JOURNAL OF VIROLOGY, Nov. 1995, p. 6712–6719 Vol. 69, No. 11
0022-538X/95/$04.00+0

Quantitative Measurement of Paramyxovirus Fusion: Differences in Requirements of Glycoproteins between Simian Virus 5 and Human Parainfluenza Virus 3 or Newcastle Disease Virus

SANGEETA BAGAI[1] AND ROBERT A. LAMB[1,2]*

Howard Hughes Medical Institute[1] and Department of Biochemistry, Molecular Biology and Cell Biology,[2] Northwestern University, Evanston, Illinois 60208-3500

Received 23 June 1995/Accepted 8 August 1995

To compare the requirements for paramyxovirus-mediated cell fusion, the fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins of simian virus 5 (SV5), human parainfluenza virus 3 (HPIV-3), and Newcastle disease virus (NDV) were expressed individually or coexpressed in either homologous or heterologous combinations in CV-1 or HeLa-T4 cells, using the vaccinia virus-T7 polymerase transient expression system. The contribution of individual glycoproteins in virus-induced membrane fusion was examined by using a quantitative assay for lipid mixing based on the relief of self-quenching (dequenching) of fluorescence of the lipid probe octadecyl rhodamine (R18) and a quantitative assay for content mixing based on the cytoplasmic activation of a reporter gene, β-galactosidase. In these assays, expression of the individual F glycoproteins did not induce significant levels of cell fusion and no cell fusion was observed in experiments when cells individually expressing homologous F or HN proteins were mixed. However, coexpression of homologous F and HN glycoproteins resulted in extensive cell fusion. The kinetics of fusion were found to be very similar for all three paramyxoviruses studied. With NDV and HPIV-3, no cell fusion was detected when F proteins were coexpressed with heterologous HN proteins or influenza virus hemagglutinin (HA). In contrast, SV5 F protein exhibited a considerable degree of fusion activity when coexpressed with either NDV or HPIV-3 HN or with influenza virus HA, although the kinetics of fusion were two- to threefold higher when the homologous SV5 F and HN proteins were coexpressed. Thus, these data indicate that among the paramyxoviruses tested, SV5 has different requirements for cell fusion.

Paramyxoviruses contain two integral membrane spike glycoproteins, the hemagglutinin-neuraminidase protein (HN) and the fusion protein (F). The assignment of specific biological activities to these glycoproteins was originally made on the basis of purification and reconstitution studies, mainly for the Sendai virus and simian virus 5 (SV5) proteins (46–48). The HN protein was found to possess receptor binding and neuraminidase activities, and the F protein is involved in virus-induced membrane fusion. Additional evidence for the biological activity of the individual F and HN proteins was obtained when the SV5 F and HN cDNAs were expressed in mammalian cells, and it was found that HN had hemadsorbing activity and F caused syncytium formation (36, 37, 39) and coexpression of the F and HN cDNAs did not markedly increase the number of syncytium (15). Perhaps the most striking example of the ability of the SV5 F protein to facilitate syncytium formation is the finding that F mutants containing single Gly to Ala changes in the fusion peptide cause a remarkable increase in syncytium formation compared with wild-type F protein (14, 54).

In contrast, for many other paramyxoviruses, the F proteins only cause syncytium formation when coexpressed with their homotypic HN glycoproteins in the same cell (8, 10, 15, 17, 29, 44, 52, 53, 55). It has been suggested that a type-specific interaction occurs between the HN and F proteins that is necessary for fusion to occur (17, 50) and that a domain(s) of HN is involved in the fusion-promoting activity (6, 25, 44). To test the notion that there are differences in the requirements for fusion between SV5 and other paramyxoviruses, we examined the biological activity of the F and HN cDNAs of several paramyxoviruses, using the same expression system and the same cell type. We found that with the SV5 F protein coexpression of the homotypic HN protein did not markedly increase the number of syncytia, whereas for human parainfluenza virus 3 (HPIV-3) and Newcastle disease virus (NDV) coexpression with the homotypic HN protein was a requirement for syncytium formation (15).

In the majority of studies performed to date, syncytium formation has been used as a measure of cell fusion and this assay suffers from difficulties arising in quantification and its low sensitivity. The present study was undertaken to define further the requirements for cell fusion induced by three different paramyxoviruses, SV5, NDV, and HPIV-3, using quantitative biophysical assays to measure the kinetics and extent of membrane fusion and the extent of content mixing. The data indicate that there are different requirements for fusion among members of the *Paramyxoviridae*.

MATERIALS AND METHODS

Cells and recombinant plasmid vectors. Monolayer cultures of the TC7 subclone of CV-1 and HeLa-T4 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% NU serum IV (Collaborative Research Laboratories, Inc., Bedford, Mass.) as described previously (37).

The SV5, NDV, and HPIV-3 F and HN cDNAs were described previously (12, 14, 35, 36) and were subcloned into pGEM3 or pBluescript SK(+). The plasmid pTF7.5 encoding the hemagglutinin (HA) cDNA of influenza virus A/Udorn/72 was described previously (51). Plasmid pGINT7 β-gal (34) containing the β-galactosidase cDNA was constructed by Richard A. Morgan and was kindly provided by Bernard Moss, National Institutes of Health. All the plasmids contained cDNA such that mRNA-sense RNA transcripts could be synthesized with bacteriophage T7 RNA polymerase by using the bacteriophage T7 RNA polymerase promoter.

* Corresponding author. Mailing address: Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 N. Campus Dr., Evanston, IL 60208-3500. Phone: (708) 491-5433. Fax: (708) 491-2467. Electronic mail address: ralamb@nwu.edu.

Vaccinia virus-based expression of foreign genes. Transient expression of the paramyxovirus envelope proteins (F or/and HN) in CV-1 or HeLa-T4 cells was performed as described previously (3, 11). Subconfluent monolayers of cells grown on 60-mm-diameter plastic dishes were infected with recombinant vaccinia virus vTF7-3 (which expresses T7 RNA polymerase) at a multiplicity of infection (MOI) of 10 PFU per cell and incubated at 37°C for 45 min. The virus inoculum was removed, and cells were washed twice with OPTI-MEM (GIBCO/BRL, Gaithersburg, Md.) and transfected with plasmid DNAs, using cationic liposomes made in our laboratory as described previously (42). cDNA encoding a glycoprotein was kept at 2.5 μg (unless otherwise noted), and the total DNA transfected was kept at 7.5 μg, where necessary adding pGEM3 vector DNA to keep the DNA amount constant. At 5 h posttransfection cells were washed twice in phosphate-buffered saline (PBS) and further treated for metabolic labeling or fusion assays.

Isotopic labeling of polypeptides, immunoprecipitation, and SDS-PAGE. Cells expressing the viral envelope proteins were incubated in cysteine- and methionine-deficient DMEM (DMEM cys−/met−) for 30 min at 5 h posttransfection and labeled with Tran[^{35}S]-label (200 μCi/ml) (ICN Radiochemicals, Irvine, Calif.) in DMEM cys−/met− for 20 min and incubated in chase medium (DMEM supplemented with 2 mM unlabeled cysteine and methionine and 2% NU serum IV) for 2 h. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors (38) and 25 mM iodoacetamide. Proteins were immunoprecipitated from cell lysates as described previously (20). Polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels under reducing conditions, and gels were processed for fluorography as described previously (20).

