JOURNAL OF VIROLOGY, Feb. 1991, p. 813–822
0022-538X/91/020813-10$02.00/0

Vol. 65, No. 2

Complementation between Avirulent Newcastle Disease Virus and a Fusion Protein Gene Expressed from a Retrovirus Vector: Requirements for Membrane Fusion

T. MORRISON,* C. McQUAIN, AND L. McGINNES

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Received 24 April 1990/Accepted 5 November 1990

The cDNA derived from the fusion gene of the virulent AV strain of Newcastle disease virus (NDV) was expressed in chicken embryo cells by using a retrovirus vector. The fusion protein expressed in this system was transported to the cell surface and was efficiently cleaved into the disulfide-linked F_1-F_2 form found in infectious virions. The cells expressing the fusion gene grew normally and could be passaged many times. Monolayers of these cells would plaque, in the absence of trypsin, avirulent NDV strains (strains which encode a fusion protein which is not cleaved in tissue culture). Fusion protein-expressing cells would not fuse if mixed with uninfected cells or uninfected cells expressing the hemagglutinin-neuraminidase (HN) protein. However, the fusion protein-expressing cells, if infected with avirulent strains of NDV, would fuse with uninfected cells, suggesting that fusion requires both the fusion protein and another viral protein expressed in the same cell. Fusion was also seen after transfection of the HN protein gene into fusion protein-expressing cells. Thus, the expressed fusion protein gene is capable of complementing the virus infection, providing an active cleaved fusion protein required for the spread of infection. However, the fusion protein does not mediate cell fusion unless the cell also expresses the HN protein. Fusion protein-expressing cells would not plaque influenza virus in the absence of trypsin, nor would influenza virus-infected fusion protein-expressing cells fuse with uninfected cells. Thus, the influenza virus HA protein will not substitute for the NDV HN protein in cell-to-cell fusion.

Newcastle disease virus (NDV) is a prototype paramyxovirus, and like most enveloped RNA viruses, the virions are composed of an envelope and a core. The NDV envelope contains the hemagglutinin-neuraminidase (HN) glycoprotein, the fusion (F) glycoprotein, and a nonglycosylated membrane protein (4). The core contains the genomic RNA and the NP, P, and L proteins (4). Viral infection is initiated by the interaction of the viral attachment protein (HN) with a sialic acid-containing receptor (4). The subsequent fusion of the viral and cellular membranes required for penetration is mediated by the fusion protein (4).

The fusion protein not only mediates fusion between the virus and the host cell membranes but also between an infected cell and an adjacent cell (2). The fusion proteins of paramyxoviruses are synthesized as a precursor, F_0, which is activated by a proteolytic cleavage, resulting in F_1 and F_2 polypeptides which are held together by disulfide bonds (34, 35). While this cleavage always occurs during growth of the virus in eggs, in tissue culture, the fusion protein of many paramyxoviruses must be cleaved extracellularly by an added protease such as trypsin (9, 25, 26). However, the fusion proteins of some viruses are cleaved in all cell types (25, 26). These readily cleaved fusion proteins have at their cleavage sites two pairs of basic amino acid residues which are recognized by ubiquitous host cell enzymes present in the *trans*-Golgi membranes or the *trans*-Golgi network (20, 24, 39). Virulent strains of NDV encode readily cleaved fusion proteins, while the avirulent strains of NDV have an uncleaved fusion protein if grown in tissue culture (26). The ability to be cleaved in tissue culture correlates with the presence of two pairs of basic amino acid residues at the cleavage site (8, 40).

The requirements for membrane fusion mediated by the paramyxovirus fusion proteins are controversial. Using various experimental approaches, it has been reported that the fusion protein alone will mediate membrane fusion (3, 6, 36). In contrast, other studies using reconstituted lipid vesicles showed that the Sendai virus fusion protein by itself would not mediate fusion (7, 10, 27, 32). However, Hsu et al. (10) showed that the addition of wheat germ agglutinin to fusion protein-containing vesicles resulted in membrane fusion, suggesting that an attachment function was necessary for cell fusion. They argued that the HN protein provides this function but that other agents could substitute. However, work by Gitman et al. (7) and Citovsky et al. (5), again using Sendai virus glycoproteins, showed that both the HN and the fusion proteins were necessary for fusion and that other attachment proteins would not substitute for the HN protein. Nussbaum et al. (28) reported that HN plays a role in fusion separate from its attachment activity. In support of the notion that the HN protein provides a specific, necessary function in fusion was the description of monoclonal antibodies directed against the HN protein which did not block virus binding but did block membrane fusion (21, 30). More recent experiments utilizing cDNA clones of paramyxovirus glycoproteins further complicate the issue. It has been reported that expression of the simian virus 5 fusion protein (29) and the measles virus fusion protein (1) results in cells which are fully competent in membrane fusion. Olmsted et al. (31) report unpublished observations suggesting that the respiratory syncytial virus fusion protein alone can also mediate membrane fusion. However, Sakai and Shibuta (33) report that expression of the parainfluenza 3 virus fusion

* Corresponding author.

protein is not sufficient for membrane fusion and that the expression of both the HN and the fusion proteins is necessary for membrane fusion.

To study the properties of the NDV fusion protein, we expressed in chick cells the cDNA derived from the fusion gene of a virulent strain of NDV, strain AV, using a retrovirus vector. Because this vector is a replication-competent retrovirus, the expression of the inserted gene occurs in virtually 100% of the cells in culture. We report that the fusion protein expressed in this system is efficiently cleaved and transported to the cell surface. These cells do not fuse, will grow normally, and can be passaged many times. These cells will plaque avirulent NDV strains in the absence of trypsin. Further, these cells will fuse if infected with the avirulent virus or if transfected with the HN protein gene. Thus, the expressed fusion protein is capable of complementing the virus infection, providing an active cleaved fusion protein required for the spread of the infection. The expressed fusion protein will also complement the HN protein gene to mediate cell-to-cell fusion.

MATERIALS AND METHODS

NDV and influenza virus. NDV strain Australia-Victoria or AV (virulent) was grown and purified as previously described (19, 24). Stocks of avirulent strains B1-Hitchner (B1) and W (8, 37) were the generous gifts of Ron Iorio. Stocks of influenza virus were the generous gift of Frank Ennis.

Construction of plasmid DNAs. The full-length fusion protein gene was assembled from two overlapping cDNA clones in pBR322 by using a restriction site in the overlapping region (32a). The GC tails generated during the cloning protocols were considered likely to be detrimental in the retroviral life cycle and were therefore removed. Figure 1A outlines the constructions used to eliminate the GC tails at both ends of the gene. Use was made of restriction sites in the 5′ and 3′ noncoding regions (*Bam*I and *Mn*I, respectively). The complete coding region of the fusion protein was assembled in pSP64 (18) by ligating three restriction fragments (*Bam*I-*Bam*I, *Bam*I-*Sph*I, and *Sph*I-*Mn*I) into a *Bam*I-*Sma*I-cut vector. Of the clones isolated, several were sequenced in the *Bam*I-*Bam*I region to determine which had the *Bam*I-*Bam*I fragment in the proper orientation (pSPFst). The plasmid DNA was transcribed with SP6 polymerase (18), and the resulting mRNA was translated to verify the integrity of the coding region of the gene (32a).

