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Newcastle Disease Virus Fusion Protein Expressed in a Fowlpox Virus Recombinant Confers Protection in Chickens

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A cDNA copy of the RNA encoding the fusion (F) protein of Newcastle disease virus (NDV) strain Texas, a velogenic strain of NDV, was obtained and the sequence was determined. The 1,792-base-pair sequence encodes a protein of 553 amino acids which has essential features previously established for the F protein of virulent NDV strains. These include the presence of three strongly hydrophobic regions and pairs of dibasic amino acids in the pentapeptide Arg-Arg-Gln-Arg-Arg preceding the putative cleavage site. When inserted into a fowlpox virus vector, a glycosylated protein was expressed and presented on the surface of infected chicken embryo fibroblast cells. The F protein expressed by the recombinant fowlpox virus was cleaved into two polypeptides. When inoculated into susceptible birds by a variety of routes, an immunological response was induced. Ocular or oral administration of the recombinant fowlpox virus gave partial protection, whereas both intramuscular and wing-web routes of inoculation gave complete protection after a single inoculation.

Newcastle disease virus (NDV) is the etiologic agent of an economically important and highly contagious disease of poultry. The disease requires control by vaccination or by quarantine, with slaughter of flocks in a confirmed outbreak. NDV is a paramyxovirus existing as a number of strains which can be classified on the basis of the severity of the disease (13, 44). Velogenic strains cause acute lethal disease in chickens of all ages. Velogenic strains may be viscerotropic, causing hemorrhagic lesions in the digestive tract, or neurotropic, causing lesions in the respiratory tract and nervous system. Mesogenic strains cause acute respiratory and sometimes lethal nervous system infections in young chickens, although mortality is rare in older birds. Lentogenic strains cause mild or inapparent respiratory tract infection. Despite the large number of NDV strains evident when classification is based on virulence characteristics, only minor antigenic differences can be detected by using standard serological tests (4, 44). Those recognizable serological differences, however, do not prevent immunity to one strain from providing cross-protective immunity against all strains.

Vaccination against Newcastle disease has been used successfully since the 1940s (3, 4). The most successful vaccines are live virus vaccines which consist of lentogenic or selected mesogenic strains produced in eggs. These live virus vaccines work well when administered correctly, but problems arise when insufficient attention is paid in the field to timing, route of inoculation, the strain of virus, and follow-up serology. In particular, it has been found that high levels of passive antibody in young chicks produced from immunized parent flocks can interfere with replication of the vaccine strain. Vaccination must therefore be timed such that the level of pre-existing immunity has waned but before young birds are put at risk of exposure to virulent field strains (4). This is complicated by the fact that broilers live to be only 6 to 8 weeks of age.

NDV is the type species of the genus *Paramyxovirus* (21). The paramyxoviruses are pleomorphic enveloped viruses possessing a nonsegmented negative-strand RNA genome. Virions are bound by a lipid bilayer membrane bearing hemagglutinin-neuraminidase (HN) and fusion (F) glycoprotein spikes (7). Both glycoproteins found on the surface of paramyxovirus particles are considered to be involved in the generation of immunity to infection (1, 23). The HN protein is involved both in virus binding to host cell receptors and in receptor-destroying activity (36). The F protein mediates membrane fusion between the membrane of the virus and the plasma membrane of the host cell or between the plasma membranes of two cells, infected and uninfected (37). The F protein is synthesized as a precursor F_0, which is subsequently cleaved into F_1 and F_2 components which are then held together by disulfide linkages (38).

It has been demonstrated that either anti-HN or anti-F antibodies can neutralize the infectivity of the paramyxovirus SV5 if added to the virus before adsorption (23). However, paramyxoviruses are capable of cell-to-cell spread by cell-fusing activity, without a requirement for released infectious virus, and only anti-F antibody is successful in preventing spread of infection of SV5 by blocking the cell-fusing activity (23). Accordingly, it has been demonstrated that a protective immune response was elicited in laboratory animals which were inoculated with vaccinia virus recombinants expressing the F protein from three paramyxoviruses: measles virus (8), rinderpest virus (2, 46), and respiratory syncytial virus (29, 45). The requirement for antibody to the F protein may therefore be critical in the development of effective paramyxovirus vaccines.

The avipox virus fowlpox virus (FPV) has recently been developed as a live viral vector for the delivery of specific poultry disease antigens (5, 40). In a previous study (40), the hemagglutinin protein of a virulent avian influenza virus strain was expressed in an FPV recombinant. After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a heterologous virulent influenza virus challenge (40). We now report work on the development of an FPV recombinant expressing the F protein from

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a velogenic NDV, strain Texas. The F protein was correctly processed by cleavage of F_0 to F_1 and F_2 and expressed on the cytoplasmic membrane of cells infected with the FPV recombinant. Chickens inoculated with the recombinant virus by a number of routes were protected against lethal challenge with NDV.

MATERIALS AND METHODS

Cells and viruses. The Texas strain of NDV is a velogenic strain. The strain of FPV designated FP-1 has been described previously (40). It is an attenuated vaccine strain useful in vaccination of day-old chickens. FP-1 and recombinant viruses were grown in chicken embryo fibroblasts (CEF) derived from 10- to 11-day-old embryonated eggs obtained from SPAFAS, Inc. (Storrs, Conn.).

Preparation of cDNA clone of F gene. The NDV strain Texas was grown in 11-day-old embryonated eggs. The virus was purified (12), and the genomic RNA was prepared by phenol-chloroform extraction. Complementary DNA was synthesized (10) by using a specific primer, 5'(TTCTGG ATCCCGGTTGGCGCCTTCTAGG), the sequence of which was determined by comparison with the published sequence of the Beaudette strain (6). The cDNAs produced were digested with *Hpa*I and inserted into the *Sca*I site of pBR322 (Fig. 1A). This ligation mixture was used to transform *Escherichia coli* HB101 and was screened for tetracycline resistance. The cDNA clones were first grouped according to size, and those with an insert large enough to contain the fusion gene (1.8 kilobases) were further analyzed by hybridization techniques by using the synthetic oligonucleotide primer as a probe. For the purpose of sequencing, appropriate cDNA clones were subcloned into the phage M13mp8 after digestion with *Bam*HI. Complete identification was performed by DNA sequencing by using the dideoxy-chain termination method (34). The clone selected for further work was designated pNDV81 (Fig. 1A).

