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Characterization of an Alternate Form of Newcastle Disease Virus Fusion Protein

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The sequence and structure of the Newcastle disease virus (NDV) fusion (F) protein are consistent with its classification as a type 1 glycoprotein. We have previously reported, however, that F protein can be detected in at least two topological forms with respect to membranes in both a cell-free protein synthesizing system containing membranes and infected COS-7 cells (J. Virol. 77:1951–1963, 2003). One form is the classical type 1 glycoprotein, while the other is a polytopic form in which approximately 200 amino acids of the amino-terminal end as well as the cytoplasmic domain (CT) are translocated across membranes. Furthermore, we detected CT sequences on surfaces of F protein-expressing cells, and antibodies specific for these sequences inhibited red blood cell fusion to hemagglutinin-neuraminidase and F protein-expressing cells, suggesting a role for surface-expressed CT sequences in cell-cell fusion. Extending these findings, we have found that the alternate form of the F protein can also be detected in infected and transfected avian cells, the natural host cells of NDV. Furthermore, the alternate form of the F protein was also found in virions released from both infected COS-7 cells and avian cells by Western analysis. Mass spectrometry confirmed its presence in virions released from avian cells. Two different polyclonal antibodies raised against sequences of the CT domain of the F protein slowed plaque formation in both avian and COS-7 cells. Antibody specific for the CT domain also inhibited single-cycle infections, as detected by immunofluorescence of viral proteins in infected cells. The potential roles of this alternate form of the NDV F protein in infection are discussed.

Newcastle disease virus (NDV) is a major agricultural pathogen that causes a fatal respiratory and neurological disease in poultry (21). This virus, a member of the *Paramyxoviridae* family, initiates infection by fusion of the viral membrane with host cell plasma membranes (13). Virus spread is facilitated by cell-cell membrane fusion. NDV, as well as other paramyxoviruses, encodes two spike glycoproteins, the hemagglutinin-neuraminidase (HN) protein, which mediates attachment of the virion to sialic acid-containing receptors, and the fusion (F) protein, which directly mediates membrane fusion (reviewed in reference 13). Primary sequence and structural analyses indicate that the F protein is a classical type 1 glycoprotein with an amino-terminal signal sequence, a hydrophobic transmembrane domain near the carboxyl terminus, and a 25- to 30-amino-acid cytoplasmic domain (CT) (13, 19). The F protein is synthesized as a precursor, F_0, which undergoes proteolytic cleavage to form disulfide-linked amino-terminal F_2 and carboxyl-terminal F_1 polypeptides, and cleavage is required for fusion activity (reviewed in reference 13).

There are now several examples of both cellular (4, 10, 15, 31) and viral glycoproteins that are found in different topological forms with respect to membranes. Examples of viral glycoproteins with alternate membrane topologies include the hepatitis B virus L protein (reference 14 and references therein), the transmissible gastroenteritis virus M protein (8), and the hepatitis C virus envelope glycoproteins (18, 23). We previously reported that synthesis of the NDV F protein in a cell-free protein-synthesizing system containing membranes resulted in at least two topological forms of the protein with respect to membranes (17). The properties of one form were entirely consistent with a type 1 fully glycosylated F protein. The other was a partially translocated or polytopic form in which approximately 200 amino acids of the amino terminus as well as the CT domain of the protein were translocated across membranes (17). Importantly, we detected this second, polytopic form of F protein in COS-7 cells expressing the F protein (17) and provided evidence that it was involved in cell-cell fusion, either directly or indirectly.

Extending these results, we report that the second form of the F protein is also found in F protein-expressing avian cells, which are the natural host cells of NDV. We have detected this second form of the F protein in virions released from both infected COS-7 and avian cells. We report evidence that this second form of the F protein may have role in virus-cell fusion.

MATERIALS AND METHODS

Cells, virus, and plasmids. COS-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, and 10% fetal calf serum. East Lansing Line (ELL-0) chicken fibroblasts (UMNSAH/DF-1), obtained from American Type Culture Collection, were maintained in DMEM supplemented with penicillin-streptomycin and 10% fetal calf serum.

NDV strain AV (virulent) and strain B1 (avirulent) stocks (21) were prepared by growth in eggs by standard protocols. AV stocks formed plaques in COS-7 cells, while B1 did not, consistent with the expected phenotypes of the two strains of NDV. The F protein gene carried by purified NDV strain B1 virus was sequenced to verify the absence of a furin recognition sequence. The NDV F and

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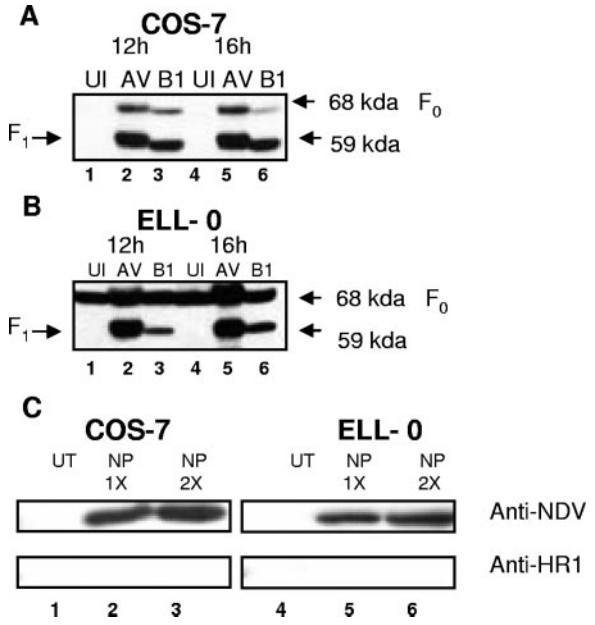

FIG. 1. Two forms of the F protein are expressed in infected avian and COS-7 cells. COS-7 (panel A) and avian cells (ELL-0) (panel B), infected with NDV strain AV (lanes 2 and 5) or strain B1 (lanes 3 and 6) or mock infected (UI) (lanes 1 and 4) for 16 h (lanes 1 to 3) or 24 h (lanes 4 to 6), were lysed, and aliquots of total extract were subjected to Western analysis as described in Materials and Methods. Blots were incubated with anti-HR1. Molecular weight markers were included in each blot and were used to determine sizes of the bands detected. Panel C shows Western analysis of 1× and 2× volume of extracts from COS-7 and avian cells transfected with pCAGGS NP (lanes 2, 3, 5, and 6). Duplicate blots were incubated with anti-NDV or anti-HR1. Untransfected (UT), lane 1.

HN genes were expressed in COS-7 and ELL-0 cells, using pCAGGS obtained from Common Access to Biotechnological Resources and Information (22).

Infections and virus purification. COS-7 or ELL-0 cells were plated at 6×10^5 per 35-mm plate and grown overnight. Cells were then infected with NDV strain AV or NDV strain B1 at a multiplicity of infection of 10. After adsorption, unbound virus was removed, and cells were washed in phosphate-buffered saline (PBS) and then incubated for 16 or 48 h. Supernatants were clarified by centrifugation at 5,000 rpm for 5 min at 4°C, and virions were pelleted through 20% sucrose by centrifugation at 40,000 rpm for 4 h at 4°C using the SW50.1 rotor (Beckman). Virus was resuspended in 100 μl of PBS.