A plasmid, pRCAS, containing a nonpermuted proviral form of a replication-competent, nontransforming derivative of the Schmidt-Ruppin A strain of Rous sarcoma virus, and pCLA12N, an adaptor plasmid which facilitates introduction of DNAs into the unique *Cla*I site of pRCAS (11–13), were the generous gifts of Stephen Hughes. To insert the fusion gene into the adaptor plasmid, pSPFst was cut with *Sac*I and *Sal*I and the resulting fragment was ligated into a *Sac*I-*Sal*I-cut pCLA12N. To insert the fusion protein gene into the retroviral vector, pCLA12NFst was cut with *Cla*I and the isolated fragment containing the fusion gene was ligated into a *Cla*I-cut pRCAS. The orientation of the insertion of the fusion gene in the viral genome was determined by *Sal*I digestion. pRFst contained the fusion gene in the sense direction, while pRantiFst contained the fusion gene in the opposite orientation.

Construction of the HN protein gene containing pRCAS DNA (pRHN) has been previously described (22).

Transfection. Early passage chicken embryo cells derived from line 0 chickens (USDA Poultry Laboratories, East Lansing, Mich.) (devoid of endogenous retroviruses) were grown in Dulbecco modified Eagle medium supplemented with 10% tryptone phosphate broth and 6% fetal calf serum. These cells were transfected with 10 μg of plasmid DNA either by the DEAE-dextran procedure followed by a 20% dimethyl sulfoxide shock (15) or with lipofectin as recommended by the manufacturer (GIBCO-Bethesda Research Laboratories). Cells were incubated with lipofectin-DNA mixtures for 5 h. Virus production was monitored with a solid-phase enzyme-linked immunosorbent assay (ELISA) for the p27 capsid protein of avian leukosis virus (ALV) (14). Typical transfected cultures produced maximal virus titers within 10 to 14 days posttransfection. All handling of virus-producing cells was conducted under PL2 containment.

Radiolabeling and immunoprecipitation of viral proteins. Unless otherwise noted, cells were radiolabeled for 2 h at 37°C in Dulbecco modified Eagle medium lacking methionine but containing 100 μCi of [^{35}S]methionine (Amersham) per ml. At the end of the labeling period, cells were washed in phosphate-buffered saline (PBS) and lysed in RSB buffer (0.01 M Tris hydrochloride [pH 7.4], 0.01 M NaCl) containing 1% Triton X-100 and 0.5% sodium deoxycholate as previously described (20, 23, 24). Nuclei were removed by centrifugation.

For NDV-infected cell extracts, chicken embryo cells were infected with NDV at a multiplicity of infection of 15, cells were radioactively labeled as described above from 5 to 7 h postinfection, and extracts were prepared as described above.

Monoclonal antibody anti-F (ROB11) was the generous gift of Ron Iorio, while anti-F (Fu1a) was the generous gift of Mark Peeples (23). Immunoprecipitation was performed as previously described (23).

Cell surface assays. Cells were plated in 35-mm dishes at low density for easy visualization of individual cells. Monolayers were washed with ice-cold PBS and incubated on ice with anti-F (ROB11) (50 μl of ascites fluid in 0.5 ml PBS) for 30 min. The monolayers were washed extensively in ice-cold PBS, and then rabbit anti-mouse immunoglobulin G coupled to beads (50 μl) (Bio-Rad) and resuspended in PBS containing 1 mg of bovine serum albumin per ml was added in a final volume of 0.5 ml. Incubation was on ice for 30 min, and then the unbound antibody was washed away with cold PBS. The monolayers were fixed with 100% methanol for 2 min and then stained with Giemsa stain (Sigma).

Fusion assays. Uninfected cells (5×10^5) were added to subconfluent monolayers of infected or uninfected cells growing on 35-mm dishes, and incubation was continued for 2 h. For fusion assays with NDV-infected cells, the cells were infected with NDV at a multiplicity of infection of 8, washed extensively after virus adsorption, and incubated for 5 h prior to the addition of uninfected cells. The cells were fixed in methanol and stained with Giemsa stain.

Plaque assays. Purified NDV of the appropriate dilution was added to monolayers of cells and incubated for 30 min at 37°C and then overlaid with 1% agar containing Dulbecco modified Eagle medium supplemented with 6% fetal calf serum. Plaque formation in the presence of trypsin was accomplished as previously described (26).

Polyacrylamide gel electrophoresis. Polypeptides were resolved in 10% polyacrylamide slab gels prepared and electrophoresed as previously described (20).