Antibodies. Monoclonal antibodies against SV5 HN (HN5a) and F (F1a) proteins were kindly provided by Rick Randall (41). SV5 F polyclonal antiserum was raised in rabbits against a mixture of three synthetic peptides specific for F_2 residues 22 to 39, 31 to 46, and 77 to 94 (14). Monoclonal antibodies against NDV F (N2710) and HN (5-2D6) proteins were provided by Mark Peeples, Rush Medical Center, Chicago, Ill. Hybridomas producing monoclonal antibodies against HPIV-3 HN were obtained from the American Type Culture Collection (HB-8934), and tissue culture supernatants were provided by Reay Paterson. Monoclonal antibody against HPIV-3 F protein was obtained from Brian Murphy, National Institutes of Health, Bethesda, Md. Goat polyclonal antiserum (SP-31) against influenza virus HA protein was described previously (51).

Labeling of RBCs with octadecyl rhodamine B. Human erythrocytes (RBCs) were labeled with the lipid probe octadecyl rhodamine B (R18) (Molecular Probes, Eugene, Ore.) as described previously (28). Fifteen microliters of R18 (1 mg/ml in ethanol) was rapidly added to human RBCs (1% hematocrit in 10 ml of PBS) by using a Hamilton syringe. After incubation at room temperature for 15 min, 30 ml of DMEM supplemented with 10% NU serum IV was added to the suspension to absorb unbound probe. The RBCs were further incubated for 20 min at room temperature and washed six times by centrifugation with 50 ml of PBS. The fluorescence intensity of intact R18 RBCs was 15 to 20% that of labeled RBCs treated with 0.1% Triton X-100 (100% fluorescence).

Binding and fusion of R18-labeled RBCs with cells expressing viral proteins. Cell monolayers expressing paramyxovirus F or/and HN proteins or influenza virus HA protein at 5 h posttransfection were washed twice with PBS and incubated overnight at 32°C in DMEM containing 10% NU serum IV. The cells were then washed with PBS, incubated for 1 h at 37°C with 50 mU per ml of neuraminidase (*Vibrio cholerae*) (Boehringer Mannheim, Indianapolis, Ind.) in DMEM, and washed twice in PBS. Three milliliters of R18-labeled RBCs (0.1% hematocrit) in PBS was added to the monolayer and incubated at 4°C for 30 min with occasional gentle agitation. Unbound RBCs were removed by six washes with PBS. The cells were then removed from the dish, using either a trypsin-EDTA solution or 50 mM EDTA in PBS at 4°C. The R18-labeled RBC-acceptor cell complexes were washed with cold PBS and placed on ice until further use.

Fluorescence changes as a result of fusion of R18-labeled RBCs with acceptor cells were measured continuously by using a spectrofluorimeter (Alpha Scan; Photon Research Inc., South Brunswick, N.J.) with 1-s time resolution at 560 and 590 nm of excitation and emission, respectively. A 570-nm cutoff filter was placed in the emission optical pathway to reduce scattering. Fifty microliters of the R18-labeled RBC-acceptor cell suspension was placed in a cuvette containing 3 ml of PBS prewarmed to 37°C. To normalize the data, percent fluorescence dequenching (% FDQ) at any time point was calculated according to the following equation: % FDQ = $100(F-F_0/F_t-F_0)$, where F_0 and F are the fluorescence intensities at time 0 and at a given time point, and F_t is the fluorescence intensity in the presence of 0.1% Triton X-100 and defined as fluorescence at "infinite" dilution of the probe (4).

Content-mixing assay. The content-mixing assay, based on the cytoplasmic activation of the reporter gene β-galactosidase, was performed essentially as described previously (34), with slight modifications. One population of CV-1 cells was infected with recombinant vaccinia virus vTF7-3 and transfected with plasmid DNA encoding the viral glycoproteins as described above. At 5 h posttransfection the cells expressing viral envelope proteins (and T7 RNA polymerase) were trypsinized, suspended in DMEM containing 2.5% NU serum IV to a density of 2×10^5/ml, and incubated overnight at 32°C in a near-horizontal position. The cells were washed twice in OPTI-MEM (containing 2.5 mM $CaCl_2$), suspended to a density of 2×10^7/ml, and treated with neuraminidase (50 mU/ml) for 1 h at 37°C. The cells were then washed and suspended in OPTI-MEM at 10^6 cells per ml.

A second population of CV-1 cells was infected with wild-type vaccinia virus (strain WR) for 45 min at 37°C and transfected with plasmid pGINT7 β-gal. At 5 h posttransfection, cells were trypsinized and treated as described above. The cells were washed with OPTI-MEM, either treated or not treated with neuraminidase, and finally suspended at 10^6/ml.

The two cell populations were mixed in triplicate by adding 100 μl of each cell population in a 96-well tissue culture plate. The plates were incubated at 37°C for 2.5 or 4 h. The cell fusion was measured at the indicated times by the colorimetric lysate assay for β-galactosidase as described previously (34). The quantity of β-galactosidase was calculated by comparing the rates of hydrolysis for each sample with that obtained for a standard preparation of *Escherichia coli* β-galactosidase (Boehringer Mannheim) and was expressed as nanograms per well. The in situ assay involving 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of cells was done as described previously (34).

Lectin analysis. Cells expressing viral fusion (F) proteins were incubated with phytohemagglutinin (PHA; 10 μg/ml), wheat germ agglutinin (WGA; 6 μg/ml), or concanavalin A (ConA; 10 μg/ml) either in the presence of R18-labeled RBCs during binding in the lipid-mixing assay or in the content-mixing assay during incubation of the two cell populations.

RESULTS

Expression of viral envelope glycoproteins. To express the envelope glycoproteins of the paramyxoviruses SV5, NDV, and HPIV-3 and influenza virus HA in CV-1 or HeLa-T4 cells, the recombinant vaccinia virus-T7 RNA polymerase system was used. To confirm the synthesis of the proteins, CV-1 cells were labeled with Tran[^{35}S]-label at 5 h posttransfection for 20 min and incubated in chase medium for 2 h to allow for cleavage of F_0 to F_1 and F_2. We have shown previously that the half-life for cleavage of SV5 F_0 when expressed using the vaccinia virus-T7 system is ~90 to 100 min (3). Proteins were immunoprecipitated with the appropriate antibodies (see Materials and Methods), and polypeptides were analyzed by SDS-PAGE (10% polyacrylamide). Examples of the coexpression of the viral glycoproteins are shown in Fig. 1. All the proteins were expressed, and a significant fraction of the F proteins was cleaved to F_1 and F_2 (F_2 is not detected on a 10% gel). As different antibodies were used, quantification of the expression of viral proteins was not done.

Induction of cell fusion: lipid-mixing assay. To compare the rate and extent of paramyxovirus F protein-induced cell fusion under various conditions, the lipophilic probe R18 was incorporated into RBCs which were then mixed with acceptor cells that expressed the viral glycoproteins and the kinetics of fluorescence dequenching of the R18 probe was measured. Although throughout data from using CV-1 cells as acceptor cells are reported, very similar data were obtained when HeLa-T4 cells were used.