Transfection. Transfections were accomplished using lipofectamine (Invitrogen) as recommended by the manufacturer. COS-7 or ELL-0 cells were plated at 3×10^5 per 35-mm plate. After 20 h, a mixture of DNA (0.5 μg) in 0.1 ml OptiMEM (Gibco/Invitrogen) and 5 μl of lipofectamine in 0.2 ml of OptiMEM was incubated at room temperature for 45 min, diluted with 0.7 ml of OptiMEM, and added to a 35-mm plate previously washed with OptiMEM. The cells were incubated for 5 h, the lipofectamine-DNA complexes were removed, and then 2 ml of supplemented DMEM was added.

Antibodies. Anti-NDV is a polyclonal antiserum raised in rabbits against UV-inactivated virions by standard methods. Anti-HR1 was raised against a glutathione *S*-transferase (GST) fusion protein that contained sequences from amino acid 130 to 173 of the F protein cloned in frame with the carboxyl terminus of GST (16). Anti-Ftail 523–553 was raised against a synthetic peptide with the sequence of the cytoplasmic domain of the fusion protein (amino acids 523 to 553) as previously described (29) and prepared by the Peptide Core Facility of the University of Massachusetts Medical School. Anti-F_2 was raised against a GST fusion protein containing the sequence between amino acids 96 and 117 cloned in frame with the carboxyl terminus of GST. Anti-Ftail 540–553 was raised against a GST fusion protein containing the sequence between amino acids 540 and 553 cloned in frame with the carboxyl terminus of GST. The GST fusion polypeptides were purified by standard methods recommended in the Novagen *Applications Guide*. Antibody was raised in rabbits by standard methods by Capralogics, Inc. (Hardwick, MA). Antibodies recognizing GST were removed from the sample by affinity purification using immobilized GST (Pierce). Antibodies raised against HR1 and Ftail sequences were recovered using protein A immunoglobulin G (IgG) purification (Immunopure Plus immobilized protein A IgG purification kit; Pierce). Actin was detected using phalloidin coupled to Alexa568 (Invitrogen/Molecular Probes).

Surface immunofluorescence. COS-7 or ELL-0 cells, grown in 35-mm plates containing glass coverslips, were infected with NDV, either strain AV or strain B1, for 12 or 16 h, respectively. COS-7 or ELL-0 cells were transfected with cDNAs as described above for 48 or 24 h, respectively. Cells were washed twice with ice-cold IF buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide, and 5 mM $CaCl_2$), blocked with IF buffer for 15 min, and incubated for 1 h at 4°C in IF buffer containing specific antibodies, washed three times with ice-cold IF buffer, and incubated for 1 h on ice with IF buffer containing Alexa 488-labeled antirabbit IgG (Molecular Probes). Cells were washed with ice-cold IF buffer, fixed with 2% paraformaldehyde, and mounted on slides using Vectashield mounting medium (Vector Labs, Inc.) for immunofluorescence microscopy.

Fluorescence images were acquired using a Nikon fluorescence microscope and Openlab software.

Flow cytometry. Transfected cells were removed from plates with 0.2 ml trypsin (50 μg/ml) (Sigma Corp.), washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% bovine serum albumin and 0.02% sodium azide) containing soybean trypsin inhibitor (2 μg/ml), and incubated for 1 h at 4°C with anti-Ftail 540–553 and anti-NDV antibodies diluted in FACS buffer. After three washes with FACS buffer, the cells were incubated for 1 h at 4°C with goat antirabbit IgG coupled to Alexa 488 (Molecular Probes) diluted in FACS buffer. After three washes with FACS buffer, the cells were resuspended in PBS containing 2% paraformaldehyde and subjected to flow cytometry.

Preparation of extracts and Western analysis. COS-7 or ELL-0 cells, infected with NDV for 12 and 16 h (multiplicity of infection of 10), were washed in PBS and lysed in RSB buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M $NaCl_2$) containing 1% Triton X-100, 0.5% sodium deoxycholate, 2 mg/ml of *N*-ethylmaleimide, and 0.2 mg/ml of DNase. Freshly made total extracts, diluted in sample buffer (125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol) with 0.7 M β-mercaptoethanol and incubated at room temperature, were loaded onto 8% or 12% polyacrylamide gels without boiling to avoid protein aggregation. After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, pH 8.2, 192 mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween 20 and 10% nonfat milk, washed with PBS-Tween 20, and incubated for 1 h at room temperature with primary antibody diluted to 1:1,000 in PBS-Tween 20. Membranes were then washed, incubated for 1 h at room temperature with secondary antibody, goat antirabbit IgG coupled to horseradish peroxidase (Amersham Biosciences) diluted to 1:40,000 in PBS-Tween 20, and then washed extensively in PBS-Tween 20. Bound antibody was detected using the ECL Western blotting detection reagent system (Amersham Biosciences).

Mass spectrometry. Protein bands were digested "in gel" according to established methods (12). Eluted peptides were separated on an LC Packings Ultimate nanoflow high-performance liquid chromatography system in the following way. Ten microliters of the peptide digest solution (approximately one-half of the total digest) was manually injected onto a micro-trap column (LC Packings Precolumn cartridge, 0.3 mm by 5 mm, C_{18} PepMap), and the trap column was manually washed with 10 μl of 0.1% formic acid prior to switching in line with the reverse-phase separating column (100-μm by 15-cm C_{18} PepMap; LC Packings). A gradient was developed from 100% solvent A (0.1% formic acid) to 60% solvent B (0.1% formic acid in acetonitrile-water [70:30]) in 60 min at a flow rate of 500 nanoliters per minute. The outlet of the column was connected to an electrospray needle (New Objective Taper tip, 20 μm). Electrospray mass spectrometry (MS) was performed on a Thermoelectron Finnigan LCQDeca ion trap mass spectrometer.

Data-dependent acquisitions were set up according to a triple-play experiment program where full MS scans from 400 Da to 2,000 Da were ongoing until an MS signal grew above a specified threshold, upon which a high-resolution scan (Zoom Scan) was performed to determine the monoisotopic mass and charge state, followed by a single MS/MS scan. Dynamic exclusion was applied to prevent repeat scans of the same peptide masses.

The raw data files were converted into mass peak lists using the LCQ_DTA program and then searched against the virus taxonomy of the NCBI nr protein database using the Mascot search engine (Matrix Science Ltd.) (www.matrixscience.com), using 1-Da mass tolerances for both the parent and fragment masses.

Neutralization assay. Confluent COS-7 or ELL-0 cells in 60-mm plates were used for plaque assays. NDV strain AV was preincubated with anti-NDV, anti-