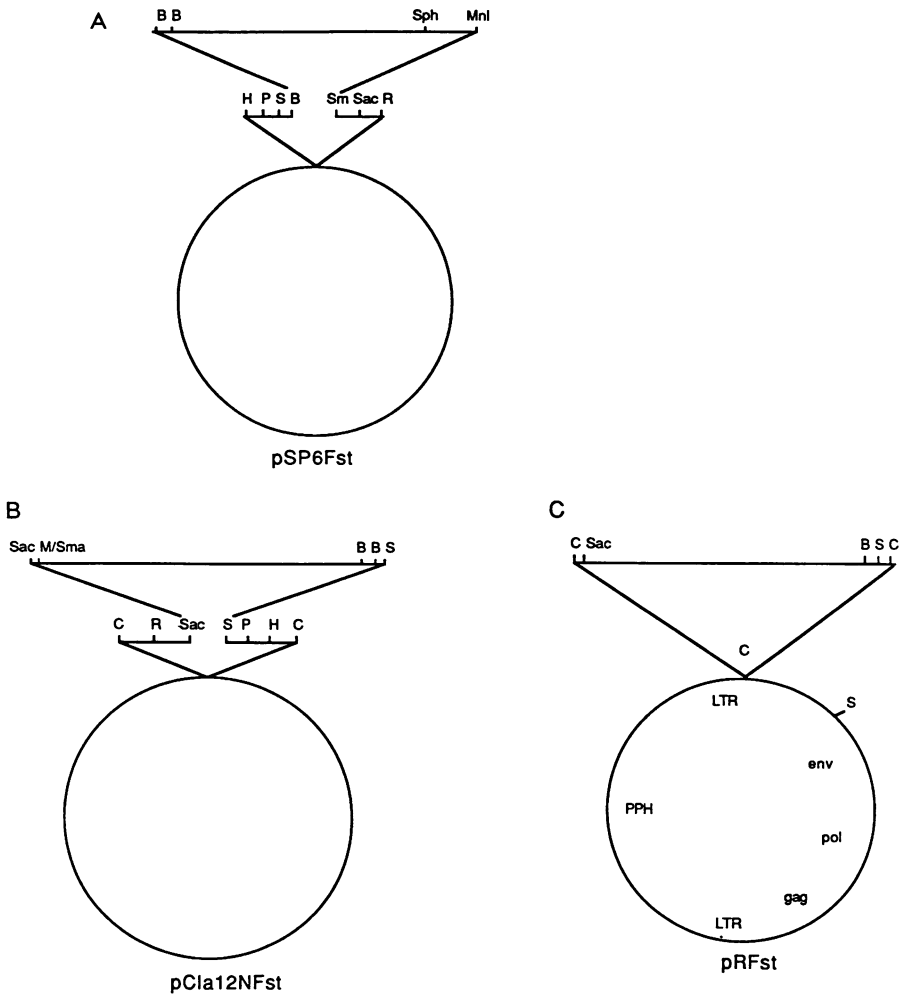

FIG. 1. Plasmid constructions. Full-length fusion protein gene was assembled from two cDNA clones in pBR322 by using a restriction site in the overlapping region (not shown). (A) To remove the GC tails from the 5′ and 3′ ends of the cDNA, we used restriction sites in the 5′ and 3′ noncoding regions. The complete coding region of the fusion protein was assembled in pSP64 by ligating three restriction fragments (*Bam*I-*Bam*I, *Bam*I-*Sph*I, and *Sph*I-*Mn*I) into a *Bam*I-*Sma*I-cut vector. (B) To insert the fusion gene into the adaptor plasmid, pSP6Fst was cut with *Sac*I and *Sal*I and the resulting fragment was ligated into a *Sac*I-*Sal*I-cut pCLA12N. (C) To insert the fusion gene into the retroviral vector, pCLA12NFst was cut with *Cla*I and the fragment containing the fusion gene was isolated and ligated into a *Cla*I-cut pRCAS. The orientation of the insertion of the fusion gene was determined by *Sal*I digestion. LTR, Long terminal repeat; PPH, bacterial plasmid; B, *Bam*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sac, *Sac*I; C, *Cla*I; M, *Mn*I.

RESULTS

Expression of fusion protein in chicken embryo cells. The full-length fusion protein gene derived from the virulent Australia-Victoria (AV) strain of NDV was assembled from two cDNA clones in pBR322 by using a restriction site in the overlapping region. The GC tails generated in the cloning protocols were removed as described in Materials and Methods to generate pSP6Fst (Fig. 1A). DNAs containing the complete coding region of the fusion protein gene were inserted in both the sense (pRFst) and antisense (pRantiFst) orientations into the Schmidt-Ruppin Rous sarcoma virus-derived vector by using the adaptor plasmid pCLA12N (Fig. 1B and C). The DNAs as well as vector DNA without an insert (pRCAS) were used to transfect early passage chicken embryo cells, and culture medium was monitored for the production of the ALV vector by using the ALV-specific ELISA (14). Maximum titers were obtained 10 to 14 days after transfection. The titers of the recombinant viruses were comparable to that of the vector lacking foreign genes. Cells transfected with the vectors containing inserts grew with the same generation time as cells transfected with the vector alone (data not shown). Cultures of cells transfected with the vector containing the fusion gene in the sense direction showed no signs of cell-to-cell fusion at any point after transfection (see below).

The expression of the fusion protein in the transfected cells was analyzed by immunoprecipitation with anti-fusion protein antibody of cytoplasmic extracts prepared from cells radiolabeled with [^{35}S]methionine. Cells transfected with the pRFst DNA synthesized a polypeptide (Fig. 2A, lanes 6 and 7) which comigrated with the fusion protein seen in NDV-infected cell extracts (lanes 3 and 4). However, cells transfected with the vector containing the antisense version of the fusion protein gene (pRantiFst) (lanes 9 and 10) or vector alone (pRCAS) (lanes 12 and 13) did not contain a fusion protein-sized polypeptide. The two different monoclonal

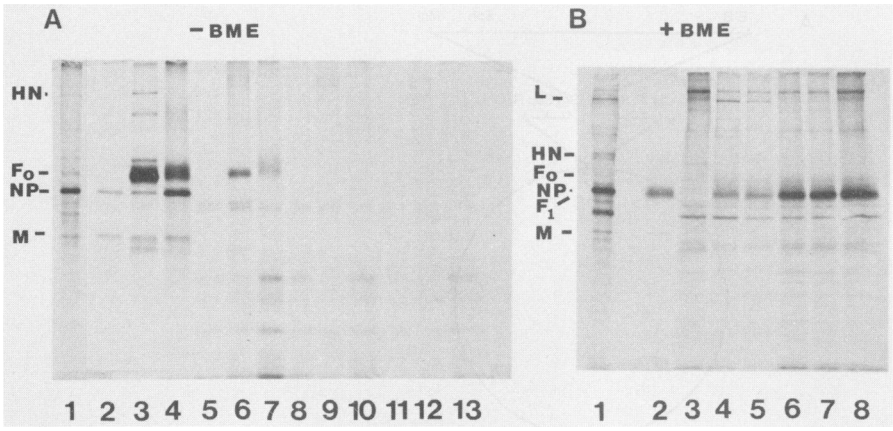

FIG. 2. Expression of the fusion protein. (A) Cells transfected with pRFst, pRantiFst, or pRCAS were radioactively labeled with [^{35}S]methionine for 2 h, and cytoplasmic extracts were prepared. Chick cells infected with NDV were similarly labeled, and cytoplasmic extracts were prepared. Fusion protein present in the extracts was immunoprecipitated with two different anti-fusion protein monoclonal antibodies. Lanes 3, 6, 9, and 12 were precipitated with antibody obtained from R. Iorio. Lanes 4, 7, 10, and 13 were precipitated with anti-fusion protein from M. Peeples (23). Lanes 2, 5, 8, and 11 show precipitated material with no added antibody. Lane 1 shows total protein in a cytoplasmic extract from NDV-infected cells, while lanes 2 to 4 show precipitates from the same NDV-infected cell lysate. Lanes 5 to 7 show precipitates from pRFst-transfected cells. Lanes 8 to 10 show precipitates from pRantiFst-transfected cells, and lanes 11 to 13 show precipitates from pRCAS-transfected chick cells. All precipitated proteins were electrophoresed in the absence of reducing agent (β-mercaptoethanol [BME]). (B) Electrophoresis of proteins in the presence of reducing agent. Cells were pulse-labeled for 15 min and subjected to a nonradioactive chase of 2 h. Lane 1 shows total extract prepared from NDV-infected cells to show the positions of all NDV proteins; lane 2 shows the immunoprecipitated material from the same infected cell extract. The majority of the material electrophoreses with the F_1 protein, which migrates under the NP protein in the total cell extracts. Lanes 4 to 8 show precipitation of the fusion protein from extracts prepared from cells transfected with pRFst for 4 days (lane 4), 5 days (lane 5), 15 days (lane 6), 18 days (lane 7), and 20 days (lane 8). Antibody from R. Iorio was used here and in all subsequent experiments. Lane 3 is precipitation of transfected cells in the absence of added antibody.

antibodies specific to the fusion protein appeared to precipitate different forms of the fusion protein. The significance of this observation is unclear and is under investigation.

In the absence of reducing agent, both the uncleaved (F_0) and the cleaved (F_1-F_2) fusion proteins migrate on polyacrylamide gels in the position of the F_0 polypeptide (17, 38). However, in the presence of reducing agent, the F_1-F_2 complex is disrupted and the F_1 polypeptide in infected cells comigrates with the NP protein (17, 38) (Fig. 2B, lanes 1 and 2). F_2 in infected cell extracts or purified virions is difficult to visualize on gels. To determine whether the fusion protein expressed from the retrovirus vector is proteolytically cleaved, the immunoprecipitates of cells subjected to a pulse-chase were electrophoresed in the presence of reducing agent (Fig. 2B). The F_1 protein was seen in both NDV-infected (lane 2) and transfected (lanes 4 to 8) cells. In addition, the same results were obtained from cells early after transfection (lanes 4 and 5) as well as from cells passaged for 15 to 20 days after transfection (lanes 6 to 8). Thus, the fusion protein expressed from the retrovirus vector is proteolytically cleaved, as is the protein synthesized in NDV-infected cells.

