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Expression at the cell surface of biologically active fusion and hemagglutinin/neuraminidase proteins of the paramyxovirus simian virus 5 from cloned cDNA

(parainfluenza viruses/eukaryotic vectors/glycoproteins/membrane fusion)

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ABSTRACT **cDNAs encoding the mRNAs for the fusion protein (F) and the hemagglutinin/neuraminidase protein (HN) of the paramyxovirus simian virus 5 have been inserted into a eukaryotic expression vector under the control of the simian virus 40 late promoter. The F and HN proteins synthesized in recombinant infected cells are indistinguishable in terms of electrophoretic mobility and glycosylation from the proteins synthesized in simian virus 5-infected cells. In addition, the expressed F and HN proteins have been shown to be anchored in the plasma membrane in a biologically active form by indirect live cell immunofluorescence, the F-mediated formation of syncytia, and the ability of HN to cause the hemadsorption of erythrocytes to the infected cell surface.**

Simian virus 5 (SV5) is a prototype of the paramyxovirus family of negative-strand RNA viruses that are known for their ability to cause cell fusion. The envelope of the SV5 virion consists of a membrane with a nonglycosylated protein [matrix (M)] associated with its inner surface and two integral membrane proteins [hemagglutinin/neuraminidase and fusion (HN and F)] that form spike-like projections on the outer surface (1). Extensive studies utilizing purified preparations of biologically active F and HN have shown that the F protein is involved in virus penetration, hemolysis, and cell fusion, whereas HN has both hemagglutinating and neuraminidase activities (2–5).

The F glycoprotein is synthesized as an inactive precursor (F_0) that is cleaved by a host cell protease to form the biologically active protein consisting of the disulfide-linked chains F_1 and F_2 (3, 6–9). Cleavage of F_0 results in the release of the NH_2 terminus of F_1 and in the case of Sendai virus this has been shown to be concurrent with a conformational change in the molecule and the exposure of new hydrophobic regions (10). Direct amino acid sequencing of the NH_2 terminus of F_1 has shown that it is very hydrophobic and that this region is highly conserved between three paramyxoviruses (SV5, Sendai virus, and Newcastle disease virus). It has been suggested that this region of the F protein is directly involved in cell fusion (11–13), and, although this suggestion is supported by the finding that oligopeptides that mimic the NH_2 terminus of F_1 are capable of inhibiting virus penetration and cell fusion (13, 14), it remains to be shown that the NH_2 terminus of F_1 mediates fusion by interacting with adjacent membranes. The complete amino acid sequence deduced from the nucleotide sequence of the F gene from three paramyxoviruses (SV5, Sendai virus, and respiratory syncytial virus) (15–17) indicates that F is extensively hydrophobic over the length of the polypeptide chain. In addition to the very hydrophobic NH_2 terminus of F_1, two other regions of the F polypeptide are especially hydrophobic: these are the NH_2-terminal signal peptide and the COOH-terminal membrane anchorage domain.

The paramyxovirus HN protein has both hemagglutinating and neuraminidase activities, in contrast to influenza virus where these activities reside on separate glycoproteins. However, the available evidence does not resolve the question as to whether HN has one or two active sites for the hemagglutinating and neuraminidase activities (4, 18).

The predicted amino acid sequence of the SV5 and Sendai virus HN proteins has been derived from the nucleotide sequence of the HN genes (19, 20). There is only a single region in the protein located near the NH_2 terminus that is sufficiently hydrophobic and of appropriate length to anchor HN in membranes. Thus, it has been suggested that HN, like the neuraminidase of influenza virus (21, 22), contains an extended signal sequence that is involved in the translocation of HN across membranes and functions as an NH_2-terminal membrane anchorage domain.

In this paper we report the expression from cloned cDNAs of biologically active SV5 F and HN proteins in eukaryotic cells. This provides a means of examining the synthesis of the F and HN proteins in the absence of the other SV5 specific proteins. In addition, it provides a system in which to investigate, among other things, (*i*) the role of regions of the F protein in the process of membrane fusion, (*ii*) the structure–function relationship of different domains of the HN protein with respect to hemagglutination and neuraminidase activity, and (*iii*) the sequences involved in the sorting and transport of these integral membrane proteins.

MATERIALS AND METHODS

Cells. Monolayer cultures of the Madin–Darby bovine kidney line of cells and the TC7 clone of CV1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Construction of the Simian Virus 40 (SV40) F and HN Recombinant Viruses. To obtain F DNA suitable for expression, clone Fc (15) was modified, as it contains 106 nucleotides of the SV5 M gene and 37 poly(A)-residues in addition to a complete cDNA copy of the F mRNA. Fc DNA was excised from the plasmid by partial *Pst* I digestion, treated with exonuclease BAL-31, and blunt-ended with T4 DNA polymerase, *Xho* I linkers were added, and the trimmed Fc DNA was cloned into a derivative of pBR322 containing an *Xho* I site at nucleotide 375. A clone containing 12 adenosine residues, the 22 intergenic nucleotides between M and F, and the complete F mRNA sequence was obtained.