Construction of an FPV insertion vector, pCE19. pCE19 was created by first inserting a 5.5-kilobase *Pvu*II fragment from FP-1 into the *Pvu*II sites of pUC9 to form pRW731.13 (Fig. 1B). The *Hin*cII site in this plasmid had previously been defined as a nonessential insertion locus. To facilitate the insertion of foreign DNA into this site, a synthetic polylinker synCE4, 5'TCGCGAGAATTCGAGCTCGGTA CCCGGGGATCCTCTAGAGTCGACCTGCAGGCATG CAAGCTTGTTAAC; synCE5, 5'GTTAACAAGCTTGCA TGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTA CCGAGCTCGAATTCTCGCGA, was cloned into the *Hin*cII site of pRW731.13 to form pCE11 (Fig. 1B). This polylinker contained recognition sites for *Nru*I, *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Hin*cII, *Acc*I, *Sal*I, *Pst*I, *Sph*I, *Hin*dIII, and *Hpa*I. This plasmid was further modified by the addition of the sequence specified by synCE11, 5'AA TTATTTTTATGAGCT, and synCE12, 5'CATAAAAAT, between the *Eco*RI and *Sac*I sites of pCE11. The resulting plasmid was designated pCE19 (Fig. 1B).

Construction of an FPV recombinant, vFP-29. A 1.8-kilobase *Bam*HI fragment containing all but 22 nucleotides from the 5' end of the F protein coding sequence was excised from pNDV81 and inserted at the *Bam*HI site of pUC18 to form pCE13 (Fig. 2). By using standard recombinant DNA techniques (20), a *Hin*dIII-*Sma*I fragment containing the vaccinia virus early-late H6 promoter previously defined (11, 32, 40, 41) was inserted into pCE13 which had been cut with *Sal*I; the ends were made blunt with the Klenow fragment of DNA *Pol*I and further cut with *Hin*dIII. The resulting plasmid was designated pCE16 (Fig. 2). To align the initiating ATG codon of the NDV fusion gene with the 3' end of the H6 promoter and to replace the 22 nucleotides lacking in pCE16, two complementary synthetic oligonucleotides, synCE20, 5'AT CCGTTAAGTTTGTATCGTAATGGGCTCCAGATCTT CTACCAGGATCCCGGTAC; synCE21, 5'CGGGATCCTG GTAGAAGATCTGGAGCCCATTACGATACAAACTT AACGGAT, were designed with *Eco*RV and *Kpn*I sites at the termini. The oligonucleotides were annealed, phosphorylated, and ligated to a *Kpn*I-*Eco*RV fragment from pCE16 to form pCE18 (Fig. 2). The final construction, pCE20 (Fig. 2), was formed by ligating a *Hin*dIII-*Sma*I fragment from pCE18 with a *Hin*dIII-*Sma*I fragment from the vector pCE19.

Plasmid pCE20 was transfected into FP-1-infected primary CEF cells by using the calcium P_i precipitation method previously described (30, 33). Progeny of the in vivo recombination were plated on CEF monolayers under agarose, and plaques were screened for the presence of the inserted NDV F protein coding sequence by in situ plaque hybridization (30, 33). Positive plaques were selected and subjected to further rounds of plaque purification until a pure population was achieved. One representative plaque was then amplified, and the resulting FPV recombinant was designated vFP-29.

Immunofluorescence. Primary CEF cells were seeded the day prior to use at a density of 500,000 cells per 35-mm dish on sterile glass cover slips. This produced a subconfluent monolayer. Cells were then infected with either the parental FP-1 or the recombinant vFP-29 virus at a multiplicity of 2 PFU per cell. Indirect immunofluorescence was performed as previously described (41) at 24 h postinfection by using an anti-NDV polyclonal serum from chickens supplied by Merieux, Inc. (Athens, Ga.) at a dilution of 1:400. The second antibody was a rabbit anti-chicken antibody obtained from Accurate Chemical and Scientific Corp. (Westbury, N.Y.) and used at a 1:200 dilution. The final antibody was a fluorescein isothiocyanate goat anti-rabbit preparation also obtained from Accurate Chemical and used at a 1:50 dilution.

Immunoprecipitation. Primary CEF monolayers were infected at a multiplicity of 10 PFU per cell with FP-1 or vFP-29. At 6 h postinfection, the overlay was changed to a medium containing no methionine for 30 min and subsequently to a methionine-free medium containing [^{35}S]methionine at 20 μCi/ml. At 24 h postinfection, cells were lysed by the addition of buffer A (1% Nonidet P-40, 10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.01% sodium azide, 500 U of aprotinin per ml, and 0.2 mg of phenylmethylsulfonyl fluoride per ml). Immunoprecipitation was performed by using the polyclonal anti-NDV serum and protein A-Sepharose CL-4B supplied by Pharmacia, Inc. (Piscataway, N.J.) as a support matrix. All incubations and washes were performed in buffer A. At the final step before removal of the antigen-antibody complex from the Sepharose beads, two washes in 2 M LiCl–0.2 M urea were performed. The complex was then solubilized by the addition of Laemmli disrupting solution, (18) and the results were analyzed by polyacrylamide gel electrophoresis.