Surface expression of fusion protein. To determine whether the fusion protein expressed from the retrovirus vector was inserted into the plasma membrane, we used monoclonal antibody. Cells transfected with pRFst and cells transfected with pRCAS were incubated with anti-fusion protein monoclonal antibody on ice. Unbound antibody was washed away, and any binding of the antibody to cell surfaces was visualized by rabbit anti-mouse monoclonal antibody coupled to beads. Cells expressing the fusion protein gene (Fig. 3A) had numerous beads attached to their surfaces, while cells transfected with pRCAS DNA (Fig. 3B) did not bind the second antibody and had no beads bound to their surfaces.

Inspection of the monolayers with the bound second antibody enabled us to quantify the numbers of cells on the plate which are expressing the fusion protein. Of 400 cells counted, 98% of the cells transfected with the pRFst bound the anti-fusion protein monoclonal antibody (Fig. 3A). This efficiency of cells expressing the fusion gene is not surprising since the vector is a replication-competent virus. Cells which did not originally receive the DNA in the transfection protocol will be infected with progeny virus which is released from the transfected cells. Thus, the fusion gene is effectively spread to all cells in the population, resulting in nearly 100% of the cells expressing the fusion gene.

Infection of fusion protein-expressing cells with NDV and influenza virus. Cells expressing the fusion protein readily plaqued NDV strain AV. Indeed, plaques of the AV strain appeared in 18 h, whereas plaques on untransfected chick cells required a 48-h incubation to be visualized. Therefore, it appeared that the expressed fusion protein-facilitated virus spread as assayed by plaque formation. To test this possibility, we plaqued two avirulent NDV strains on the fusion protein-expressing cells. Both NDV B1 and NDV W are avirulent strains which will not plaque on chick cells unless trypsin is included in the monolayer (8, 40). Both these strains readily plaqued on fusion protein-expressing cells in the absence of trypsin (Table 1). Indeed, the efficiency of plaque formation was as high as that on chick cells in the presence of trypsin. In addition, appearance of plaques on chick cells in the presence of trypsin required 48 to 72 h, while plaques on fusion protein-expressing cells in the absence of trypsin were readily seen in 18 h. Strains B1 and W did not plaque in the absence of trypsin on cells transfected

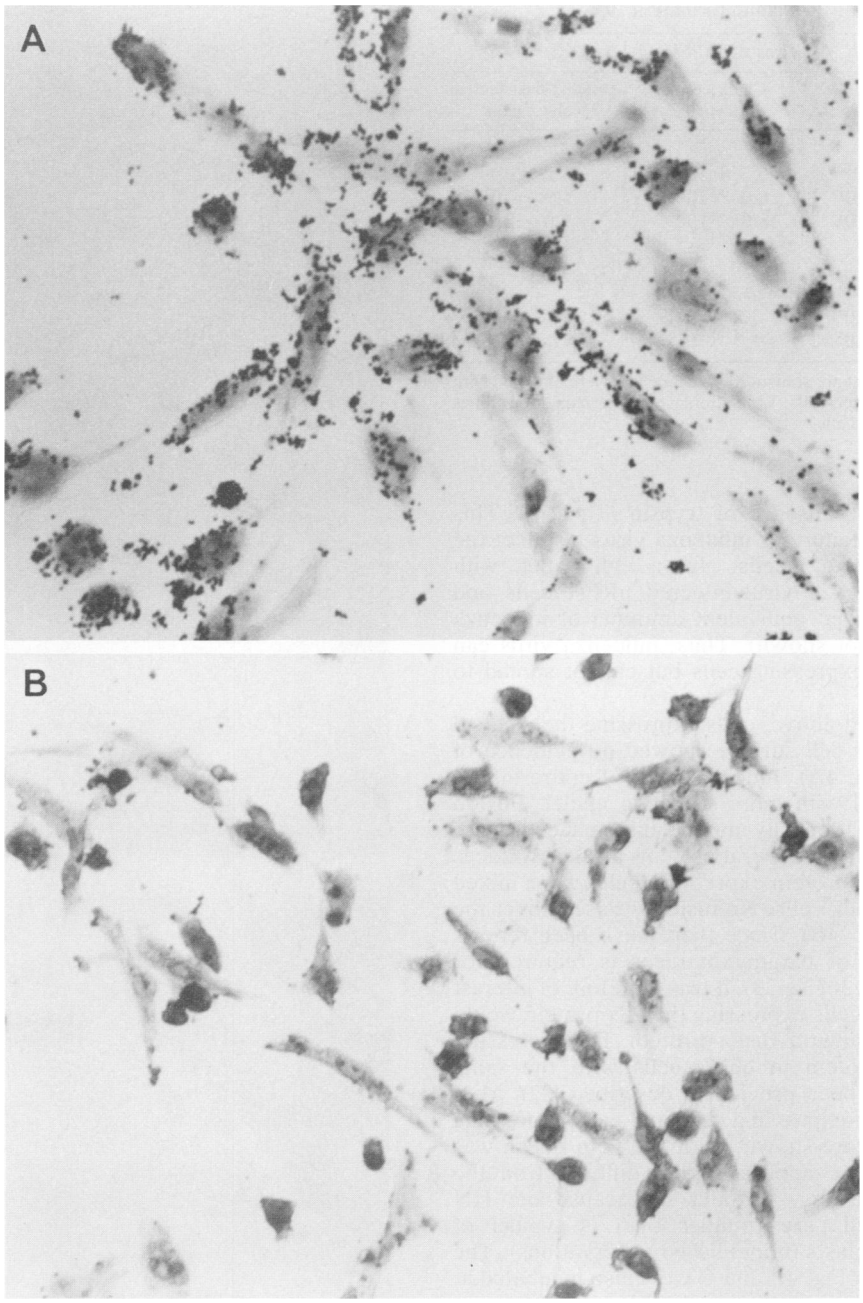

FIG. 3. Surface expression of the fusion protein. Cells transfected with pRFst (A) and cells transfected with pRCAS (B) were plated at low density. Monolayers were washed with PBS and incubated with monoclonal antibody specific for the fusion protein. Incubation was on ice for 30 min. Unbound antibody was removed, the monolayers were washed extensively with cold PBS, and anti-mouse immunoglobulin G coupled to beads was added. After incubation on ice for 30 min, unbound antibody was removed and the monolayers were washed extensively with PBS and then fixed with methanol and stained with Giemsa stain. The field shown in panel A was selected to show one of the few cells not covered with beads.

with the retrovirus alone. Thus, the expression of the retrovirus *env* glycoprotein is not responsible for this result.