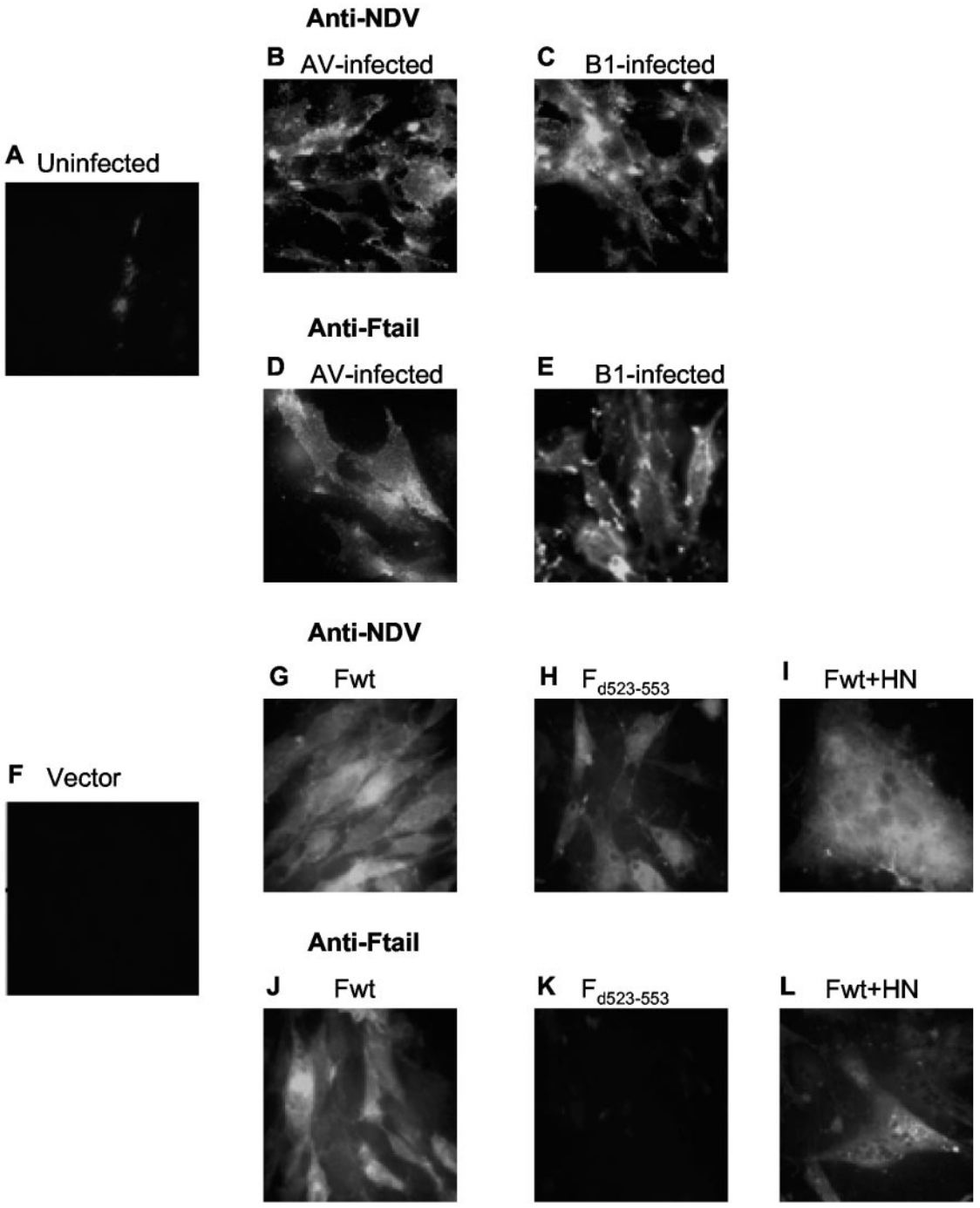

FIG. 2. Detection of cytoplasmic tail sequences on the cell surfaces of transfected and infected avian cells by immunofluorescence. Panels B-E show results with NDV-infected avian cells. Panels F-L show results with transfected avian cells. Uninfected avian cells (panel A) and avian cells infected with NDV strain AV (panels B and D) or strain B1 (panels C and E) was incubated with anti-NDV (panels A to C) or anti-Ftail 540–553 (panels A, D, and E). Infected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s. Avian cells transfected with vector alone (panel F) or transfected with pCAGGS-Fwt (panels G and J), pCAGGS-Fd523-553 (panels H and K), or pCAGGS-Fwt and pCAGGS-HN (panels I and L), were incubated with anti-NDV (panels F-I) and anti-Ftail 540–553 (panels F and J to L). Transfected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s. Images were acquired using a ×60 objective and OPEN LAB software.

Ftail antibodies, or preimmune sera for 1 h at room temperature prior to adsorption. The virus and antibody mixes were then added to cells and incubated for 45 min at 37°C. After adsorption, the virus-antibody mixture was removed and cells were washed once in PBS. For plaque assays, cells were then overlayered with agar diluted to 1% in DMEM and supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, sodium bicarbonate, and 10% fetal bovine serum. Plaques were counted after 48 h of incubation at 37°C. Cells were fixed in methanol and stained with Giemsa stain (Sigma) diluted 1:20 in