Chicken inoculations with recombinant FPV and NDV challenge. Groups of 21-day-old chickens obtained from specific-pathogen-free eggs were divided into five groups and treated as follows. For group I, 10 chickens were inoculated with 4.0 $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$) in 0.1 ml by the oral route. For group II, 10 chickens were inoculated with 4.0 $\log_{10}$ $TCID_{50}$ in 0.1 ml by the ocular

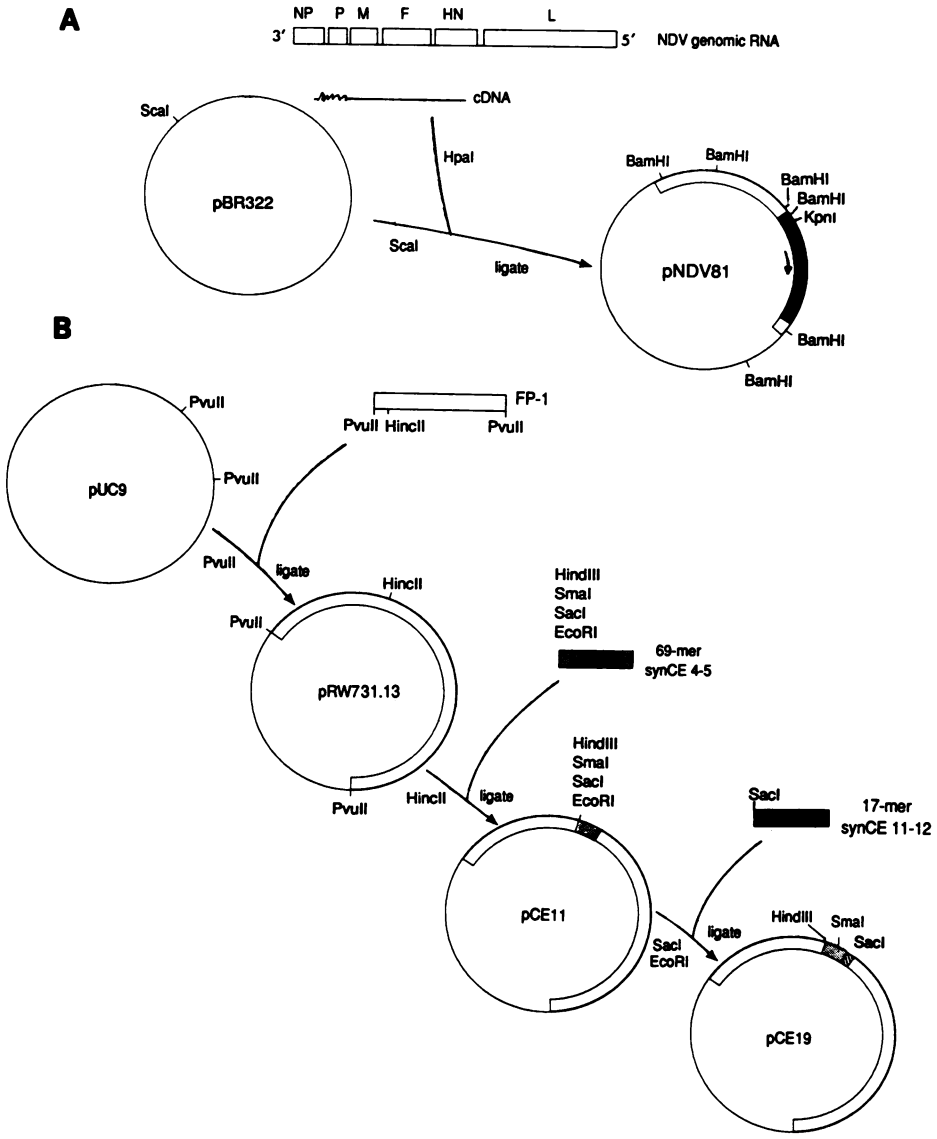

FIG. 1. Synthesis of NDV F cDNA and construction of fowlpox insertion vector. (A) The cDNA cloning procedure was as described in Materials and Methods. The genomic RNA of NDV is shown, with the primer (wavy line) used to synthesize the cDNA (straight line) indicated beneath the NDV genome. The NDV cDNA was digested with *Hpa*I and inserted into the *Sca*I site of pBR322. The NDV fusion sequence is shown as a dark block; additional NDV sequences are shown as an open block. The arrow indicates the direction of transcription. (B) A 5.5-kilobase *Pvu*II fragment of FP-1 (open block) was cloned into the *Pvu*II sites of pUC9 to form pRW731.13. A synthetic polylinker (synCE4-5 [stippled block], described in Materials and Methods) was inserted into the *Hin*cII site of pRW731.13 to form pCE11. A further modification was made to pCE11 by the addition of synCE11-12 (striped block) (see Materials and Methods), which was cloned into the *Eco*RI and *Sac*I sites to form pCE19.

route. For group III, 20 chickens were inoculated with 4.0 $\log_{10}$ $TCID_{50}$ in 0.01 ml by wing-web scarification. For group IV, 20 chickens were inoculated with 4.0 $\log_{10}$ $TCID_{50}$ in 0.2 ml by the intramuscular route. For group V, 20 chickens were left as uninoculated controls. At 28 days postinoculation, groups I and II as well as 10 birds each from groups III, IV, and V were challenged as described below. The remaining chickens in each group were reinoculated at 28 days in an identical manner to the first inoculation. At 49 days after the primary inoculation or 21 days after the booster inoculation, these birds were also challenged with the velogenic NDV Texas strain. The challenge consisted of 5.0 $\log_{10}$ 50% egg infectious doses in 0.5 ml by the intramuscular route. Deaths were recorded, and surviving birds were maintained to 14 days postchallenge. At this time, healthy birds without nervous signs were judged protected. Individual serum samples were collected from each bird before the initial inoculation, at 14 days, before challenge or boost at 28 and 42 days, and before challenge at 49 days. These sera were tested for the presence of NDV-neutralizing antibodies in a serum neutralization test and for anti-FPV antibodies in an enzyme-linked immunosorbent assay (ELISA).

ELISA. Sera were tested for the presence of anti-FPV antibodies by using an ELISA performed essentially as previously described (41), with FPV purified from host cell contaminants by sucrose gradient centrifugation as an antigen source.