Influenza virus (H1N1 or H7N7) also will not plaque on tissue culture cells in the absence of trypsin, again because the HA glycoprotein remains uncleaved (16). The uncleaved HA protein does, however, have cell-binding activity (16). Therefore, it was of interest to determine whether the fusion protein expressed from the retrovirus would complement influenza virus infection for plaque formation. It has been shown that influenza virus which fuses at the cell surface after acid treatment will not initiate infection (41). However, it seemed possible that the presence of the cell surface influenza virus HA protein with the fusion protein might stimulate cell-to-cell fusion and the spread of the influenza virus infection to adjacent cells, resulting in plaque formation. However, no plaques were observed on fusion protein-

TABLE 1. Plaque formation

Virus (strain)	Virus titer (PFU/ml)[a] on:		
	Chick cells	Chick cells + trypsin	pRFst-transfected chick cells
NDV			
AV	6.3×10^{10}	6.3×10^{10}	3.2×10^{10}
B1	$<1.0 \times 10^{1}$	6.0×10^{10}	5.9×10^{10}
W	$<1.0 \times 10^{1}$	8.0×10^{10}	9.0×10^{10}
Influenza virus			
H1N1	$<1.0 \times 10^{1}$	3.2×10^{8}	$<1.0 \times 10^{1}$
H7N7	$<1.0 \times 10^{1}$	1.3×10^{7}	$<1.0 \times 10^{1}$

[a] Results of one experiment are shown. Identical results were obtained with two different sets of transfected cells. Virus titers were determined four times with each set of transfected cells.

expressing cells in the absence of trypsin (Table 1). This result is not due to a failure of influenza virus to infect the fusion protein-expressing cells. In a 2-h label with [^{35}S]methionine, influenza virus-infected pRFst cells and pRCAS cells synthesized equivalent amounts of influenza virus proteins (data not shown). Thus, influenza virus can infect fusion protein-expressing cells but cannot spread to adjacent cells.

Cell fusion. As noted above, cells expressing the cleaved fusion protein at their cell surface showed no evidence of cell-to-cell fusion (Fig. 4A). Indeed, scans of entire monolayers showed no cell with more than two nuclei. Fusion assays are standardly done by mixing infected cells with uninfected cells containing no viral proteins at their surfaces (2). Therefore, fusion protein-expressing cells were mixed with untransfected chick cells. No fusion was seen over the entire monolayer (Fig. 4B). Since there have been reports that the HN protein of paramyxoviruses is required for fusion (5, 7, 21, 27, 28, 30, 32, 33), it was therefore of interest to determine whether cells expressing the HN protein would fuse with cells expressing the fusion protein. The expression of the NDV HN protein in chick cells with the same retrovirus vector has been previously described (22). Mixtures of fusion protein-expressing cells and cells expressing the NDV HN protein are shown in Fig. 4C. No fusion was observed. The monolayer appears slightly different from the monolayers shown in Fig. 4A and B because the HN protein-expressing cells are rounder than is typical of chicken embryo fibroblasts (unpublished observations). The cell mixtures shown in Fig. 4B and C were also incubated at pH 5 and pH 8.5, and again no fusion was observed (data not shown). In addition, treatment of pRFst-transfected cells or untransfected cells with neuraminidase prior to the fusion assay did not stimulate fusion (data not shown).

Fusion was, however, observed if the fusion protein-expressing cells were infected with the avirulent NDV B1 strain and mixed with uninfected cells (Fig. 5A). This fusion was comparable or even more extensive than that seen after virulent (AV) NDV infection of chick cells (Fig. 5B). As expected, fusion was not observed when chick cells transfected with pRCAS and infected with NDV strain B1 were mixed with uninfected cells (Fig. 5C). In addition, influenza virus-infected fusion protein-expressing cells showed no evidence of fusion when mixed with uninfected cells (data not shown).

Since fusion protein-expressing cells would fuse only after infection with NDV strain B1, it was possible that the B1 virus was providing an additional factor required for cell fusion. Because of past reports of the requirement for HN protein in cell fusion, it seemed likely that expression of the HN protein was required. To test this idea, we transfected fusion protein-expressing cells with the NDV HN gene (pRHN DNA). After this transfection, numerous regions of cell fusion were seen (for example, Fig. 6A). However,

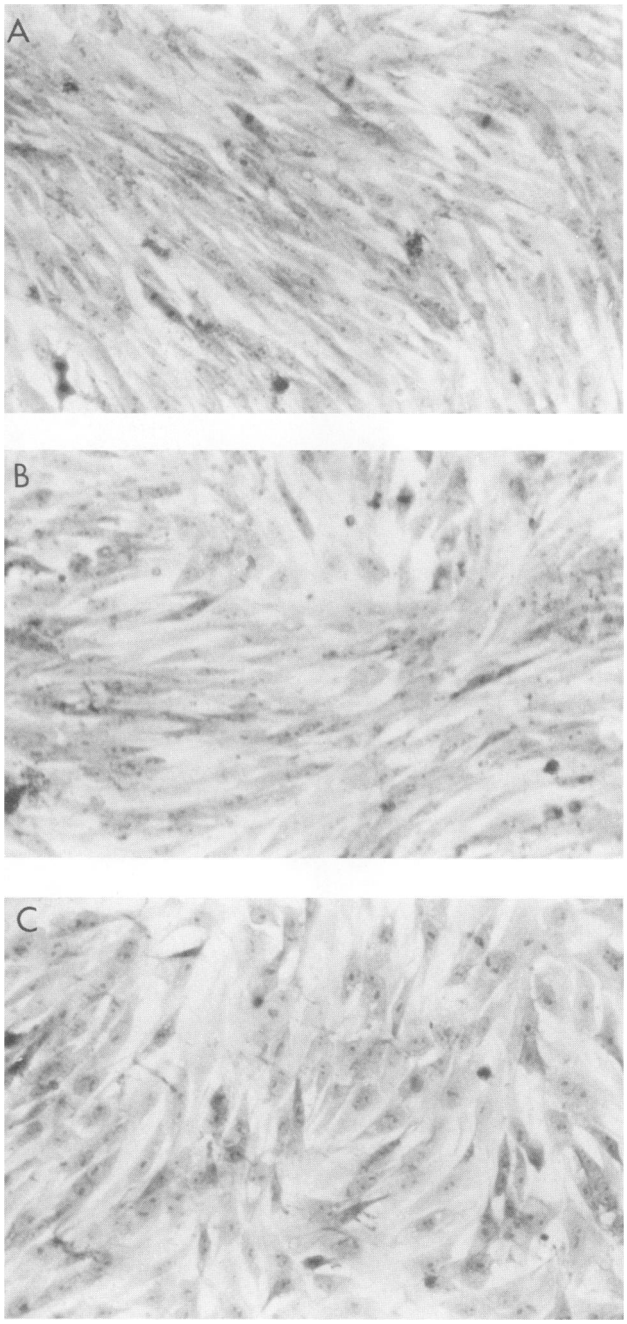

FIG. 4. Fusion assays with fusion protein-expressing cells. (A) Confluent monolayer of cells expressing F_1-F_2. No uninfected cells were added. (B) Cells expressing F_1-F_2 mixed with uninfected chick cells as described in Materials and Methods. (C) Cells expressing F_1-F_2 mixed with cells expressing the HN protein. In each case, the entire monolayer was scanned for evidence of fusion. No cells containing more than two nuclei were observed.