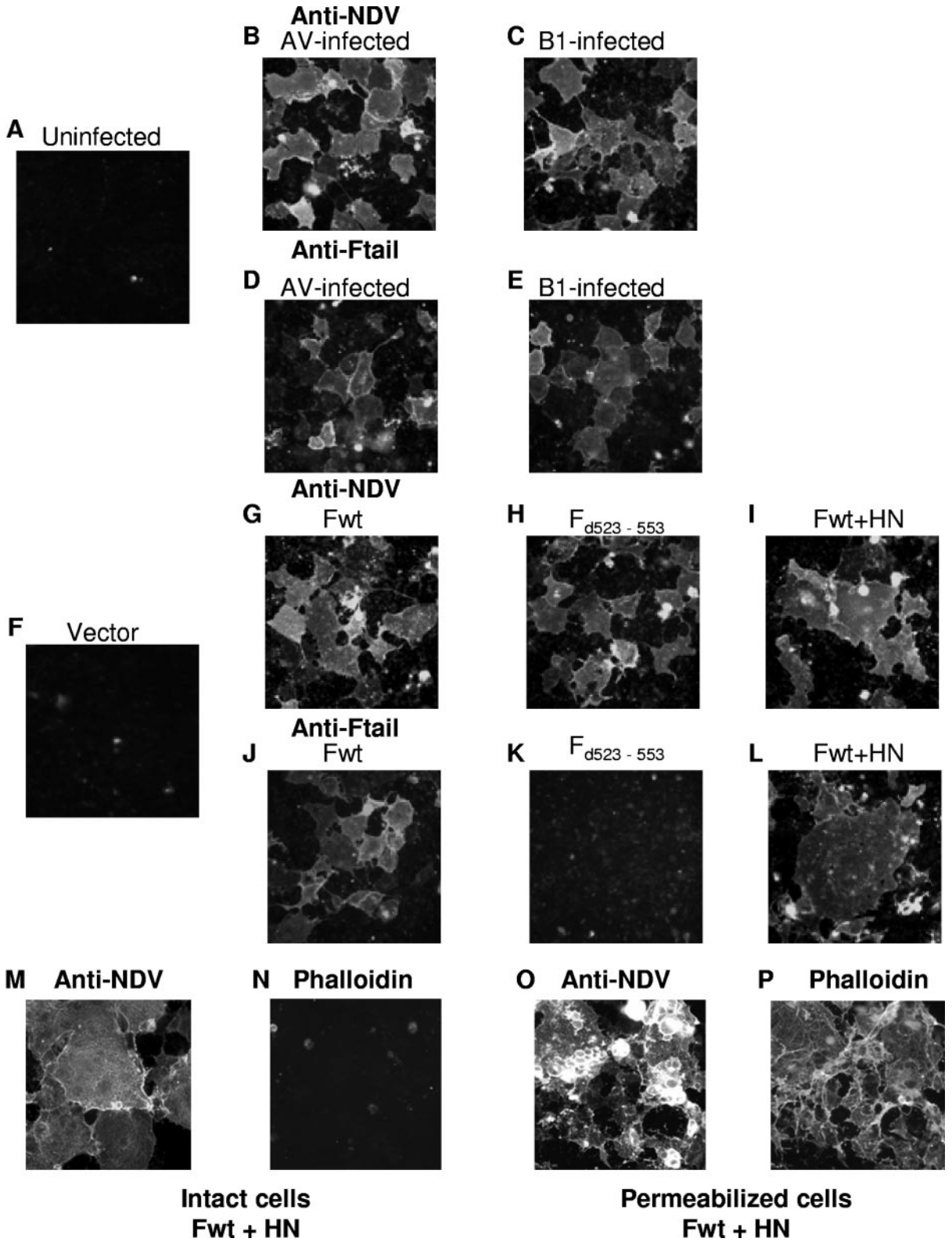

FIG. 3. Detection of cytoplasmic tail sequences on the cell surfaces of infected and transfected COS-7 cells by immunofluorescence. Panels B to E show results with NDV-infected COS-7 cells. Panels F to P show results with transfected COS-7 cells. Uninfected COS-7 cells (panel A) and COS-7 cells infected with NDV strain AV (panels B and D) or strain B1 (panels C and E) were incubated with anti-NDV (panels A, B, and C) or anti-Ftail 540–553 (panels A, D, and E). Infected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s. COS-7 cells transfected with vector alone (panel F), transfected with pCAGGS-Fwt (panels G and J), pCAGGS-Fd523-553 (panels H and K), or pCAGGS-Fwt and pCAGGS-HN (panels I and L, M, N, O, and P) were incubated with anti-NDV (panels F to I) or anti-Ftail 540–553 (panels F and J to L). Transfected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s using OPEN LAB software. Intact cells expressing the HN and F proteins were incubated with anti-NDV and phalloidin-Alexa568 (panels M and N, respectively). Cells expressing HN and F proteins were incubated in 0.1% Triton X-100 and then anti-NDV and phalloidin-Alexa568 (panels O and P, respectively).

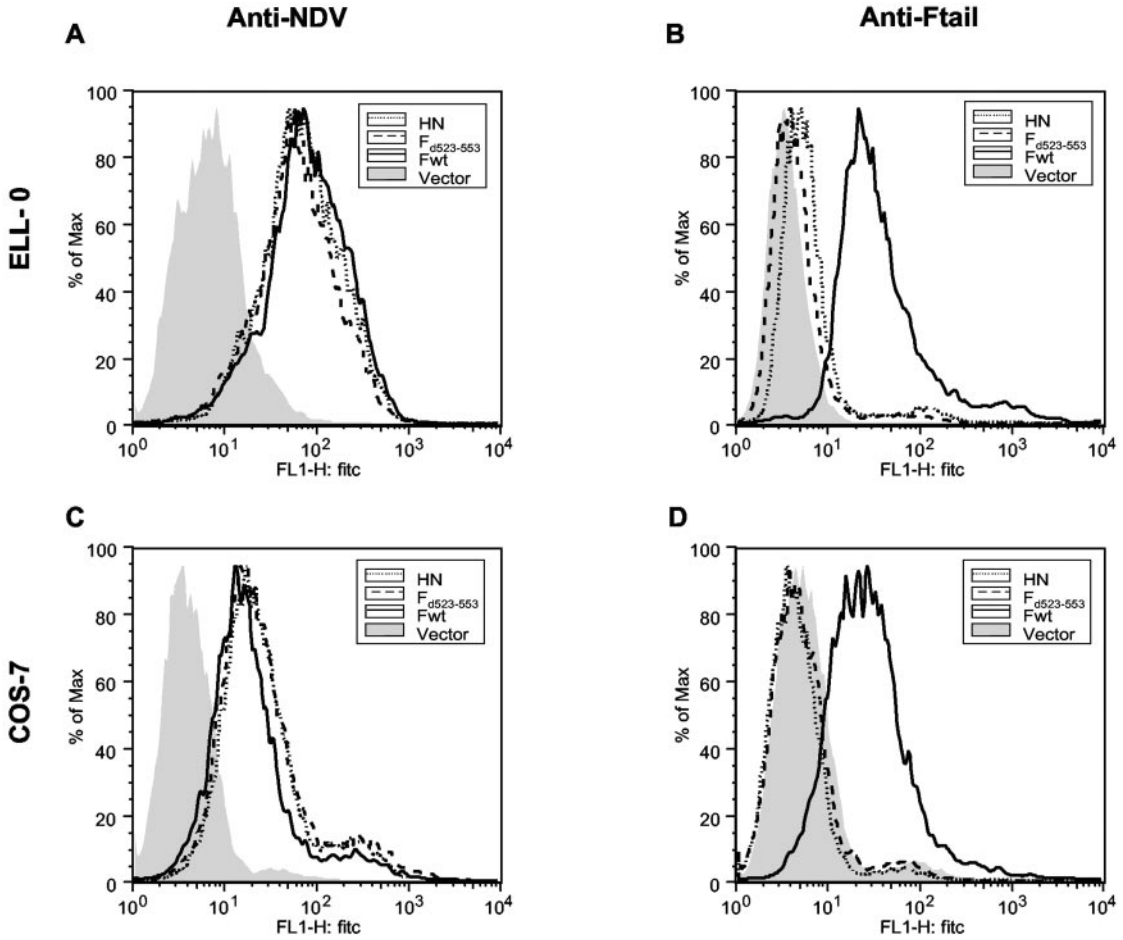

FIG. 4. Detection of cytoplasmic tail sequences on the surfaces of cells transfected with Fwt cDNA by flow cytometry. Avian cells (panels A and B) and COS-7 cells (panels C and D) transfected with pCAGGS vector alone, pCAGGS-Fwt, pCAGGS-$Fd_{523–553}$, or pCAGGS-HN were incubated with anti-NDV (panels A and C) or anti-Ftail 540–553 (panels B and D). FL1-H, fluoroscein isothiocyanate.

distilled water for photography. For immunofluorescence detection of infected cells, complete medium was added after removal of unbound virus and antibody, and cells were incubated for 9 h at 37°C. Surface immunofluorescence was accomplished as described above.

RESULTS

Two forms of F protein are expressed in infected avian cells. Because a second form of the paramyxovirus F protein has not been previously found in other systems, it seemed possible that the alternate form of the NDV F protein could be an unusual property of protein expression in COS-7 cells. Thus, we asked if this second form of the NDV F protein could be detected in avian cells, cells that are the natural host cell for NDV.

Unambiguous detection of the second, partially translocated form of the F protein by Western analysis required the expression of an F protein that was not cleaved, since the partially translocated F protein comigrated on polyacrylamide gels with the F_0 cleavage product, F_1 protein (17). Therefore, we utilized the B1 strain of NDV, a strain that encodes an F protein missing a furin recognition sequence at the cleavage site. The B1 F protein, synthesized in tissue culture cells, is not proteolytically cleaved (13, 19, 21). NDV strain AV encodes an F protein with a furin recognition site, and the F protein present in cells infected by NDV strain AV is proteolytically cleaved into F_1 and F_2 (13, 19, 21).

Avian cells (ELL-0 cells), as well as COS-7 cells, were infected with NDV strain B1 or NDV strain AV for 12 or 16 h, and the F proteins present in the resulting cell extracts were characterized by Western analysis using F-protein-specific antibodies. As previously reported (17), two forms of the F protein from B1-infected COS-7 cells were detected (Fig. 1A, lanes 3 and 6), the fully translocated F_0 protein (68 kDa) and a 59-kDa protein. As expected, extracts from AV-infected COS-7 cells contained two polypeptides, a 68-kDa F_0 and a 59-kDa F_1 (Fig. 1A, lanes 2 and 5). Proteins from ELL-0 cells contained a 68-kDa host band, which obscured the F_0 polypeptide from both B1- and AV-infected cells (Fig. 1B, lanes 1 to 6). However, ELL-0 cell infection with B1 virus resulted in the synthesis of significant amounts of a 59-kDa polypeptide (Fig. 1B, lanes 3 and 6), a polypeptide the size of the partially translocated F protein. These results show that the second form of the F protein can be detected in B1-infected avian cells.

