was identified as being responsible for counteracting the IFN-induced antiviral state, whereas in SV5 it was the V protein that accounted for inhibiting IFN signaling by targeting STAT1 for proteasome degradation. Thus, the key determinant in SeV and SV5 pathogenicity appears to be the prevention of the IFN-mediated antiviral response. In contrast, treatment of HeLa cells with 1,000 IU of IFN produced no difference in IFN sensitivity between wild-type MV and V-deficient MV, suggesting that the IFN system probably does not play a major role in limiting the spread of MV that lacks V protein (27). It is possible that the V proteins of different members of the *Paramyxoviridae* function differently, perhaps in a host-specific manner, to overcome the antiviral effect of the immune system. In agreement with this, Didcock et al. (10) demonstrated that SV5 blocks IFN signaling in human but not in murine cells, showing that the action is host cell specific. This property may prevent one virus from crossing species barriers and causing disease in another species.

In most parts of the world, chickens and turkeys have to be protected against the ravages of ND by ND vaccines administered to hatched birds through drinking water, aerosols, or eye drops or by parenteral routes. In recent years, the in ovo technology using automated multiple-head injectors to deliver vaccines in embryonated eggs has largely replaced certain post-hatching poultry vaccines. Vaccination is generally carried out

at day 18 of embryonation and provides a labor-saving alternative to posthatching vaccination. Moreover, in ovo vaccination facilitates administration of a uniform dose of vaccine into each egg. Most posthatching NDV vaccines are based on lentogenic NDV strains that are safe for hatched chickens. Currently, however, there is no live ND vaccine that can be administered in ovo, mainly due to high embryo mortality and very low hatchability, even with the highly attenuated NDV strains. Thus, further attenuation of lentogenic NDV strains was necessary to render it safe for use as an embryo vaccine without losing immunogenicity. In the present study we succeeded in generating a recombinant NDV that is dramatically attenuated for chicken embryos. When the vaccine was administered at day 18, hatchability was not substantially affected, and hatched chickens reached body weights similar to those of control chickens (Table 2). NDV-P1 was able to induce a sufficient immune response to fully protect SPF chickens from a lethal challenge despite its reduced replication in embryonated eggs. NDV-P1 was able to confer protection not only in SPF chickens without maternally derived antibody but also in commercial chickens with high levels of maternal antibody (Tables 3 and 4). It is remarkable that NDV-P1 can provide protection in the face of the high levels of maternally derived antibody present at the time of administration and to confer protection in 85% of the chickens. The level of protection is dose dependent, and a relatively higher dose is required in commercial chickens with neutralizing maternal antibodies to achieve a high degree of protection than is required in SPF animals. Since passive immunity levels vary from flock to flock, the dose selected for practical use should remain safe in SPF chickens, in order to make sure that vaccination does not have adverse effects in animals with low levels of maternal antibody.

The attenuated mutant virus NDV-P1 not only is an attractive candidate embryo vaccine but also provides some insight into the effects of reduced levels of V protein expression in virus replication and pathogenicity. The phenotype of NDV-P1 and the inability of NDV-Δ6 and NDV-Vstop to propagate in 9- to 11-day-old chicken embryos demonstrated that genetic manipulation directed toward reducing V protein expression rather than abolishing it completely is the more promising strategy for developing a viable attenuated NDV. Such an attenuated virus is also an attractive vaccine vector for the expression of immune-stimulatory proteins or heterologous antigens derived from other poultry pathogens. Furthermore, scientific interest in NDV therapy is currently reviving, since NDV is remarkably effective in selectively killing tumor cells in humans and animals (30, 34). The possibility of generating recombinant NDV will conceivably facilitate the design of a safe and effective NDV-based anticancer therapy for humans and animals.

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