Serum neutralization. The immune response to the F

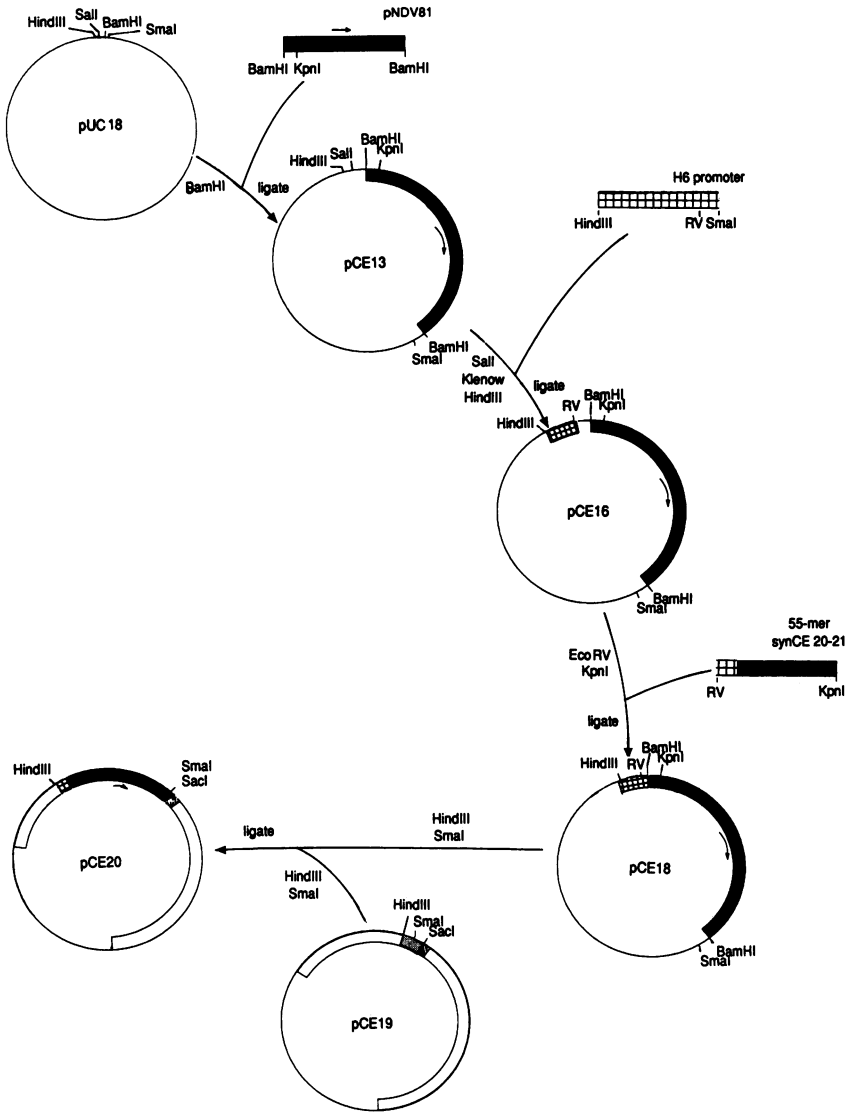

FIG. 2. Construction of insertion vector pCE20. A *Bam*HI fragment from pNDV81 containing the NDV F sequence (dark block) was cloned into the *Bam*HI site of pUC18 to form pCE13. The arrow denotes the direction of transcription. After digestion with *Sal*I, the ends of pCE13 were made blunt with the Klenow fragment of DNA *Pol*I and further cut with *Hin*dIII, and the vaccinia virus early-late H6 promoter *Hin*dIII-*Sma*I fragment (hatched block) was inserted. The resulting plasmid, pCE16, was digested with *Eco*RV and *Kpn*I, and a synthetic oligonucleotide (synCE20-21, described in Materials and Methods) was inserted to form pCE18. The H6 promoter-NDV F sequence was transferred from pCE18 as a *Hin*dIII-*Sma*I fragment into pCE19. The resulting plasmid, pCE20, was then used in in vitro recombination to generate the recombinant FPV vFP29.

protein of NDV was detected in a serum neutralization test. By using a microdilution plate system, sera were diluted in serial fourfold dilutions beginning at 1:4. Each serum dilution was mixed with an equal volume containing 100 $TCID_{50}$ of the cell-adapted Hitchner B1 strain of NDV (14). After incubation at 38°C for 30 min, a primary chicken kidney cell suspension was added and the plates were further incubated in an atmosphere of 5% CO_2 at 38°C. The test was read when complete cytopathic effect was observed in the negative control serum and after verification of the virus positive control at 100 $TCID_{50}$. The titer of the serum was $\log_{10}$ of the inverted endpoint of the serum dilution showing complete neutralization of cytopathic effect. For each group, the results were expressed as the geometric mean titer.

RESULTS

In NDV-infected cells, the F glycoprotein is anchored in the membrane via a hydrophobic transmembrane region near the carboxyl terminus and requires posttranslational cleavage of a precursor, F_0, into two disulfide-linked polypeptides, F_1 and F_2. Cleavage of F_0 is important in determining the pathogenicity of a given NDV strain (15, 27, 28), and the sequence of amino acids at the cleavage site is therefore critical in determining viral virulence. A comparison of the sequence of the F_0 protein from a number of virulent and avirulent strains (6, 9, 19, 22, 42) has demonstrated that virulent strains possess the highly basic sequence Arg-Arg-Gln-Arg/Lys-Arg in the pentapeptide immediately pre-

ceding the cleavage site. Avirulent strains possess the sequence Gly-Arg/Lys-Gln-Gly/Ser-Arg. It has been proposed that this highly basic sequence is essential for maintaining the cleavage site in an exposed conformation accessible to host cell proteases in a wide variety of tissues. In avirulent strains, the F_0 protein is cleaved only in a limited variety of tissues (embryonated eggs or the chorioallantoic membrane) or by trypsin, possibly because the cleavage site in these strains is less accessible to cellular proteases.