To establish the assay system, cells were transfected with different ratios of F and HN DNAs (2.5 μg:2.5 μg; 5 μg:2.5 μg; 7.5 μg:2.5 μg; F:HN DNA, with total DNA made up to 10 μg with pGEM3 vector DNA). The effect on the lipid-mixing activity of cells transfected with different F:HN DNA ratios was studied. It was found that the initial rate of R18 dequenching was ~five- to sixfold lower at an F:HN DNA ratio of 1:1 than when an F:HN DNA ratio of 2:1 or 3:1 was used (Fig. 2). However, the extent of fusion after 600 s of mixing was not greatly different. Similar data were obtained when the homotypic NDV or HPIV-3 F and HN DNAs were used. Thus, as the rates of fusion were similar at 2:1 and 3:1 F:HN DNA ratios, in all subsequent experiments cells were transfected with F:HN DNAs at a ratio of 2:1 (microgram to microgram).

When cells expressing the SV5, NDV, or HPIV-3 F protein alone were mixed with R18-labeled erythrocytes, no fluorescence dequenching was detected (Fig. 3). However, when the homotypic HN proteins were coexpressed in the cells with the homotypic F proteins fluorescence dequenching occurred rap-

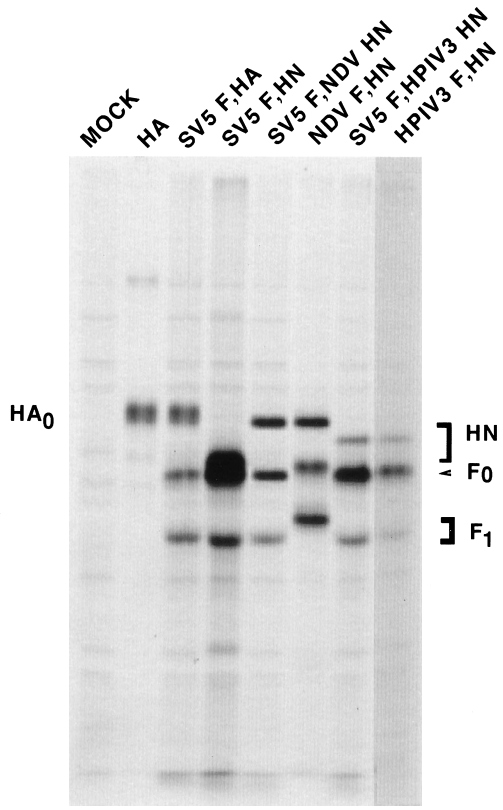


FIG. 1. Coexpression of viral envelope glycoproteins. To coexpress the viral envelope proteins, CV-1 cells were infected with vaccinia virus vTF7-3 at 10 PFU per cell for 45 min at 37°C and then transfected with plasmid DNAs encoding the glycoproteins either individually or in combinations as described in Materials and Methods. Transfected cells were metabolically labeled with Tran[^{35}S]-label (200 μCi/ml) at 5 h posttransfection for 20 min and incubated in the chase medium for 2 h at 37°C. Detergent lysates of labeled proteins were immunoprecipitated by addition of the appropriate monoclonal or polyclonal antibodies for the specific proteins being expressed, and polypeptides were analyzed by SDS-PAGE and fluorography on a 10% gel under reducing conditions. Lanes: Mock, polypeptides immunoprecipitated with a mixture of anti-SV5 F and HN sera from vTF7-3-infected but untransfected cells; HA, cells expressing influenza virus HA protein; SV5 F,HA, cells coexpressing the SV5 F and influenza virus HA proteins; SV5 F,HN, cells coexpressing the SV5 F and HN proteins; SV5 F,NDV HN, cells coexpressing the SV5 F and NDV HN proteins; NDV F,HN, cells coexpressing the NDV F and HN proteins; SV5 F,HPIV3 HN, cells coexpressing SV5 F and HPIV-3 HN proteins; HPIV3 F,HN, cells coexpressing the HPIV-3 F and HN proteins. Lane HPIV3 F,HN is from a two times longer exposure of the autoradiograph.

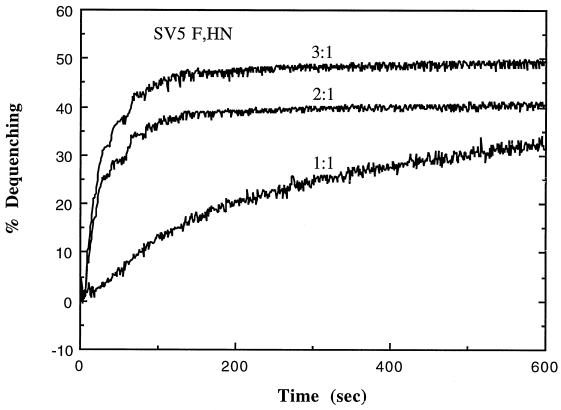


FIG. 2. Kinetics of fluorescence dequenching of R18-labeled RBCs with cells coexpressing SV5 F and HN glycoproteins. R18-labeled RBCs were bound to CV-1 cells transfected with various ratios of SV5 F and HN cDNAs (microgram to microgram, with HN DNA held at 2.5 μg and total DNA made up to 10 μg with pGEM3 vector DNA), washed, and resuspended with trypsin-EDTA as described in Materials and Methods. Fifty microliters of the R18-labeled RBC-acceptor cell complex was injected into 3 ml of PBS, pH 7.4 (prewarmed at 37°C), and the fluorescence measurements were made as described in Materials and Methods.

idly (Fig. 3); the kinetics of fusion were comparable for SV5, NDV, and HPIV-3.

The SV5 F protein-mediated fluorescence dequenching was inhibited by a 4°C preincubation of R18-labeled RBC-acceptor cell suspensions with monoclonal antibody specific to the SV5 F protein (Fig. 3A), suggesting that the observed fluorescence increase on coexpression of SV5 F and HN reflects fusion activity of the F protein and is not an artifact as a result of nonspecific dye transfer. To determine whether cell fusion required the expression of both the F and HN proteins in the same acceptor cell, CV-1 cells were transfected separately with the F and HN genes and then mixed. No fluorescence dequenching was detected when cells individually expressing the homologous SV5, NDV, or HPIV-3 F and HN proteins were mixed and incubated with R18-labeled RBCs in the lipid-mixing assay (data for SV5 F plus HN is shown in Fig. 3A).