To eliminate the possibility that anti-F protein antibody cross-reacted with NP, which is also 59 kDa, avian and COS-7

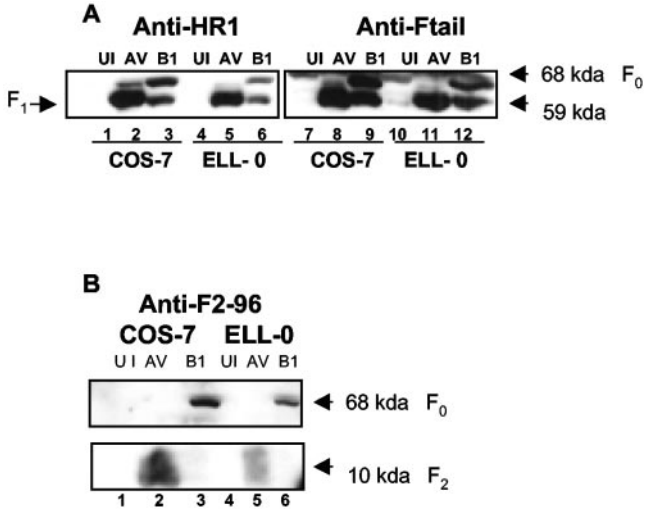

FIG. 5. Two forms of the F protein are present in virions. NDV strain AV (panel A, lanes 2, 5, 8, and 11, and panel B, lanes 2 and 5) and strain B1 (panel A, lanes 3, 6, 9, and 12, and panel B, lanes 3 and 6), generated from COS-7 (panel A, lanes 2, 3, 8, and 9, and panel B, lanes 2, and 3) and avian cells (panel A, lanes 5, 6, 11, and 12, and panel B, lanes 5 and 6, were purified as described in Materials and Methods. Virus particles were subjected to Western analysis using anti-HR1 antibody (panel A, lanes 1 to 6), anti-Ftail antibody (panel A, lanes 7 to 12), or anti-F_2 antibody (panel B, lanes 1 to 6). Uninfected (UI) supernatant, panel A, lanes 1, 4, 7, and 10, and panel B, lanes 1 and 4.

cells were transfected with NP cDNA, and proteins present in the resulting cell extracts were detected by Western analysis using anti-NDV and anti-HR1 antibodies. The results show that NP is recognized only by anti-NDV antibody and not by anti-HR1 antibody (Fig. 1C). Thus, the 59-kDa protein detected with anti-F antibodies in B1-infected cells is not due to a cross-reaction of the antibodies with NP.

Surface expression of cytoplasmic tail sequences. Our analysis of the cell-free translation products directed by F-protein mRNA also showed that the F protein cytoplasmic tail was translocated across membranes (17), leading to the proposal that the partially translocated F protein may be a polytopic protein with the carboxyl terminus as well as the amino terminus of the protein exposed on cell surfaces. In support of this idea, we found that CT sequences could be detected on surfaces of COS-7 cells expressing the NDV F protein using a polyclonal antibody raised against a peptide with the F protein CT sequence (amino acids 523 to 553) (17).

We asked if F-protein CT sequences could be detected on infected or transfected avian cell surfaces (Fig. 2). We also raised another polyclonal antibody specific for the carboxyl-terminal half of the F-protein CT domain (amino acids 540 to 553) to eliminate the possibility that our previous results were due to a nonspecific effect of the anti-Ftail 523–553 antibody (Fig. 2 and 3).

ELL-0 and COS-7 cells were infected with NDV AV or B1 strains. In addition, ELL-0 and COS-7 cells were transfected with vector alone, wild-type F (Fwt) protein cDNA, cDNA encoding an F protein with a cytoplasmic tail deletion ($F_{d523-553}$), or a mixture of Fwt-protein and HN-protein cDNAs. Intact cells were incubated with anti-Ftail 540–553 antibody, and binding of antibody was assessed by cell surface immunofluorescence. Both infected and transfected ELL-0 cells, as well as COS-7 cells, bound this anti-Ftail antibody (Fig. 2 and 3, panels D, E, J, and L). In contrast, binding of anti-Ftail antibody to cells expressing the $F_{d523-553}$ protein was negative (Fig. 2 and 3, panel K), although these cells were positive for anti-NDV antibody (Fig. 2 and 3, panel H), which further confirms specificity of the anti-Ftail antibody for the CT sequences. None of the antibodies bound to uninfected or vector-transfected cells (panels A and F). Binding of anti-NDV antibody was detected in parallel cultures (Fig. 2 and 3, panels B, C, G, H, and I).

Detection of CT sequences on surfaces of infected or transfected cells was not due to permeabilization of cells during antibody binding. Under the same conditions, fluorescence-labeled phalloidin, which binds to actin, did not stain intact cells (Fig. 3N), although phalloidin readily stained actin after permeabilizing cells in 0.1% Triton X-100 (Fig. 3P).

To confirm expression of CT sequences on avian cell surfaces, binding of the anti-Ftail 540-553 antibody was also analyzed by flow cytometry. Figure 4 shows that cells expressing the F protein bound anti-Ftail 540-553 antibody, while cells expressing the $F_{d523-553}$ or HN proteins were negative (Fig. 4B and D). These results show that CT sequences can be detected on the surfaces of both avian and COS-7 cells expressing the NDV F protein using two different anti-Ftail antibodies.

Two forms of F protein are in virions. We next asked if the polytopic form of the F protein was present in virions. NDV strain B1 virions were generated from infected ELL-0 or COS-7 cells. NDV strain AV virions were also prepared in parallel. Figure 5 shows Western analysis of B1 virion proteins, as well as AV virion proteins, using two different F-protein-specific polyclonal antibodies. F-protein-specific bands detected were similar to those found in the infected cell extracts. As expected, F_0 and F_1 polypeptides were detected in AV virions by using anti-HR1 and anti-Ftail antibodies (Fig. 5A). Similarly, F_0 was detected in B1 virions grown from both COS-7 and ELL-0 cells using anti-HR1 and anti-Ftail antibodies. Interestingly, a 59-kDa polypeptide was also detected in B1 virions by both anti-HR1 and anti-Ftail antibodies (Fig. 5A). To determine if the 59-kDa polypeptide was due to unexpected cleavage of the F_0 protein, we used anti-F_2 antibody, an antibody specific to the F_2 polypeptide. Detection of the F_2

TABLE 1. F-protein-specific peptides in p59 band isolated from virions

Peptide[a]	Location in F protein	Domain
IPVPLMLITRVMLILSCIR	F_2	Signal sequence
SPTNVPAPLMLTVR	F_2	Signal sequence
QNAANILR	F_1	HR1 domain
LKESIAATNEAVHEVTNGLSQ LAVAVGK	F_1	HR1 domain
MQQFVNGQFNNTAQELDCIK	F_1	HR1 domain
TLLWLGNNTLDQMR	F_1	CT domain

[a] Virions were purified from the supernatants of NDV strain B1-infected avian cells and proteins in the purified virions electrophoresed on 8% polyacrylamide gels in the presence of a reducing agent. The proteins in the gel were stained with Coomassie blue dye, and the p59-sized stained band was excised and subjected to analysis by mass spectrometry as described in Materials and Methods.