An alternate hypothesis is also available. It has been shown that precursors to hormones are cleaved at pairs of dibasic amino acids in the trans-Golgi membrane by specific endopeptidases (39). Since the NDV fusion protein is also cleaved in the vicinity of the trans-Golgi membrane (26), it is conceivable that cleavage of the NDV fusion sequence occurs via the same enzymes (25, 31).

Nucleotide and amino acid sequence analysis of the NDV F gene. The nucleotide sequence and the deduced amino acid sequence of the F gene of NDV strain Texas is shown in Fig. 3. Previous publications of sequences of NDV genes have defined consensus mRNA start sites as ACGGGTAGAAG (16, 24) and polyadenylation signals as TT/AAGAAAAAA (6, 19, 24). These signals are conserved in the Texas F_0 sequence and define a gene of 1,792 bases encoding 553 amino acids (Fig. 3), in agreement with previous estimates (6, 19, 24). There are six potential asparagine-linked glycosylation sites in the sequence. One of these sequences, Asn-Asn-Thr at residues 541 to 543, occurs in the presumed C-terminal cytoplasmic tail of the protein and is probably not glycosylated. The putative cleavage site of F_0 to F_1 and F_2 is indicated in Fig. 3 by an arrow before residue 117. The preceding pentapeptide (residues 112 to 116) has the sequence Arg-Arg-Gln-Arg-Arg and conforms to the sequence previously found to be a requirement for virulent NDV strains.

The hydropathic profile of the NDV F protein was determined by using the method of Kyte and Doolittle (17) (results not shown). In agreement with previously published data (6, 19, 24), three areas of high hydrophobicity were seen, corresponding to (i) the presumed signal peptide, (ii) the N terminus of the F_1 protein, which has been demonstrated to be directly involved in the fusogenic activity of the protein, and (iii) the presumed membrane-spanning region.

A comparison has been made between amino acid sequences of the F glycoprotein of the Texas strain and previously published sequences of the Beaudette C strain (6) and the Texas GB (1948) strain (35). The analysis is shown in Fig. 4. Excluding the 25 amino acids of the signal sequence, there are 32 amino acid changes between Texas (line 1) and Beaudette C (line 2), representing 94% homology. The 32 amino acid changes are spread throughout the protein. The same 32 amino acid changes occur in the Texas GB strain (line 3), with one additional change of Leu to Ser at residue 197. All three isolates have the optimal sequence in the pentapeptide preceding the cleavage site between F_1 and F_2. Both Beaudette C and Texas GB (1948) have a lysine at position 115, while Texas has an Arg at this position.

Immunofluorescence and immunoprecipitation analyses of NDF-F protein expressed in an FPV recombinant. In order to determine that the F protein produced by the FPV recombinant vFP29 was expressed on the infected cell surface, as in NDV-infected cells, immunofluorescence experiments were performed. Primary CEF monolayers were infected with parental (FP-1) or recombinant (vFP-29) viruses, and immunofluorescence was performed at 24 h postinfection by using an anti-NDV polyclonal chicken serum. The results are shown in Fig. 5. Cells infected with the recombinant vFP-29 showed strong surface fluorescent staining (Fig. 5d). In cells infected with the parental virus FP-1, no fluorescence was detected (Fig. 5b).

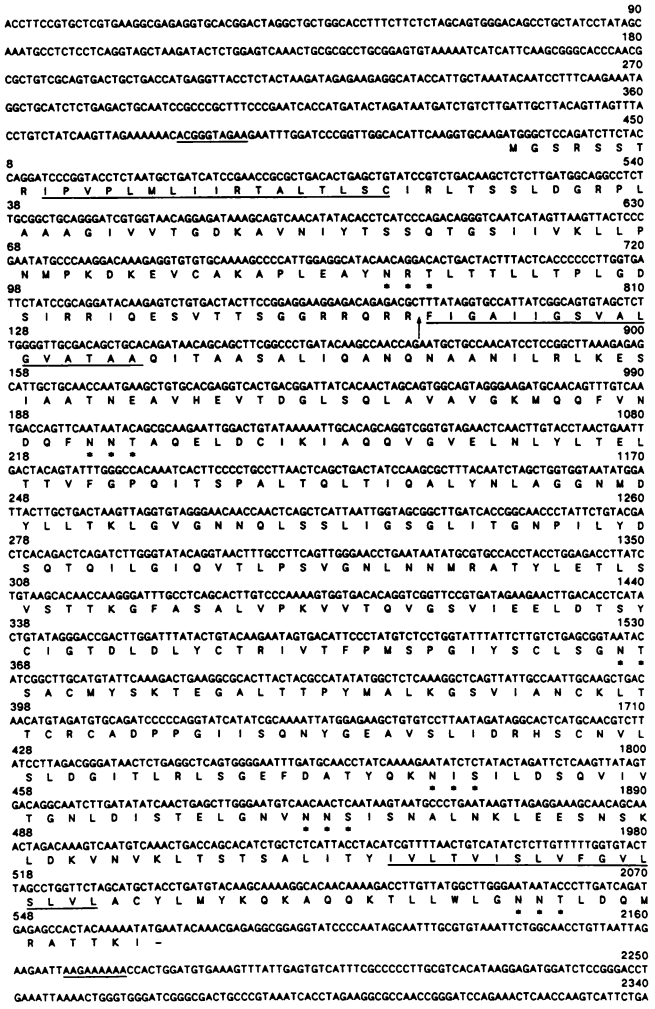

FIG. 3. Nucleotide sequence and deduced amino acid sequence of pNDV-81 containing the open reading frame encoding NDV strain Texas F_0 protein. Numbering in the left- and right-hand margins pertains to amino acid and nucleic acid sequences, respectively. The consensus mRNA start site and polyadenylation signal are underlined. Amino acids in the signal peptide, the N-terminal portion of the F_1 protein, and the membrane-spanning region are also underlined. The cleavage site is indicated by the arrow. The six potential glycosylation sites where the consensus sequence (N-X-S/T) is used are denoted by asterisks.