To obtain further quantitative information concerning the role of paramyxovirus HN proteins in cell fusion, the F proteins of SV5, NDV, and HPIV-3 were coexpressed in cells with heterologous HN proteins. Fusion activity was also tested when the paramyxovirus F proteins were coexpressed with HA, the sialic acid-binding receptor protein of influenza virus. Coexpression of NDV and HPIV-3 F proteins with heterologous HN or HA proteins did not lead to any significant fluorescence dequenching (data for coexpression of NDV F plus SV5 HN is shown in Fig. 4). In contrast, when SV5 F protein was coexpressed with NDV or HPIV-3 HN protein or influenza virus HA, significant fluorescence dequenching was observed (Fig. 4). The rates of fluorescence dequenching were calculated from the maximum slopes of curves as exemplified in Fig. 2 to 4, and the rates were normalized to the maximum extent for a given R18-labeled RBC-acceptor cell population. As shown in Fig. 5, differences were found in both the rates (panel A) and extent of fusion (panel B) between cells coexpressing different viral proteins and the R18-labeled RBCs. The rates and extent of fusion on coexpression of the homologous paramyxovirus F and HN proteins of SV5, NDV, and HPIV-3 were similar. Although the rate of fusion of SV5 F protein coexpressed with either HA or NDV or HPIV-3 HN proteins was 12, 44, and 24%, respectively, of that for the homologous SV5 F and HN proteins, the extent of fusion of SV5 F protein coexpressed with either HA or NDV HN or HPIV-3 HN proteins was 61, 40, and 34%, respectively. The cells expressing influenza virus HA alone did not show fusion with R18-labeled RBCs at pH 7.4 (Fig. 4), indicating that HA provided only the binding function in fusion of cells expressing SV5 F and influenza virus HA protein. To investigate whether lectins could substitute for the attachment function provided by the HN or HA proteins in SV5-mediated fusion, lectins ConA, WGA, or PHA were added to the R18-labeled RBC-acceptor cell suspensions during binding at 4°C. No significant increase in the fluorescence was detected (Fig. 4), indicating that in this assay system, lectins cannot replace the function of the HN proteins or HA in SV5 F protein-mediated cell fusion. Addition of lectins to the R18-labeled RBC-acceptor cell complexes expressing NDV or HPIV-3 F proteins did not cause detectable fluorescence dequenching.

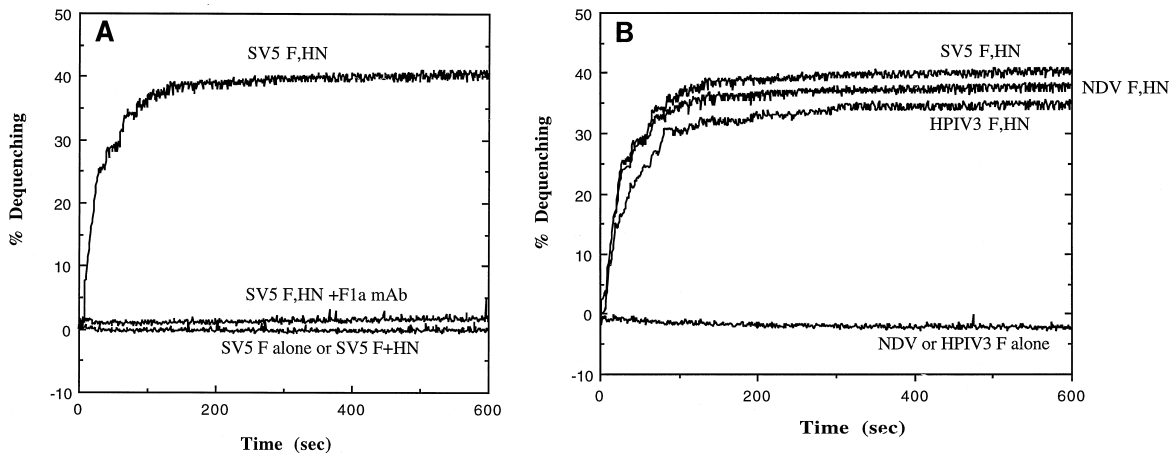


FIG. 3. Kinetics of fusion of R18-labeled RBCs with cells coexpressing paramyxovirus envelope proteins. R18-labeled RBCs were bound to CV-1 cells expressing paramyxovirus envelope proteins, and fluorescence measurements were performed as described in Materials and Methods. (A) SV5 F alone; cells expressing the SV5 F protein; SV5 F,HN, cells coexpressing SV5 F and HN proteins; SV5 F,HN +F1a mAb, cells coexpressing SV5 F and HN proteins with the fusion assay performed in the presence of monoclonal antibody F1a specific for the F protein; SV5 F+HN, cells expressing F or HN protein individually and mixed in equal numbers during incubation with R18-labeled RBCs. (B) SV5 F,HN, as described above; NDV F,HN, cells coexpressing NDV F and HN proteins; HPIV3 F,HN, cells coexpressing HPIV-3 F and HN proteins; F alone, a single tracing that represents data obtained from two different experiments for cells expressing either NDV or HPIV-3 F alone.

Content-mixing assay. Cell fusion involves mixing of both the outer leaflet membrane lipids (as measured by the R18 assay) and the mixing of inner leaflet membrane lipids with concomitant mixing of aqueous contents of donor and recipient cells (18). To measure content mixing we employed the reporter gene (β-galactosidase)-activation assay (34). For the content-mixing assay two populations of cells were used. First, the vaccinia virus-T7 expression system was used to express the various combinations of homologous and heterologous viral envelope proteins. This cell population was treated with neuraminidase to eliminate cell fusion within this population. A second cell population was transfected with a plasmid containing the *lacZ* gene, such that *lacZ* was under the control of the T7 RNA polymerase promoter, and this population of cells was either untreated or treated with neuraminidase. The two populations of cells were mixed (1:1 ratio) and incubated at 37°C for either 2.5 h or 4 h. The β-galactosidase activity was analyzed in situ by using the X-Gal staining procedure (Fig. 6, top), and the data were quantified by spectroscopy using aliquots of cell lysates (Fig. 6, bottom). The results of the content mixing assay directly correlated with that of lipid mixing analyses. A significant level of β-galactosidase was produced, and heavily blue-stained syncytia were observed when cells expressing T7 RNA polymerase and the homologous F and HN proteins of either SV5, NDV, or HPIV-3 were mixed with cells that contained the *lacZ* plasmid and that were not treated with neuraminidase. Only background levels of β-galactosidase were detected when cells expressing either F protein alone or HN protein alone or when individual populations of cell expressing individual homologous F and HN proteins were mixed before the assay. Similarly, no β-galactosidase was produced when NDV or HPIV-3 F protein was coexpressed with heterologous HN protein or influenza virus HA. On the other hand, coexpression of SV5 F with NDV or HPIV-3 HN or influenza virus HA protein resulted in production of significant amounts of β-galactosidase activity and blue-stained cells. The extent of β-galactosidase produced was, however, two- to threefold higher when homologous SV5 F and HN proteins were coexpressed. Incubation of cells coexpressing SV5 F and HN proteins with partner cells in the presence of SV5-specific monoclonal antibody F1a resulted in background levels of β-galactosidase activity both using the in situ method and using spectrophotometric analysis indicating the specificity of the fusion process (Figs. 6). Neuraminidase treatment of the partner cells greatly reduced β-galactosidase production when the treated cells were mixed with cells coexpressing homologous paramyxovirus (SV5, NDV, and HPIV-3) proteins, thus confirming the notion that specific binding through glycoprotein-sialic acid receptor interactions is required between two cell