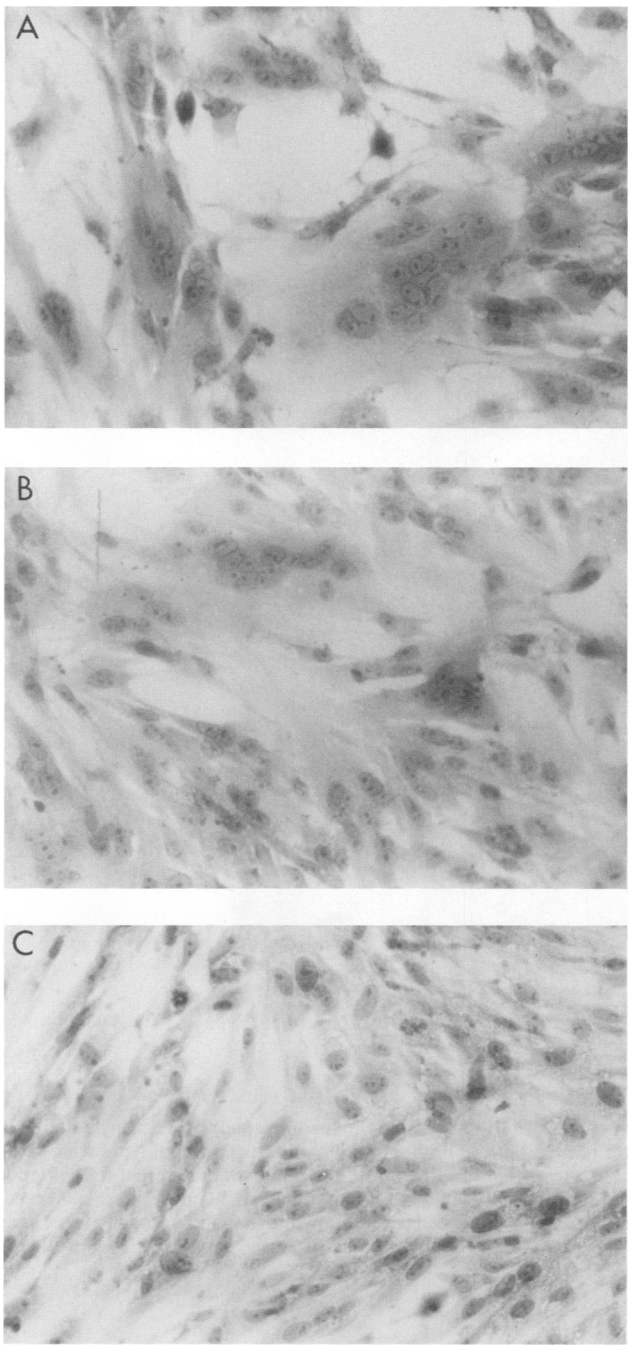

FIG. 5. Fusion assays with F_1-F_2-expressing cells infected with NDV strain B1. (A) Cells expressing F_1-F_2 and infected with NDV strain B1 for 5 h were mixed with uninfected cells and incubated for 2 h. Most of the monolayer was involved in cell fusion. (B) Chick cells (untransfected) infected with NDV strain AV for 5 h were mixed with uninfected cells and incubated for 2 h. (C) Cells transfected with pRCAS and infected with NDV strain B1 for 5 h were mixed with uninfected cells and incubated for 2 h. No evidence for cell fusion was seen over the entire monolayer.

transfection of fusion protein-expressing cells with the vector alone (pRCAS) did not result in any cell fusion (Fig. 6B). Thus, the fusion protein-expressing cells are capable of cell-to-cell fusion if the HN protein is expressed in the same cell.

DISCUSSION

The coding region of the fusion protein gene derived from a virulent strain of NDV, strain AV, was inserted into the genome of an ALV derived from Rous sarcoma virus (11–13). The fusion protein gene was placed in the position occupied by the *src* gene and is presumably expressed as the *src* gene in Rous sarcoma virus. Cells transfected with DNA derived from this vector express a protein which is probably the fusion protein since it is precipitated by antibody specific for the fusion protein and it comigrates with the fusion protein synthesized in NDV-infected cells.

Use of a retroviral vector allows the expression of a gene in cells that will continue to grow and divide through multiple passages. The vector used here was a replication-competent virus (11). Therefore, transfected cells will release infectious virus which can then infect untransfected cells, spreading the inserted gene throughout the culture. Indeed, nearly all cells derived from the transfection of the chick cells with the fusion protein gene-containing vector expressed the fusion protein. Such a system allows one to easily assess the properties of cells expressing the fusion protein and the ability of these cells to support the growth of avirulent NDV.

The fusion protein gene used here was derived from the virulent NDV strain, AV. The fusion protein synthesized in cells infected with this virus was proteolytically cleaved in the *trans*-Golgi or *trans*-Golgi network and in the absence of added trypsin (24). The protein expressed from the cDNA derived from this gene was also proteolytically cleaved in the absence of added trypsin. Furthermore, the expressed protein could be detected at the cell surface by monoclonal antibody.

Cells expressing the fusion protein grew normally and appeared identical to cells transfected with the retrovirus alone. The fusion protein-expressing cells have been maintained through 30 passages, as have cells transfected with pRantiFst and pRCAS. Even though the expressed fusion protein was cleaved, no cell-to-cell fusion was observed at any time after transfection. Nor was fusion observed when untransfected chick cells were mixed with cells expressing the fusion protein. However, cells expressing the fusion protein could fuse if the cells were infected with an avirulent strain of NDV. Avirulent strains of NDV have fusion proteins that are not cleaved in tissue culture in the absence of trypsin and are, therefore, unable to mediate fusion (25, 26). Indeed, pRCAS-transfected cells infected with strain B1 did not fuse; therefore, the fusion observed after infection of pRFst-transfected cells with NDV strain B1 must be due to the cleaved fusion protein expressed from the retrovirus vector. Thus, fusion is only observed in cells expressing a cleaved fusion protein and another viral protein contributed by the NDV B1 infection. It has been reported that the parainfluenza 3 virus fusion protein will not mediate fusion unless the cells also express the HN protein (33). Some earlier work also suggested a necessary role for the HN protein (5, 7, 21, 27, 28, 32). Indeed, the NDV fusion protein-expressing cells will fuse following transfection of the HN protein gene, demonstrating that both the fusion protein and the HN protein are required for membrane