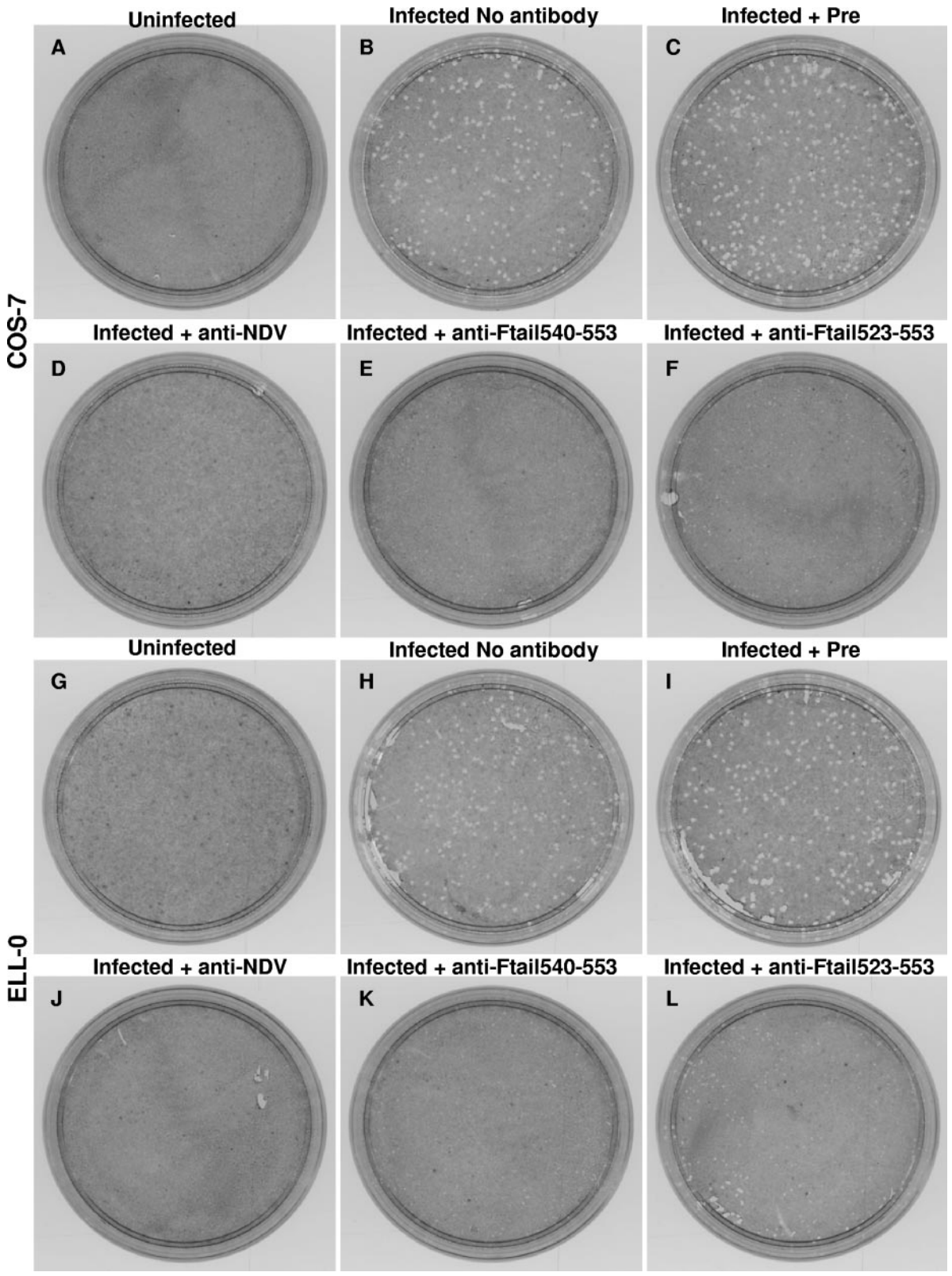

FIG. 6. Anti-Ftail antibodies alter plaque morphology. COS-7 (panels A to F) and avian cells (panels G to L) were infected with untreated NDV strain AV (panels B and H) or NDV that was preincubated with preimmune sera (panels C and I), anti-NDV (panels D and J), anti-Ftail 540–553 (panels E and K), or anti-Ftail 523–553 (panels F and L). Uninfected monolayers, panels A and G. Plaques were counted and stained with 1:20 Giemsa and distilled water after 48 h of incubation at 37°C.

TABLE 2. Effect of anti-NDV and anti-Ftail antibodies on plaque formation in COS-7 and ELL-0 cells

Treatment[a]	Titer of virus			
	COS-7		ELL-0	
	Expt 1	Expt 2	Expt 1	Expt 2
UI	0	0	0	0
No Ab	3.45×10^{10}	5.33×10^{10}	4.53×10^{10}	3.78×10^{10}
Pre	3.25×10^{10}	5.13×10^{10}	4.25×10^{10}	3.75×10^{10}
Anti-NDV	$<1.0 \times 10^{9}$	$<1.0 \times 10^{9}$	$<1.0 \times 10^{9}$	$<1.0 \times 10^{9}$
Anti-Ftail				
540–553[b]	5.00×10^{8}	3.00×10^{9}	3.40×10^{10}	3.08×10^{8}
523–553[b]	2.15×10^{10}	2.18×10^{10}	3.78×10^{10}	3.75×10^{10}

[a] Dilutions of 10^{-9} for NDV strain AV were preincubated with anti-NDV and anti-Ftail antibodies for 1-h prior to adsorption. No.Ab, without antibody; Pre, preincubated with preimmune sera; UI, mock infected. Antibody was 150 μg protein per ml.
[b] Plaques are pinpoint.

polypeptide in B1 virions would suggest that the 59-kDa polypeptide was due to cleavage of the F_0 polypeptide. Figure 5B, shows that while the F protein from the AV virions was cleaved, generating an F_2 polypeptide, F_2 is not present in B1 virions, indicating that the F protein was not cleaved in these virions.

To verify this conclusion, NDV strain B1 virions were purified from supernatants of avian infected cells, and the polypeptides that migrated with a molecular weight of 59 kDa were analyzed by mass spectrometry. As expected peptides derived from the viral NP and P proteins were present in the band. In addition, F-protein-specific peptides were present (Table 1), supporting the conclusion that a form of the F protein comigrated with the NP and P proteins. Furthermore, peptides from both the F_2 and F_1 regions of the protein were detected. F_1-specific peptides detected were from the CT domain of the F protein and from the HR1 domain. Surprisingly, F_2 peptides detected were from the signal sequence of the F protein. These findings suggest that the p59 F polypeptide contains sequences from the uncleaved F_0 polypeptide and that the signal sequence was not cleaved from the alternate form of the F protein. These results are consistent with the presence of a second form of F protein, an uncleaved protein with a molecular weight of 59 kDa.