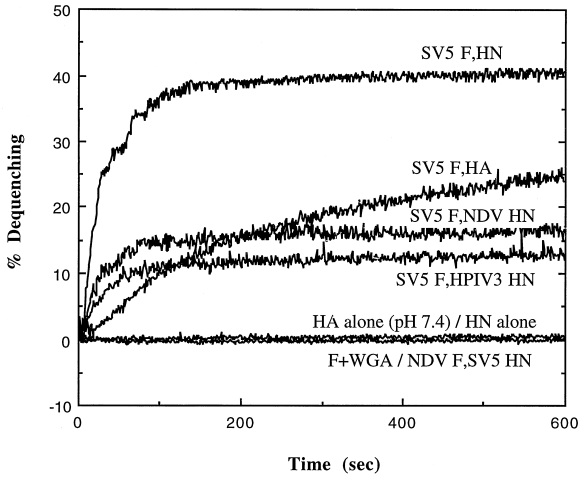


FIG. 4. Effect of coexpression of heterologous viral glycoproteins on paramyxovirus-induced cell fusion. R18-labeled RBCs were bound to CV-1 cells coexpressing viral envelope proteins, and fluorescence measurements were done as described in the legend to Fig. 2. SV5 F,HN, cells coexpressing SV5 F and HN proteins; SV5 F,HA, cells coexpressing SV5 F and influenza virus HA; SV5 F,NDV HN, cells coexpressing SV5 F and NDV HN; SV5 F,HPIV3 HN, cells coexpressing SV5 F and HPIV-3 HN; NDV F,SV5 HN, cells coexpressing NDV F and SV5 HN; HA alone, cells expressing influenza virus HA; HN alone, cells expressing SV5, NDV, or HPIV-3 HN only; F+WGA, a single tracing that represents data obtained from three different experiments for cells expressing either SV5, NDV, or HPIV-3 F protein in the presence of lectin WGA at a concentration of 6 μg/ml. Other combinations of viral proteins, such as, SV5 F,lectins; HPIV-3 F,SV5 HN; HPIV-3 F,NDV HN; HPIV-3 F,HA; HPIV-3 F,lectins; NDV F,HPIV-3 HN; NDV F,HA; and NDV F,lectins, yielded data that were essentially identical to that shown for NDV F,SV5 HN, i.e., no detectable fusion (lectins is used to describe either WGA, PHA, or ConA).

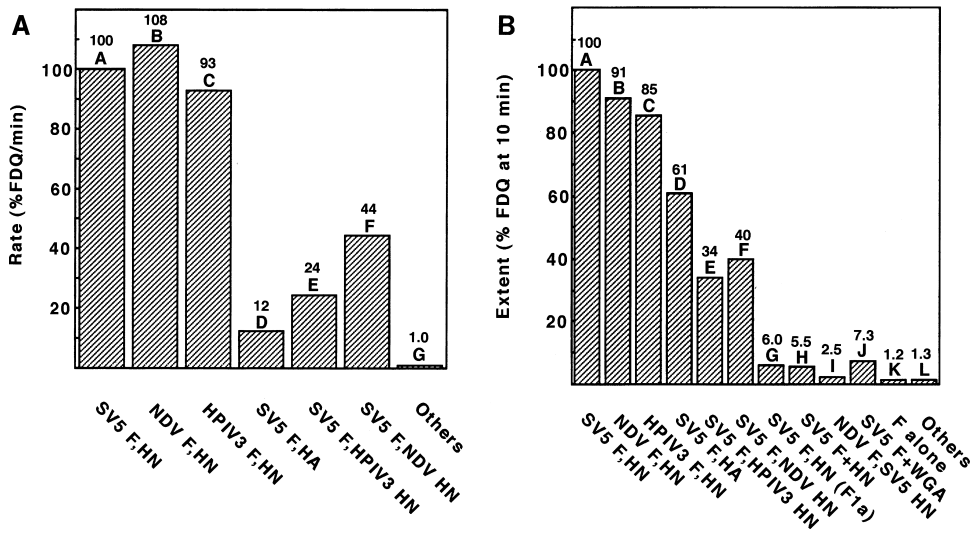


FIG. 5. Quantification of the rates and extent of fusion of R18-labeled RBCs with cells expressing various combinations of paramyxovirus envelope glycoproteins. (A) The rate of fluorescence dequenching was calculated from maximum slopes of curves, such as those shown in Fig. 2 to 4, and normalized to the maximum rate of SV5 F,HN fusion. The data are the averages of four independent experiments. Bar G represents the average data for any combination not shown in this histogram of SV5, NDV, or HPIV-3 F and HN (or lectins PHA, WGA, or Con A) as described in Fig. 2 to 4. (B) The maximum extent of dequenching at 10 min was calculated from kinetics curves analogous to those shown in Fig. 2 to 4. The values are the averages of four independent experiments. Histograms A through L represent the different permutations of glycoproteins described in the legends to Fig. 2 to 4. SV5 F,HN (F1a), coexpression of SV5 F and HN in the presence of monoclonal antibody F1a. Others, the combinations SV5 F,lectins; HPIV-3 F,SV5 HN; HPIV-3 F,NDV HN; HPIV-3 F,HA; HPIV-3 F,lectins; NDV F,HPIV-3 HN; NDV F,HA; and NDV F,lectins yielded data that was essentially identical to that shown in bar I (NDV F,SV5 HN). Data are normalized to SV5 F,HN.

populations. Mock-infected cells did not show any β-galactosidase production.

DISCUSSION

It is becoming increasingly clear that among different members of the *Paramyxoviridae* there are different requirements for fusion. In addition, it is clear that there is an important role for the cell type in the fusion process (reviewed in reference 18). Whereas with SV5, and in some reports measles virus, it has been shown that the F protein alone can cause syncytium formation (1, 2, 14, 15, 36, 37, 39, 54), for many other paramyxoviruses (including measles virus) it has been shown that the F protein only causes syncytium formation when coexpressed with the homotypic HN glycoprotein (6, 8, 10, 12, 15, 17, 29, 44, 52, 53, 55). It has been suggested that for those viruses for which F and HN have to be coexpressed for fusion to occur, a type-specific interaction occurs between the HN and F proteins (17, 50).

Although the requirement for coexpression of F and HN proteins for syncytium formation for many paramyxoviruses except SV5 is well established, the role of HN in the early fusion events is not understood. Data obtained from reconstituting purified Sendai virus F protein into vesicles indicate that the F protein in the presence of an attachment factor (lectin) is sufficient for fusion (4, 16, 49), although the presence of the Sendai virus HN protein in the reconstituted vesicles enhances the rate of fusion of virosomes with target cells (4). Furthermore, for virus-cell fusion it is noted that a Sendai virus mutant deficient in HN is able to infect HepG2 cells through binding to the asialoglycoprotein receptor (24). In contrast, data obtained from examination of cell-cell fusion for HPIV-2, HPIV-3, and NDV suggest that the role of HN is more complex than mediating the juxtapositioning of membranes. Although a primary requirement is the presence of the HN receptor sialic acid on cell surfaces, neither lectins, influenza virus HA, nor heterotypic HN protein can substitute for coexpression of the homotypic HN protein for fusion to occur (12, 15, 17, 30–33). At the present time the differences between fusion of viruses, virosomes, and liposomes to cells and cell-cell fusion are not understood; however, they are related yet different processes. An alternative explanation for the different requirements is that either purification of the F protein or the reconstitution process alters the protein conformation of F and renders it competent to initiate fusion.

In our studies we have used quantitative fusion assays to study the requirements for membrane fusion mediated by the paramyxoviruses SV5, NDV, and HPIV-3. These fusion assays offer highly sensitive direct methods for obtaining quantitative information on the fusion process in its very early stages (13, 23, 45). When the F proteins of the paramyxoviruses SV5, NDV, and HPIV-3 were expressed alone, even though they were cleaved to the fusion-active form $F_1 + F_2$, no cell fusion was detected with the quantitative assays. However, extensive fusion resulted upon coexpression of the homotypic F and HN proteins. The absence of detectable cell fusion on expression of the SV5 F protein alone in this assay system was not unexpected because the fusion assay is performed with a suspension of cells (as opposed to the confluent monolayer cells of syncytium assays). For fusion to occur, a binding moiety is needed to bring the donor and acceptor cells into close contact and the HN protein fulfills this role.