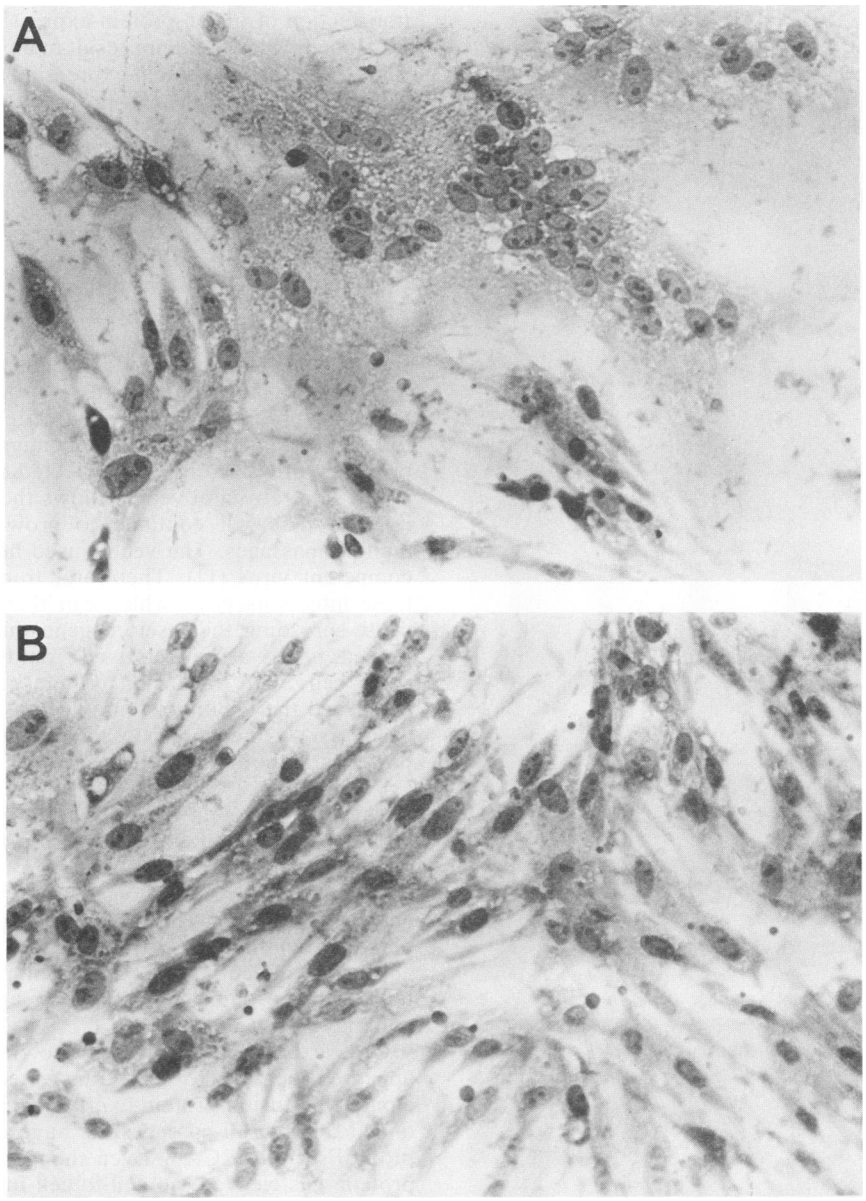

FIG. 6. Fusion assays with F_1-F_2-expressing cells transfected with pRHN DNA. Cells expressing the fusion protein were transfected with pRHN DNA (A) or pRCAS (B) by using lipofectin. Twenty-four hours after removal of the lipofectin-DNA mixture, monolayers were fixed and stained with Giemsa stain as described in Materials and Methods. For each transfection, the entire monolayer was scanned for evidence of cell-to-cell fusion. Following transfection with pRHN DNA, numerous regions of cell fusion containing from 5 to 30 nuclei were observed. A typical region of fusion is shown in panel A. Following transfection with pRCAS DNA, no cells containing more than two nuclei were seen over the entire monolayer. A typical field is shown in panel B. Similar results were obtained with three separate lines of fusion protein-expressing cells derived from three separate transfections with pRFst DNA.

fusion in this system. Significantly, the HN protein must be in the same cell as the cleaved fusion protein since mixtures of cells expressing the HN protein and cells expressing the fusion protein did not fuse.

The nature of the contribution of the HN protein in fusion in this system is not clear. It has been previously suggested that the HN protein serves to position the membranes in the close proximity required for fusion (10). If so, other viral attachment proteins should also function. Our results suggest that other viral attachment glycoproteins expressed in the same cell will not substitute for the HN protein. The vector used to express the fusion protein was a replication-competent ALV which expresses the *env* protein. Thus, cell surfaces also contained the ALV glycoprotein. This protein was unable to mediate cell-to-cell interactions required for cell fusion. Nor could the influenza virus HA protein substitute for the HN protein. Influenza virus HA protein expressed during infection of chick cells is not cleaved but is active in cell binding (16). The HA protein present in influenza virus-infected fusion protein-expressing cells should therefore mediate binding to adjacent cells. However, no fusion was observed between uninfected cells and

influenza virus-infected fusion protein-expressing cells. These results argue for a specific role of the HN protein in fusion beyond the cell attachment activity of the protein.

As described above, earlier work has resulted in conflicting conclusions about the requirements for membrane fusion. The most direct test is the expression of the individual genes in cells. Again, conflicting results were obtained. It has been reported that the expression of the simian virus 5 (29), the measles virus (1), or the respiratory syncytial virus (31) fusion protein alone is sufficient to mediate cell-to-cell fusion. In contrast, our results and those of Sakai and Shibuta (33) argue for a necessary role of the HN protein. The reasons for these different results are unclear except that in each case different vectors and different cell types were used to express the viral proteins. It is possible that a requirement for the HN protein varies with the cell line used.

Another possible explanation for these conflicting results is related to differences in the viruses. It is possible that fusion requires two activities. For some viruses, such as simian virus 5 and measles virus, the fusion protein may carry both activities, while in other viruses such as NDV, the fusion protein may carry one activity and the HN protein may carry the other. Resolution of the problem will require further characterization of different fusion proteins with the same vectors and the same cells.

Cells expressing the fusion protein readily plaqued avirulent NDV in the absence of trypsin. Further, the efficiency of plating was equivalent to that observed in the presence of trypsin. Thus, the expressed fusion protein can complement the avirulent virus, providing a cleaved fusion protein required for plaque formation. This result underscores the importance of the cleaved fusion protein in the cell-to-cell spread of infection required for plaque formation.

In summary, we expressed the fusion protein gene derived from a virulent NDV strain using a retroviral vector. The expressed protein could complement infection by an avirulent NDV strain, resulting in cell fusion and plaque formation in the absence of trypsin. Our results suggest that the HN protein is required for fusion, that other viral attachment proteins will not substitute, and that the HN protein must reside in the same membrane as the fusion protein.

ACKNOWLEDGMENTS

We gratefully acknowledge the invaluable assistance of Harriet Robinson and David Brown. Thanks also to Stephen Hughes for providing the vectors used and Ron Iorio and Mark Peeples for the anti-fusion protein antibodies used.

This work was supported by Public Health Service grant GM 37745 from the National Institutes of Health.