Cleavage of the F_0 peptide to the disulfide-linked F_1 and F_2 forms has been shown to be an essential feature of virulent NDV strains (27, 28). To confirm that the protein expressed in the FPV recombinant was correctly processed, immunoprecipitation experiments were performed by using [^{35}S] methionine-labeled lysates of CEF cells infected with parental and recombinant viruses.

The expected values for apparent molecular weights of the glycosylated forms of F_1 and F_2 are 54.7 and 10.3 kilodaltons, respectively (6). In order to demonstrate the presence of both F_1 and F_2 components, products of immunoprecipitation were analyzed on a 15% polyacrylamide gel. The

FIG. 4. A comparison of the deduced amino acid sequences of the F proteins of strains of NDV. The sequence of the F protein of the Texas strain is shown in line 1. Line 2 shows the sequence of the F protein of Beaudette C (6), and line 3 shows the sequence of Texas GB (35). The residue number is given at the right of the figure. A dash indicates an identical residue.

results are shown in Fig. 6. Lanes a and b illustrate immunoprecipitation of uninfected (lane a) and FP-1-infected (lane b) CEF cells. Lane c demonstrates specific immunoprecipitation from vFP-29-infected CEF cells of the F_1 and F_2 glycoproteins, with a calculated apparent molecular mass of 51 kilodaltons for F_1. No reliable molecular mass could be derived from this gel for F_2. The difference in efficiency of labeling of the two components is probably due to the presence in the F_1 component of eight methionine residues, while the F_2 component contains only one methionine residue. In order to more precisely estimate the size of the F_1 component, the products of the immunoprecipitation were analyzed on a 10% polyacrylamide gel under conditions in which the F_2 component had migrated off the gel (results not shown). The calculated molecular mass of the F_1 component was 56.5 kilodaltons, in agreement with published values (6). No F_0 precursor was detected in these experiments.

Immunogenic responses to NDV-F expressed by FPV recombinants. To determine whether the F protein expressed in the FPV recombinant vFP-29 was immunogenic, groups of susceptible 21-day-old chickens were inoculated with 10^4 $TCID_{50}$ of the recombinant FPV by a number of routes (see Materials and Methods). Following inoculation, serial bleeds were monitored for the presence of anti-F antibodies by a serum neutralization test and for anti-FPV antibodies by ELISA. The results of the serological testing are shown in Fig. 7 and 8.

When the anti-FPV response was monitored (Fig. 7), a significant response was observed when vFP-29 was inoculated by the wing-web or intramuscular route. The sera from all birds were positive for anti-F antibodies after the first inoculation, and a booster effect was evident after the second inoculation. With the oral and ocular routes, an antibody response was observed after the second inoculation, but only 6 of 10 birds were positive after oral inoculation and only 7 of 10 birds were positive after ocular inoculation.

When the anti-F response was monitored by a serum neutralization test (Fig. 8), antibody titers were low for oral, ocular, and wing-web routes after a single inoculation, while an intramuscular inoculation induced a higher neutralizing antibody response (Fig. 8). After a second inoculation, an increased neutralizing antibody titer was clearly observed for intramuscular and wing-web routes. The oral and ocular routes showed only a small increase in neutralizing titer, with 2 or 10 birds positive for the oral route and 4 of 10 positive for the ocular route. By the intramuscular route, 8 of 10 chickens were positive after only a single inoculation.

Protection of immunized birds against NDV challenge. Groups of chickens (10 per group) were vaccinated with either a primary or a primary-plus-booster inoculation of the recombinant FPV vFP-29 and subsequently challenged with a dose of 5.0 $\log_{10}$ 50% egg infectious doses of the Texas strain by the intramuscular route. The results of the challenge experiment are shown in Table 1. Nonvaccinated control birds were all susceptible to challenge. For birds inoculated by the oral and ocular routes, which were challenged only after two inoculations, the levels of protection were 50 and 90%, respectively. Chickens inoculated by the wing-web or intramuscular route were completely protected after either a primary or a primary-plus-booster inoculation.

DISCUSSION

A cDNA clone of the RNA sequence encoding the F protein of NDV strain Texas was obtained, and the sequence was determined. The gene is 1,792 base pairs long and encodes a protein of 553 amino acids possessing features in common with previously published sequences. In particular, the deduced amino acid sequence shows three hydrophobic areas corresponding to the signal sequence, the N-terminal portion of the F_1 protein, and the membrane-spanning region of the F_2 protein. The sequence also showed pairs of dibasic amino acids in the pentapeptide preceding the putative cleavage site between F_1 and F_2. This sequence has been shown to be characteristic of virulent NDV strains.

NDV strains have been classified on the basis of virulence characteristics. On the basis of mean death time in eggs, Texas GB would be classified as velogenic (4, 35). Beaudette C is described as mesogenic (4, 6) and has a slightly longer death time in eggs. Texas is velogenic, killing 5-week-old chickens in 4 to 6 days (unpublished data).

The amino acid sequence of the F_0 protein of the Texas GB (35) and Beaudette C (6) strains has previously been published, and the proteins have been shown to share strong

TABLE 1. Efficacy of protection of birds inoculated with recombinant vFP-29 against lethal virus challenge[a]

Inoculation route	Time (days)			No. of survivors/ total
	0	28	49	
Oral	+[b]	+	Challenge	5/10
Ocular	+	+	Challenge	9/10
Wing-web	+	Challenge		10/10
	+	+	Challenge	10/10
Intramuscular	+	Challenge		10/10
	+	+	Challenge	10/10
Controls		Challenge		0/10
			Challenge	0/10

[a] Chickens previously inoculated with one or two doses of recombinant vFP-29 were challenged at 28 days (for birds receiving only one inoculation) or 49 days (for birds receiving two inoculations) with 5 $\log_{10}$ $TCID_{50}$ of NDV strain Texas in 0.5 ml by the intramuscular route. Mortality was recorded daily, and the final result was assessed at 14 days after challenge when birds without nervous symptoms were judged protected.

[b] Day of inoculation.