Whereas neither a heterotypic HN protein, influenza virus HA, nor lectins could replace the homotypic HN for fusion to occur for NDV and HPIV-3, confirming earlier data obtained when syncytium formation was measured (9, 12, 17, 29, 31), this was not the case for SV5. With SV5, cell fusion was detected upon coexpression of the SV5 F protein with heterologous HN proteins or influenza virus HA, although an enhancement in the fusion rate and extent was observed on coexpression of the homotypic F and HN proteins. Thus, these data suggest that SV5-mediated cell fusion has requirements that differ from those of NDV and HPIV-3, and the findings reported here augment those found previously with the syncy-

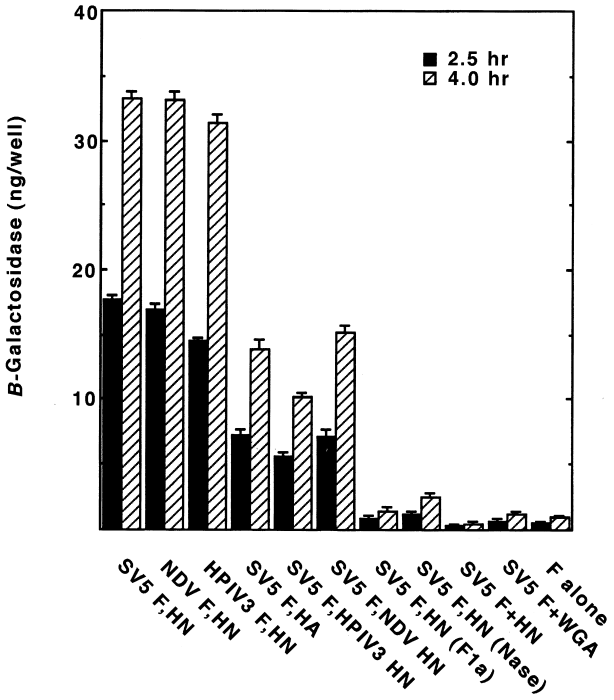


FIG. 6. Reporter gene activation assay (content mixing) for paramyxovirus-mediated cell fusion. One culture of CV-1 cells was infected with recombinant vaccinia virus vTF7-3, which encodes T7 RNA polymerase, and cotransfected with plasmid DNA containing the cDNAs for the paramyxovirus envelope proteins. Cells were then treated with bacterial neuraminidase as described in Materials and Methods. A second culture of CV-1 cells was infected with wild-type vaccinia virus (strain WR) and transfected with plasmid pGINT7 β-gal which contains the β-galactosidase gene and, where indicated, the cells were treated with neuraminidase (Nase). The cell populations were washed and suspended in OPTI-MEM containing $CaCl_2$ (2.5 mM) at 10^6 cells per ml. Mixtures of the two populations of cells (0.1 ml each) were plated in duplicate 96-well plates and incubated at 37°C. (Top) Following incubation at 37°C for 4 h, samples were analyzed by using in situ X-Gal staining (Materials and Methods). Cells were photographed with a Kodak Digital 420 camera at 10× magnification on a Nikon inverted phase-contrast microscope. (A) CV-1 cells coexpressing SV5 F and HN proteins. (B) Cells coexpressing SV5 F and influenza virus HA. (C) Cells coexpressing SV5 F and HN in the presence of monoclonal antibody F1a. (D) Vaccinia virus vTF7-3-infected but untransfected cells. (Bottom) At the indicated times, samples were analyzed by the colorimetric lysate assay described in Materials and Methods. Each point represents an average of three experiments plus or minus standard deviation. Designation of histogram bars is as described in the legend to Fig. 5A. SV5 F,HN (Nase), cells coexpressing SV5 F and HN and incubated with neuraminidase-treated target cells.

tium formation assay when it was found that expression of the SV5 F protein alone causes syncytium formation (14, 15, 36, 37, 39, 54). Other evidence pointing toward the sufficiency of the SV5 F protein in cell fusion comes from the finding that anti-HN antiserum was not able to prevent spread of SV5 by cell-cell fusion in tissue culture (26), whereas for Sendai virus anti-HN monoclonal antibodies, although allowing agglutination to occur, interfered with F protein-mediated fusion (27). Furthermore, there is a difference in the requirement for a sialic acid receptor for HN in the fusion process between SV5 and HPIV-3 (31). Treatment of SV5-infected cells with neuraminidase prevents cell fusion in cells infected at a low MOI, and the same result was found for HPIV-3. However, whereas neuraminidase treatment prevents fusion in cells infected with a high MOI of HPIV-3, treatment of cells infected with SV5 at a high MOI with neuraminidase had no effect on the degree of syncytium formation (31).

As discussed above, one function of HN is to bring the donor and acceptor cells into alignment for fusion to occur. It seems likely that the molecular architecture of the proteins is important as both heterotypic HN proteins and influenza virus HA but not lectins, support SV5 F protein-mediated fusion. In this regard it is interesting to note that electron microscopic and X-ray structural information indicate that the distal tips of HN and HA are 120 to 140 Å (1 Å = 0.1 nm) from the membrane and thus the sialic acid-binding pocket may be a similar distance from the membrane (reviewed in references 21 and 22), whereas ConA is a very different size (42 by 40 by 39 Å) (5, 40).

It seems unlikely that the F protein hydrophobic fusion peptides are freely exposed to an aqueous environment after cleavage-activation (either in the *trans*-Golgi network or at the

cell surface) as aggregation of the F protein oligomers would most likely occur. Thus, it has been hypothesized that the cleaved F protein undergoes a conformational change to expose the fusion peptide at the right time and in the right place (19, 50). By analogy to influenza virus HA, it has been suggested that the trimeric paramyxovirus F proteins (43) undergo a conformational change to form a related coiled-coil structure (7, 19). However, unlike influenza virus for which the biologically relevant trigger to the conformational change is low pH, the trigger for the proposed paramyxovirus conformational change has not been identified. One model for a tightly regulated trigger for a conformational change in F that has been proposed is that upon binding of sialic acid by HN, the HN protein undergoes a conformational change, which through a protein-protein interaction triggers the conformational change in F (19, 50). The question arises as to why the requirement for HN is not so stringent for SV5-mediated cell fusion, when it appears to be absolutely necessary for most of the other paramyxoviruses. For SV5, for which coexpression of HN only weakly influences cell-cell fusion, an F conformational change may be hair-triggered by contact of F with the target membrane or triggered after docking of F with an unrecognized receptor on the target membrane (19).