REFERENCES

1. **Alkhatib, G., C. Richardson, and S.-H. Shen.** 1988. Intracellular processing, glycosylation, and cell-surface expression of the measles virus fusion protein (F) encoded by a recombinant adenovirus. Virology **175**:2662–2670.
2. **Bratt, M. A., and W. R. Gallagher.** 1969. Preliminary analysis of the requirements for fusion from within and fusion from without by Newcastle disease virus. Proc. Natl. Acad. Sci. USA **64**:536–540.
3. **Bundo-Morita, K., S. Gibson, and J. Lenard.** 1987. Estimation by radiation inactivation of the size of functional units governing Sendai and influenza virus fusion. Biochemistry **26**:6223–6227.
4. **Choppin, P. A., and R. W. Compans.** 1975. Reproduction of paramyxoviruses. Compr. Virol. **4**:95–178.
5. **Citovsky, V., P. Yanai, and A. Loyter.** 1986. The use of circular dichroism to study conformational changes induced in Sendai virus envelope glycoproteins. J. Biol. Chem. **261**:2235–2239.
6. **Gibson, S., K. Bundo-Morita, A. Portner, and J. Lenard.** 1988. Fusion of a Sendai mutant deficient in HN protein (ts271) with cardiolipin liposomes. Virology **163**:226–229.
7. **Gitman, A. G., and A. Loyter.** 1984. Construction of fusogenic vesicles bearing specific antibodies. J. Biol. Chem. **259**:9813–9820.
8. **Glickman, R. L., R. J. Sydall, R. M. Iorio, J. P. Sheehan, and M. A. Bratt.** 1988. Quantitative basic residue requirements in the cleavage activation site of the fusion glycoprotein as a determinant of virulence of Newcastle disease virus. J. Virol. **62**:354–356.
9. **Homma, M., and M. Ohuchi.** 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. III. Structural differences of Sendai viruses grown in eggs and in tissue culture cells. J. Virol. **12**:1457–1465.
10. **Hsu, M.-C., A. Scheid, and P. W. Choppin.** 1979. Reconstitution of membranes with individual paramyxovirus glycoproteins and phospholipid in cholate solution. Virology **95**:476–491.
11. **Hughes, S., and E. Kosik.** 1984. Mutagenesis of the region between env and src of the SR-A strain of Rous sarcoma virus for the purpose of constructing helper independent vectors. Virology **136**:89–99.
12. **Hughes, S., K. Mellstrom, E. Kosik, R. Tamanoi, and J. Brugge.** 1984. Mutation of a termination codon affects *src* initiation. Mol. Cell. Biol. **4**:1738–1746.
13. **Hughes, S. H., J. J. Greenhouse, C. J. Petropoulos, and P. Sutrave.** 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. **61**: 3004–3012.
14. **Hunt, L. A., D. W. Brown, H. L. Robinson, C. W. Naeve, and R. G. Webster.** 1988. Retrovirus-expressed hemagglutinin protects against lethal influenza virus infections. J. Virol. **62**:3014–3019.
15. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4**:1172–1174.
16. **Kingsbury, D. W.** 1985. Orthomyxoviruses and paramyxoviruses and their replication, p. 1157–1178. *In* B. N. Fields et al., (ed), Virology. Raven Press, New York.
17. **Markwell, M. K., and C. F. Fox.** 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. J. Virol. **33**:152–166.
18. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
19. **McGinnes, L. W., and T. G. Morrison.** 1986. Nucleotide sequence of the gene encoding the Newcastle disease virus fusion protein and comparisons of paramyxovirus fusion protein sequences. Virus Res. **5**:343–356.
20. **McGinnes, L. W., A. Semerjian, and T. G. Morrison.** 1985. Conformational changes in Newcastle disease virus fusion glycoprotein during intracellular transport. J. Virol. **56**:341–348.
21. **Miura, N., T. Uchida, and Y. Okada.** 1982. HVJ (Sendai virus)-induced envelope fusion and cell fusion are blocked by monoclonal anti-HN protein antibody that does not inhibit hemagglutinin activity of HVJ. Exp. Cell Res. **141**:409–420.
22. **Morrison, T. G., and L. W. McGinnes.** 1989. Avian cells expressing the Newcastle disease virus HN protein are resistant to NDV infection. Virology **171**:10–17.
23. **Morrison, T. G., M. E. Peeples, and L. W. McGinnes.** 1987. Conformational change in a viral glycoprotein during maturation due to disulfide bond disruption. Proc. Natl. Acad. Sci. USA **84**:1020–1029.
24. **Morrison, T. G., L. Ward, and A. Semerjian.** 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein J. Virol. **53**:851–857.
25. **Nagai, Y., and H.-D. Klenk.** 1977. Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. Virology **77**:125–134.

26. **Nagai, Y., H.-D. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoprotein and its significance for virulence of NDV. Virology **72**:494–508.
27. **Nakanishi, M., T. Uchida, J. Kim, and Y. Okada.** 1982. Glycoproteins of Sendai virus (HVJ) have a critical ratio for fusion between virus envelopes and cell membranes. Exp. Cell Res. **142**:95–101.
28. **Nussbaum, O., N. Zakai, and A. Loyter.** 1984. Membrane bound antiviral antibodies as receptors for Sendai virions in receptor depleted erythrocytes. Virology **138**:185–197.
29. **Paterson, R. G., M. S. Shaughnessy, and R. A. Lamb.** 1989. Analysis of the relationship between cleavability of paramyxovirus fusion protein and length of the connecting peptide. J. Virol. **63**:1293–1301.
30. **Portner, A., R. A. Scroggs, and D. W. Metzger.** 1987. Distinct functions of antigenic sites of the HN glycoprotein of Sendai virus. Virology **158**:61–68.
31. **Olmsted, R. A., N. Elango, G. A. Prince, B. R. Murphy, P. R. Johnson, B. Moss, R. M. Chanock, and P. L. Collins.** 1986. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc. Natl. Acad. Sci. USA **83**:7462–7466.
32. **Ozawa, M., A. Asano, and Y. Okada.** 1979. Biological activities of glycoproteins of HVJ (Sendai virus) studied by reconstitution of hybrid envelope and by conconavalin A-mediated binding: a new function of HANA protein and structural requirements for F protein in hemolysis. Virology **99**:197–202.
32a. **Reitter, J. N., and T. G. Morrison.** Unpublished data.
33. **Sakai, Y., and H. Shibuta.** 1989. Syncytium formation by recombinant vaccinia viruses carrying bovine parainfluenza 3 virus envelope protein genes. J. Virol. **63**:3661–3668.
34. **Scheid, A., and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57**:470–490.
35. **Scheid, A., and P. W. Choppin.** 1977. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology **80**:54–66.
36. **Sechoy, O., J. R. Philippot, and A. Bienvenue.** 1986. Preparation and characterization of F-protein vesicles isolated from Sendai virus by means of octylglucoside. Biochim. Biophys. Acta **857**:1–12.
37. **Sheehan, J. P., R. W. Iorio, R. J. Syddall, R. L. Glickman, and M. A. Bratt.** 1987. Reducing agent sensitive dimerization of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus correlates with the presence of cysteine at residue 123. Virology **161**:603–606.
38. **Smith, G. W., and L. E. Hightower.** 1981. Identification of the P proteins and other disulfide-linked and phosphorylated proteins of Newcastle disease virus. J. Virol. **37**:256–267.
39. **Steiner, D., P. Quinn, S. Chan, J. Marsh, and D. Tager.** 1980. Processing mechanisms of the biosynthesis of proteins. Ann. N.Y. Acad. Sci. **343**:1–16.
40. **Toyoda, T., T. Sakaguchi, K. Imai, N. M. Inocencio, B. Gotoh, M. Hamaguchi, and Y. Nagai.** 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. Virology **158**:242–247.
41. **Webster, R. G., L. E. Brown, and D. C. Jackson.** 1983. Changes in the antigenicity of the hemagglutinin molecule of H3 influenza virus at acid pH. Virology **126**:587–599.