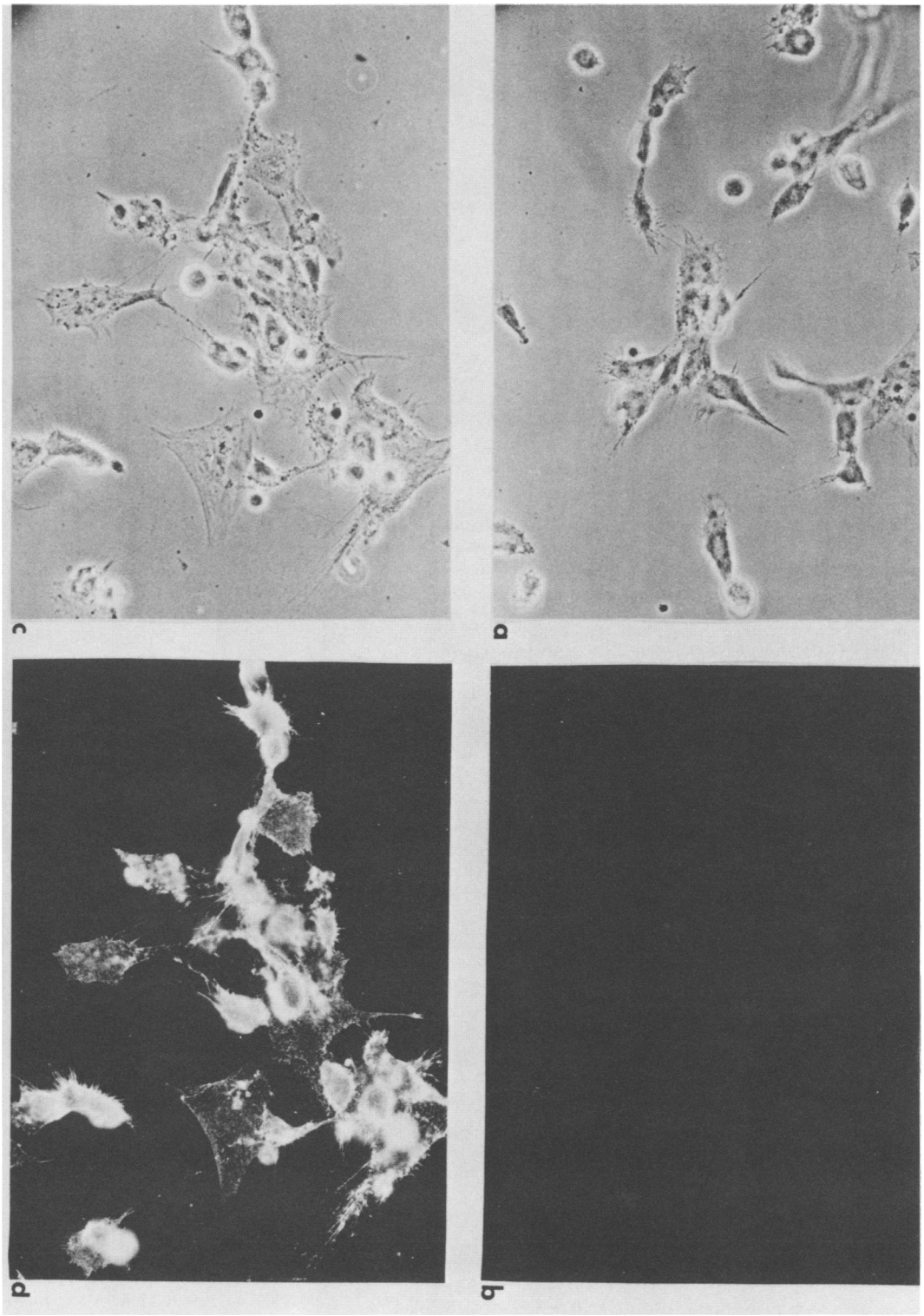

FIG. 5. Expression of the NDV F protein on the surface of cells infected with vFP-29. Primary CEF cells were infected with parental or recombinant viruses at 2 PFU per cell. Immunofluorescence was performed 24 h postinfection by using a polyclonal anti-NDV chicken serum. The figure shows identical fields under phase-contrast (a and c) and fluorescence (b and d) microscopy. (a and b) FP-1-infected cells; (c and d) vFP-29-infected cells.

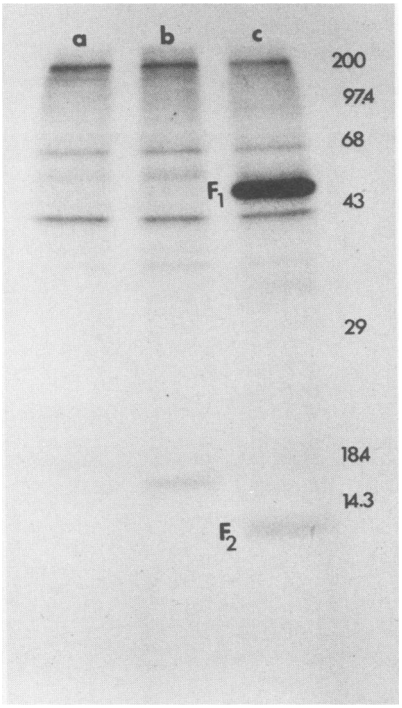

FIG. 6. Immunoprecipitation of F protein from vFP-29-infected CEF cells. CEF cells were infected at 10 PFU per cell with parental FP-1 and recombinant vFP29 viruses. [^{35}S]methionine was added at 6 h postinfection, and the incorporation was allowed to proceed until 24 h postinfection, when cultures were harvested and cells were lysed with a nonionic detergent. Immunoprecipitations were performed by using the polyclonal anti-NDV chicken serum and protein A bound to Sepharose CL-4B as a support matrix. Lanes: a, uninfected CEF cells; b, FP-1-infected CEF cells; c, vFP-29-infected CEF cells.

homology, with only two amino acid differences. In the present study, when the deduced amino acid sequence of Texas was compared with these previously published sequences, it was found that there was a homology level of 94.8% (excluding signal peptide sequence) between Texas and Beaudette C and (94.6%) between Texas and Texas GB. In a comparative study of the F protein of the lentogenic La Sota strain and the mesogenic Beaudette C, Le et al. (19) again found a strong sequence conservation of 97.8% between the two proteins. The authors postulated that amino acid changes occurring near cysteine residues in the F_1 portion of the La Sota strain may alter protein conformation and thus be involved in moderation of virulence. On comparison of the Beaudette C sequence with the Texas sequence, a number of amino acid changes do occur in this region of the protein (residues 340, 396, 402, 403, 421, and 422) close to cysteine residues. However, all the changes occurring in Beaudette C also occur in Texas GB, which does not show moderation of virulence. The sequence similarity of these strains is interesting in view of their variance in virulence characteristics. It may imply the involvement of other viral proteins, including the HN, in determining virulence.