ACKNOWLEDGMENTS

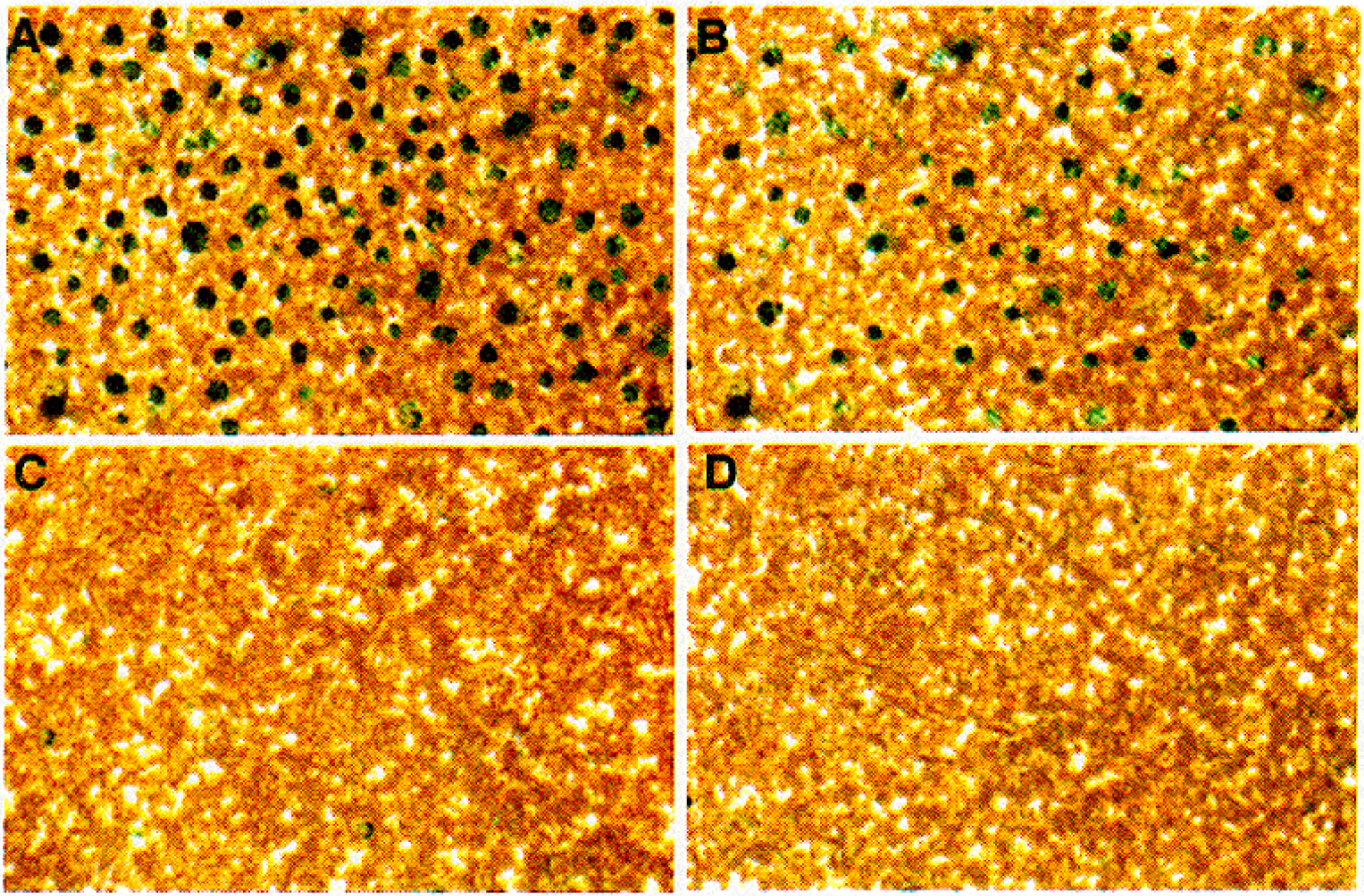
We are very grateful to Ruby MacDonald (Northwestern University) for her advice and for allowing us to use her time-resolving fluorimeter. We thank Bernard Moss (National Institutes of Health) for providing vTF7-3 and pGINT7 β-gal and the members of the Lamb laboratory for helpful discussions.