Effect of anti-Ftail antibodies on plaque formation. Since the second form of the F protein was detected in virions generated from both infected avian and COS-7 cells, we asked if it has a role in plaque formation. We asked if anti-Ftail antibodies could neutralize virus infectivity in a plaque assay. Egg-grown NDV strain AV was incubated with preimmune sera, anti-NDV, or anti-Ftail antibody (both anti-Ftail 523–553 and anti-Ftail 540–553) and then plated on monolayers of avian or COS-7 cells. After adsorption, unbound virus and antibody were removed and the cells were washed prior to addition of agar overlays. Thus, effects of the antibody should reflect effects on the initial virus cell interaction and not subsequent cell-cell fusion. After 40 h, plaques were visible on control plates (Fig. 6B, C, H, and I). As expected, anti-NDV neutralized virus infectivity, and no plaques were observed (Fig. 6D and J). Anti-Ftail 540–553 reduced the titer approximately 2- to 18-fold in COS-7 cells and 20-fold in ELL-0 cells compared to that for the preimmune sera and no-antibody controls (Table 2). Most importantly, however, plaques formed after incubation with both anti-Ftail antibodies were pinpoint (Fig. 6E, F, K, and L), much smaller than those produced by untreated virus or virus incubated with preimmune serum (Fig. 6B, C, H, and I). These results suggest that the anti-Ftail antibodies slow plaque formation. This result is not likely due to a nonspecific effect of antibody raised against a GST fusion protein. Incubation of virus with antibody raised against HR2 sequence coupled to a GST protein did not inhibit plaque size or number (unpublished observations).

Effect of anti-Ftail antisera on a single cycle of infection. The effect of anti-Ftail antibodies on plaque size suggested that the anti-Ftail antibodies, bound prior to virus attachment, inhibited virus entry. To test this possibility by an alternative approach, effects of anti-Ftail antibody on a single cycle of infection were monitored by immunofluorescence. Virus incubated with anti-Ftail antibody, anti-NDV antibody, anti-β-galactosidase, or preimmune sera, as described in the legend to Fig. 6, was then bound to cell surfaces. After adsorption, unbound virus and antibody were removed and cells were washed and incubated for 9 h at 37°C. Infected cells were detected by the presence of proteins on cell surfaces that bound anti-NDV antibody. Figure 7C and D show the presence of virus proteins on infected cell surfaces after infection with untreated virus, while uninfected cells were negative for anti-NDV staining (panels A and B). Incubation of virus in preimmune serum or nonspecific antiserum had no effect on the infection (panels E, F, G, and H), while incubation with anti-NDV antibody inhibited infection as expected (panels I and J). Incubation of virus with anti-Ftail 540–553 inhibited infection (Fig. 7K and L), consistent with results of the plaque assay.

This inhibition by anti-Ftail antibody was not likely due to an inhibition of virus binding. Cells cotransfected with HN- and F-protein cDNAs were incubated with avian red blood cells in the presence or absence of antibody. As expected, anti-NDV blocked the binding of red blood cells (RBCs) to these monolayers, while binding of RBCs in the presence of anti-Ftail antibodies was unaffected (Table 3).

DISCUSSION

We have previously reported that the NDV F protein, synthesized in a cell-free protein-synthesizing system containing membranes, inserted itself into membranes in at least two ways (17). One form of the protein (p68) was typical of a type 1 glycoprotein anchored in membranes by a transmembrane domain located near the carboxyl terminus. The second form (p59) had properties consistent with the partial translocation of the protein, translocation of the F_2 domain and the amino terminus of the F_1 domain. In addition, we reported evidence for the translocation of the CT domain of the protein. Based on these observations, we suggested that the second form of the F protein was a polytopic protein that spanned membranes at least twice. We also reported the detection of this second form of the F protein in infected and transfected COS-7 cells. A polypeptide consistent with the size of this second form was detected by Western analysis of proteins in extracts of infected cells. In addition, CT sequences were detected on the surfaces of cells expressing the NDV F protein.

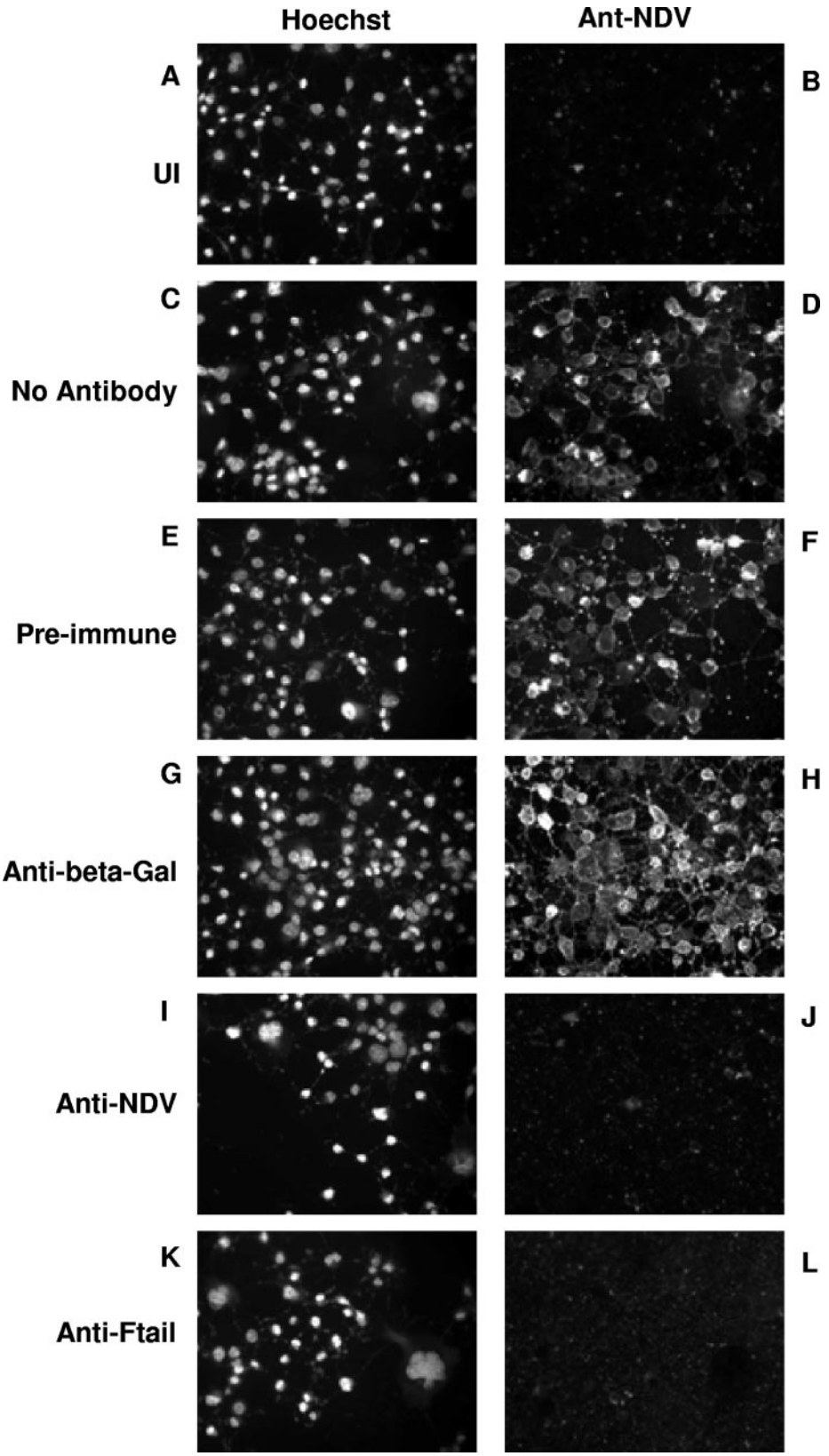

FIG. 7. Anti-Ftail antibodies block infection. COS-7 cells were infected at a multiplicity of infection of 10 with untreated egg-grown NDV strain B1 (panels C and D) or NDV strain B1 that was preincubated with preimmune sera (panels E and F), anti-β-galactosidase (panels G and H), anti-NDV (panels I and J), or anti-Ftail 540–553 (panels K and L) as described in Materials and Methods. Uninfected monolayers are shown in panels A and B. After 9 h of infection, cells were incubated with Hoechst stain, in order to visualize nuclei of all cells in the monolayer, and then anti-NDV antibody. Binding of anti-NDV antibody was visualized by Alexa 568 coupled to goat antirabbit antisera. Images were acquired with a ×20 objective using OPEN Lab software. Hoechst-stained images were digitally exposed for 0.5 s, and anti-NDV images were digitally exposed for 2.5 s. Identical results were obtained in two separate experiments.