An FPV-based recombinant virus which expresses the F protein of the NDV Texas strain has been developed. In CEF cells, the F protein is cleaved into the F_1 and F_2 components and an immunologically recognizable protein is expressed on the surface of infected cells. No cell-fusing activity was evident in CEF cells infected with the recombinant virus (results not shown).

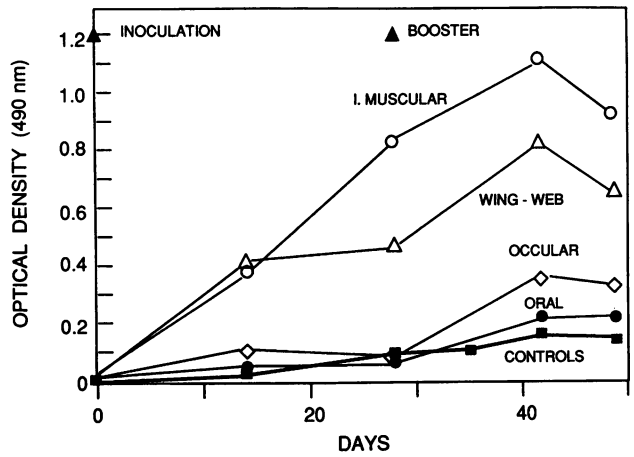

FIG. 7. Serological response of chickens inoculated with recombinant vFP-29. Chickens were inoculated with 4.0 $\log_{10}$ $TCID_{50}$ of vFP-29 by the routes indicated and with a booster by the same routes at day 28. The birds were bled at 0, 14, 28, 42, and 49 days. Sera were tested for the presence of anti-FPV antibodies by ELISA by using as an antigen source FPV purified from tissue culture contaminants by sucrose gradient centrifugation. The ELISA titer was expressed as an optical density read at 490 nm.

When chickens are inoculated, a slow immune response is observed when the inoculation is introduced by the ocular or oral route with respect to the development of both anti-NDV F antibodies and anti-FPV antibodies. The response is low even after a second inoculation by these routes. A much stronger response is seen after delivery of the FPV recombinant by the wing-web or intramuscular route, and this response rises sharply after a booster dose.

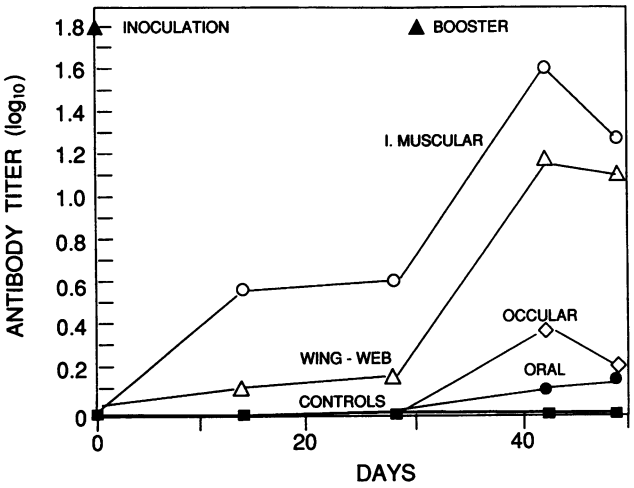

FIG. 8. Serological response of chickens inoculated with recombinant vFP-29. Chickens were inoculated with vFP-29 as indicated in Materials and Methods and in the legend to Fig. 7. The immune response against NDV F protein was measured in a microdilution serum neutralization test by using the Hitchner B1 strain of NDV and primary chicken kidney cells. Microdilution plates were read after complete cytopathic effect was observed in the negative serum control. The titer of the sera was expressed as $\log_{10}$ of the inverted endpoint dilution showing complete neutralization of cytopathic effect. For each group, the results were expressed as the geometric mean titer.

On challenge, 90% of birds receiving the recombinant by the ocular route were protected after two inoculations and 50% of birds receiving two doses of the recombinant by the oral route were also shown to be protected. Significantly, birds inoculated by either the wing-web or intramuscular route were fully protected after either a primary or booster inoculation. The chickens were symptomless after vaccination and upon challenge were completely disease free.

In previous studies (40) in which an FPV recombinant expressing the HA molecule of a virulent avian influenza virus was used, birds were protected against homologous challenge despite relatively low levels of hemagglutinin-specific antibody and against heterologous challenge in the absence of detectable hemagglutinin or serum-neutralizing antibody against the heterologous challenge virus. The relative contributions of detectable humoral antibody and cellular responses in that study (40) as well as in the study presented here remain to be assessed. Previous results on the role of antibody to the F protein in inducing immunity to NDV have been mixed. In common with previous work on the paramyxovirus SV-5 (23), Avery and Niven (1) found that antibody to either the HN or F_0 protein could fully neutralize infectivity of NDV. However, Umino (43) found that antibody to the HN protein was much more efficient at neutralizing infectivity than was antibody to the fusion protein. The present study indicates the efficacy of delivery of the NDV F protein in inducing immunity against a lethal virus challenge and may provide the basis for a useful vaccine against this disease.

This and other studies from our laboratory (40, 41) indicated the utility of FPV-based vectors for delivering protective antigens for diseases in poultry as well as in nonavian species.

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