S.B. is an Associate and R.A.L. is an Investigator of the Howard Hughes Medical Institute. This research was supported by Public Health Service Research grant AI-23173 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Alkhatib, G., C. Richardson, and S. H. Shen.** 1990. Intracellular processing, glycosylation, and cell-surface expression of the measles virus fusion protein (F) encoded by a recombinant adenovirus. Virology **175:**262–270.
2. **Alkhatib, G., S.-H. Shen, D. Briedis, C. Richardson, B. Massie, R. Weinberg, D. Smith, J. Taylor, E. Paoletti, and J. Roder.** 1994. Functional analysis of N-linked glycosylation mutants of the measles virus fusion protein synthesized by recombinant vaccinia virus vectors. J. Virol. **68:**1522–1531.
3. **Bagai, S., and R. A. Lamb.** 1995. Individual roles of N-linked oligosaccharide chains in intracellular transport of the paramyxovirus SV5 fusion protein. Virology **209:**250–256.
4. **Bagai, S., A. Puri, R. Blumenthal, and D. P. Sarkar.** 1993. Hemagglutinin-neuraminidase enhances F protein-mediated fusion of reconstituted Sendai virus envelopes with cells. J. Virol. **67:**3312–3318.
5. **Becker, J. W., G. J. Reeke, J. L. Wang, B. A. Cunningham, and G. M. Edelman.** 1975. The covalent and three-dimensional structure of concanavalin A. III. Structure of the monomer and its interactions with metals and saccharides. J. Biol. Chem. **250:**1513–1524.
6. **Bousse, T., T. Takimoto, W. L. Gorman, T. Takahashi, and A. Portner.** 1994. Regions on the hemagglutinin-neuraminidase proteins of human parainfluenza virus type-1 and Sendai virus important for membrane fusion. Virology **204:**506–514.
7. **Carr, C. M., and P. S. Kim.** 1993. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell **73:**823–832.
8. **Cattaneo, R., and J. K. Rose.** 1993. Cell fusion by the envelope glycoproteins of persistent measles viruses which cause lethal human brain disease. J. Virol. **67:**1493–1502.
9. **Deng, R., Z. Wang, A. M. Mirza, and R. M. Iorio.** 1995. Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. Virology **209:**457–469.
10. **Ebata, S. N., M. J. Coté, C. Y. Kang, and K. Dimock.** 1991. The fusion and hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus 3 are both required for fusion. Virology **183:**437–441.
11. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
12. **Heminway, B. R., Y. Yu, and M. S. Galinski.** 1994. Paramyxovirus mediated cell fusion requires co-expression of both the fusion and hemagglutinin-neuraminidase glycoproteins. Virus Res. **31:**1–16.
13. **Hoekstra, D., T. de Boer, K. Klappe, and J. Wilschut.** 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry **23:**5675–5681.
14. **Horvath, C. M., and R. A. Lamb.** 1992. Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. J. Virol. **66:**2443–2455.
15. **Horvath, C. M., R. G. Paterson, M. A. Shaughnessy, R. Wood, and R. A. Lamb.** 1992. Biological activity of paramyxovirus fusion proteins: factors influencing formation of syncytia. J. Virol. **66:**4564–4569.
16. **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.
17. **Hu, X., R. Ray, and R. W. Compans.** 1992. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. J. Virol. **66:**1528–1534.
18. **Kemble, G. W., T. Danielli, and J. M. White.** 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. Cell **76:**383–391.
19. **Lamb, R. A.** 1993. Paramyxovirus fusion: a hypothesis for change. Virology **197:**1–11.
20. **Lamb, R. A., P. R. Etkind, and P. W. Choppin.** 1978. Evidence for a ninth influenza viral polypeptide. Virology **91:**60–78.
21. **Lamb, R. A., and D. Kolakofsky.** 1996. *Paramyxoviridae*: the viruses and their replication, p. 1177–1204. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed. Raven Press, New York.
22. **Lamb, R. A., and R. M. Krug.** 1996. *Orthomyxoviridae*: the viruses and their replication, p. 1353–1394. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed. Raven Press, New York.
23. **Loyter, A., V. Citovsky, and R. Blumenthal.** 1988. The use of fluorescence dequenching methods to follow viral fusion events. Methods Biochem. Anal. **33:**128–164.
24. **Markwell, M. A., A. Portner, and A. L. Schwartz.** 1985. An alternative route of infection for viruses: entry by means of the asialoglycoprotein receptor of a Sendai virus mutant lacking its attachment protein. Proc. Natl. Acad. Sci. USA **82:**978–982.
25. **McGinnes, L., T. Sergel, and T. Morrison.** 1993. Mutations in the transmembrane domain of the HN protein of Newcastle disease virus affect the structure and activity of the protein. Virology **196:**101–110.
26. **Merz, D. C., A. Scheid, and P. W. Choppin.** 1979. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J. Exp. Med. **151:**275–288.
27. **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.
28. **Morris, S. J., D. P. Sarkar, J. M. White, and R. Blumenthal.** 1989. Kinetics of pH-dependent fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells. J. Biol. Chem. **264:**3972–3978.
29. **Morrison, T., C. McQuain, and L. McGinnes.** 1991. Complementation between avirulent Newcastle disease virus and a fusion protein gene expressed from a retrovirus vector: requirements for membrane fusion. J. Virol. **65:** 813–822.
30. **Morrison, T., and A. Portner.** 1991. Structure, function, and intracellular processing of the glycoproteins of *Paramyxoviridae*, p. 347–382. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
31. **Moscona, A., and R. W. Peluso.** 1992. Fusion properties of cells infected with human parainfluenza virus type 3: receptor requirements for viral spread and virus-mediated membrane fusion. J. Virol. **66:**6280–6287.
32. **Moscona, A., and R. W. Peluso.** 1991. Fusion properties of cells persistently infected with human parainfluenza virus type 3: participation of hemagglutinin-neuraminidase in membrane fusion. J. Virol. **65:**2773–2777.
33. **Moscona, A., and R. W. Peluso.** 1993. Relative affinity of the human parainfluenza virus type 3 hemagglutinin-neuraminidase for sialic acid correlates with virus-induced fusion activity. J. Virol. **67:**6463–6468.
34. **Nussbaum, O., C. C. Broder, and E. A. Berger.** 1994. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. J. Virol. **68:**5411–5422.
35. **Paterson, R. G., T. J. R. Harris, and R. A. Lamb.** 1984. Analysis and gene assignment of mRNAs of a paramyxovirus, simian virus 5. Virology **138:**310–323.
36. **Paterson, R. G., T. J. R. Harris, and R. A. Lamb.** 1984. Fusion protein of the paramyxovirus simian virus 5: nucleotide sequence of mRNA predicts a highly hydrophobic glycoprotein. Proc. Natl. Acad. Sci. USA **81:**6706–6710.
37. **Paterson, R. G., S. W. Hiebert, and R. A. Lamb.** 1985. Expression at the cell surface of biologically active fusion and hemagglutinin/neuraminidase proteins of the paramyxovirus simian virus 5 from cloned cDNA. Proc. Natl. Acad. Sci. USA **82:**7520–7524.
38. **Paterson, R. G., R. A. Lamb, B. Moss, and B. R. Murphy.** 1987. Comparison of the relative roles of the F and HN surface glycoproteins of the paramyxovirus simian virus 5 in inducing protective immunity. J. Virol. **61:**1972–1977.
39. **Paterson, R. G., M. A. Shaughnessy, and R. A. Lamb.** 1989. Analysis of the

relationship between cleavability of a paramyxovirus fusion protein and length of the connecting peptide. J. Virol. **63:**1293–1301.
40. **Quiocho, F. A., G. J. Reeke, J. W. Becker, W. N. Lipscomb, and G. M. Edelman.** 1971. Structure of concanavalin A at 4 Å resolution. Proc. Natl. Acad. Sci. USA **68:**1853–1857.
41. **Randall, R. E., D. F. Young, K. K. Goswami, and W. C. Russell.** 1987. Isolation and characterization of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. J. Gen. Virol. **68:**2769–2780.
42. **Rose, J. K., L. Buonocore, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechnology **10:**520–525.
43. **Russell, R., R. G. Paterson, and R. A. Lamb.** 1994. Studies with cross-linking reagents on the oligomeric form of the paramyxovirus fusion protein. Virology **199:**160–168.
44. **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.
45. **Sarkar, D. P., S. J. Morris, O. Eidelman, J. Zimmerberg, and R. Blumenthal.** 1989. Initial stages of influenza hemagglutinin-induced cell fusion monitored simultaneously by two fluorescent events: cytoplasmic continuity and lipid mixing. J. Cell Biol. **109:**113–122.
46. **Scheid, A., L. A. Caliguiri, R. W. Compans, and P. W. Choppin.** 1972. Isolation of paramyxovirus glycoproteins. Association of both hemagglutinating and neuraminidase activities with the larger SV5 glycoprotein. Virology **50:**640–652.
47. **Scheid, A., and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57:**475–490.
48. **Scheid, A., and P. W. Choppin.** 1974. The hemagglutinating and neuraminidase protein of a paramyxovirus: interactions with neuraminic acid in affinity chromatography. Virology **62:**125–133.
49. **Sechoy, O., M. Vidal, J. R. Phillippot, and A. Bienvenue.** 1989. Interactions of human lymphoblasts with targeted vesicles containing Sendai virus envelope proteins. Exp. Cell Res. **185:**122–131.
50. **Sergel, T., L. W. McGinnes, M. E. Peeples, and T. G. Morrison.** 1993. The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. Virology **193:**717–726.
51. **Simpson, D. A., and R. A. Lamb.** 1992. Alterations to influenza virus hemagglutinin cytoplasmic tail modulate virus infectivity. J. Virol. **66:**790–803.
52. **Tanabayashi, K., K. Takeuchi, K. Okazaki, M. Hishiyama, and A. Yamada.** 1992. Expression of mumps virus glycoproteins in mammalian cells from cloned cDNAs: both F and HN proteins are required for cell fusion. Virology **187:**801–804.
53. **Taylor, J., S. Pincus, J. Tartaglia, C. Richardson, G. Alkhatib, D. Briedis, M. Appel, E. Norton, and E. Paoletti.** 1991. Vaccinia virus recombinants expressing either the measles virus fusion or hemagglutinin glycoprotein protect dogs against canine distemper virus challenge. J. Virol. **65:**4263–4274.
54. **Ward, C. D., R. G. Paterson, and R. A. Lamb.** 1995. Mutants of the paramyxovirus SV5 fusion protein: regulated and extensive syncytium formation. Virology **209:**242–249.
55. **Wild, T. F., E. Malvoisin, and R. Buckland.** 1991. Measles virus: both the haemagglutinin and fusion glycoproteins are required for fusion. J. Gen. Virol. **72:**439–442.


A
B
C
D