TABLE 3. Effect of anti-Ftail antibodies on attachment activity of HN[a]

Cells	Antibody	% RBC bound
Vector-transfected cells	None	0
HN+F cDNA-transfected cells	None	100
	Anti-NDV	10 ± 5
	Anti-HR1	128 ± 50
	Anti-Ftail	108 ± 40

[a] COS-7 cells were cotransfected with cDNAs encoding the HN and F (HN+F) proteins or with a comparable amount of vector DNA. At 40 h post-transfection, avian red blood cells were incubated with cells in the presence of the indicated antibodies at 4°C for 30 min. Binding was quantified by measuring released hemoglobin after lysis of the attached red blood cells as previously described (24).

Because of the unexpected nature of our findings and because this second form of the F protein has not been found in other paramyxovirus systems, we were concerned that formation of a polytopic NDV F protein in COS-7 cells could be attributed to a translocation defect in primate cells of a glycoprotein normally expressed in avian cells. However, as described above, we detected this second form of the F protein in avian cells, both in infected cells and in cells transfected with F-protein cDNA. First, we detected by Western analysis a polypeptide the size of the polytopic F protein (p59), as well as the fully translocated F_0 protein (p68), in tissue culture-grown avian cells infected with an avirulent strain of NDV, strain B1, as well as in purified virions derived from these cells. Cells infected with this virus should express only uncleaved protein (13, 19–21), and indeed, virus released from cells infected with this virus does not form plaques in the absence of trypsin. However, it was possible that the F protein was cleaved, accounting for the p59 form of the protein. To verify that the p59 polypeptide was the F protein and that it included the full-length polypeptide rather than only a cleaved form of the protein, we analyzed, by mass spectroscopy, the p59 material from tissue culture-derived, purified virus particles. As expected, the material contained NP and P protein. Significantly, F-protein-specific peptides were detected in this band, indicating that detection of F-protein sequences by Western analysis was not due to a cross-reaction of anti-F-protein antibodies to another protein. Furthermore, peptides from the F_2 region of the protein, the HR1 domain, and the CT domain were detected, indicating that sequences from the entire F_0 protein were present in this polypeptide.

Interestingly, the F_2 sequences detected in the p59 material by mass spectroscopy were from the signal sequence of the protein, suggesting that the signal sequence was not cleaved from this polypeptide. This surprising finding suggests that the second form of the F protein is anchored in membranes not only by sequences in the HR1 domain and the transmembrane domain but also the signal sequence. The presence of peptides from the signal sequence also indicates that the material is not from contaminating fully translocated F_0 protein (p68), since it has been reported that the signal sequence is cleaved from the fully translocated F protein (9). It should be noted that the two peptides detected from the signal sequence are overlapping but have several amino acid differences. Heterogeneity in the signal sequence is not unexpected, since comparisons of signal sequences from F proteins of many different strains of NDV have shown that the sequence of this region of the F protein is highly variable (28).

Another indication for the existence of the alternate form of the F protein was the detection, on avian cell surfaces, of sequences from the CT domain of the F protein of both the AV and B1 strains of NDV. To eliminate the possibility that our previous detection of surface-expressed CT sequences in COS-7 cells (17) was due to an unusual cross-reactivity of that anti-Ftail antibody preparation, we prepared a second polyclonal antibody by different protocols using only the carboxyl-terminal half of the F protein CT domain as an immunogen. This antibody also detected surface-expressed F-protein CT sequences. Furthermore, deletion of the CT domain of the F protein eliminated the binding of both antibodies to transfected ELL-0 and COS-7 cell surfaces (17; these results) and shows that the antibodies were not binding to an unrelated polypeptide. Thus, the existence of an unusual form of the F protein is indicated by Western analysis and mass spectroscopy of proteins in cell extracts and virions and by detection of CT sequences on infected and transfected cell surfaces.

The functional significance of a second form of the F protein is a key question. It is possible that the alternate F protein is an aberrant form that has no direct role in the virus infection. Alternatively, the expression of this form of the F protein may serve to down-regulate the expression of the fully translocated F protein while not having a direct role in infection. However, detection of the second form of the F protein in purified virions released from both infected avian cells and COS-7 cells led us to explore a role of this polypeptide in membrane fusion.

Current models for type 1 fusion proteins propose that these proteins are initially folded into a metastable conformation (2, 7, 11). Upon activation of fusion, it is proposed that these proteins undergo a series of conformational changes that involve, first, the insertion of a fusion peptide into the target membrane, followed by the association of heptad repeat domains into a very stable, coiled-coil structure. The structure of this coiled coil and the anchoring of the protein to both the target and viral membrane by the fusion peptide and the transmembrane domain, respectively, result in pulling the target and viral membrane into close proximity (2, 7, 11). The membrane merger proceeds first by hemifusion and then by pore formation and expansion (6).

We previously reported that antibody specific for CT sequences inhibited red blood cell fusion to cells expressing the NDV HN and F proteins (17). The antibody inhibited hemifusion, suggesting that antibody binding interfered with the initial stages in the onset of fusion. Here we explored the effects of anti-Ftail antibodies on virus entry. To limit effects of the antibody to virus-cell fusion and not cell-cell fusion, we removed unbound antibody after virus binding. We showed that two different antibodies specific for CT sequences considerably slowed plaque formation, suggesting a role of surface-exposed CT sequences in virus-cell fusion. Furthermore, in a single cycle of infection, preincubation of anti-Ftail antibody with virions eliminated infection. These combined results suggest that antibody bound to the surface-exposed CT sequences inhibited both virus-cell fusion (Fig. 6 and 7) and cell-cell fusion (17).

Since the antibody inhibited hemifusion, initial steps of fusion are affected. It is possible that binding of the antibody to

CT sequences sterically inhibits conformational changes in the fully translocated F protein required for initiation of fusion and that the CT domain has no direct role in the fusion process. Alternatively, the CT domain could be involved in the formation of the metastable form of the fully translocated F protein or the activation of that form required for fusion. The presence of the anti-Ftail antibody could inhibit this activation.

Previous reports indicate that CT domains of paramyxovirus F proteins do have a role in fusion (1, 5, 25–27, 30). Deletion of the CT domain of the NDV F protein interferes with complete fusion (25). It has been proposed that this domain affects the conformation of the ectodomain (25, 30). Surface-exposed CT domains may also have additional roles. Clarification of the role of surface-expressed CT domains in fusion as well as potential roles of the amino-terminal regions of the alternate F protein requires further investigation.

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