HN DNA was derived from clone HN177 (19). The HN177 DNA insert was excised from the plasmid by partial *Pst* I

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Abbreviations: SV5, simian virus 5; F, fusion protein; HN, hemagglutinin/neuraminidase protein; M, matrix protein; SV40, simian virus 40.

digestion, and the SH region (23), which maps before the position of the 5′ end of the HN mRNA, was removed by digestion with *Nae* I, which cleaves the DNA at a unique site within the 5′ noncoding region of the HN mRNA. The large HN DNA fragment was isolated and *Xho* I linkers were added.

The SV40 late-region replacement vector pSV103 was adapted from pSV-2330 (24) and is constructed such that foreign cDNA can be inserted under the control of the SV40 late promoter and polyadenylylation signals via an *Xho* I site, which replaces the late region between the unique *Hae* II and *Bam*HI sites. The pSV103 vector also contains a 183-nucleotide deletion (SV nucleotides 336–519) spanning the agno protein initiation codon (25). The SV40 sequences in pSV103 are linked by means of a *Sac* I site (replacing the unique *Taq* I site at SV nucleotide 4739) to a pBR322 derivative in which a *Sac* I site replaces the region from *Eco*RI to *Bam*HI.

The modified F and HN DNAs were inserted into the *Xho* I site of pSV103 (Fig. 1), and recombinant molecules containing the F and HN DNAs in both the mRNA (SV-Fm, SV-HNm) and viral RNA (SV-Fv, SV-HNv) sense, with respect to SV40 late-region transcription, were isolated.

The SV-F and SV-HN DNAs were released from the bacterial plasmid sequences by *Sac* I digestion and the DNAs were circularized by using T4 DNA ligase. Four micrograms of total *Sac* I-digested DNA and 4 μg of an SV40 early-region deletion mutant (dl1055) to act as helper virus (26) were introduced into 2×10^7 CV1 cells by DEAE-dextran-mediated transfection (27). After 10 days, virus stocks were made as described (28).

Radioisotopic Labeling of Polypeptides in Infected Cells, Immunoprecipitation, and Polyacrylamide Gel Electrophoresis. The W3 strain of SV5 was grown in Madin–Darby bovine kidney cells as described (8). Monolayer cultures of CV1 cells were used for all biochemical experiments. SV5-infected cells were labeled at 15–17 hr after infection with 75 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml in methionine-free DMEM for 2 hr or with 250 μCi of [^{3}H]glucosamine per ml in 80% phosphate-buffered saline (P_i/NaCl)/20% DMEM for 2 hr with a 1-hr chase in DMEM following the glucosamine labeling period.

Cells infected with the SV40 F and HN recombinant viruses (SV-F and SV-HN) were labeled at 40–48 hr after infection with 250 μCi of [^{35}S]methionine per ml in methionine-free DMEM for 3–4 hr or with 250 μCi of [^{3}H]glucosamine per ml in 80% P_i/NaCl/20% DMEM for 3 hr with a chase as described above. Tunicamycin was added to cells at 1.0 μg/ml 2 hr prior to labeling with [^{35}S]methionine as described above. Immunoprecipitation was carried out as described (29) by using monospecific antisera to purified SV5 F and HN proteins (30, 31). Samples were prepared for electrophoresis and analyzed on 15% polyacrylamide gels (29).

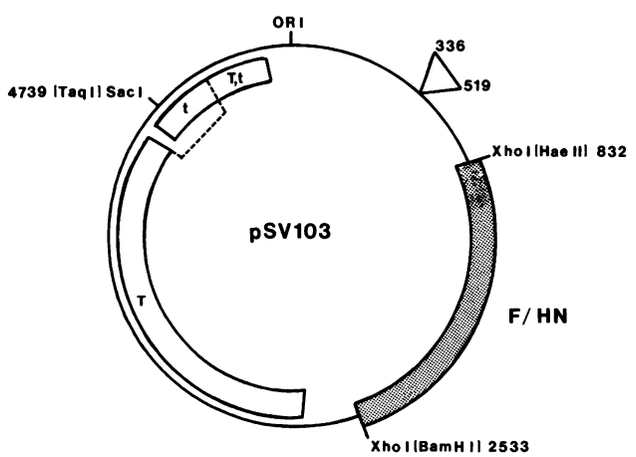

FIG. 1. Schematic diagram of the pSV103 F or HN vector. The stippled bar in pSV103 represents either the SV5 F or HN DNA and the open bars represent the mRNAs for the SV40 large and small T antigens with the broken line defining the region spliced out of the mRNA for large T antigen. ORI, origin.

Indirect Immunofluorescence. Indirect immunofluorescence on live cells was done as described (32) by using monospecific antibodies to the purified SV5 F and HN proteins (30, 31). Antisera were absorbed by incubation with uninfected CV1 cells prior to use in immunofluorescence.

Syncytium Formation and Hemadsorption. Monolayers of CV1 cells were infected with first-passage lytic stocks of SV-F. Virus was absorbed for 3 hr at 37°C, and the inoculum was removed and replaced with DMEM supplemented with 2% fetal calf serum. Syncytia were photographed at 3–4 days after infection with a Nikon Diaphot-TMD inverted microscope and Kodak Plus-X-pan film.

CV1 cells were infected with first-passage lytic stocks of SV-HN as described above. At 48 hr after infection cells were washed several times with P_i/NaCl, incubated with fowl erythrocytes (0.5% in P_i/NaCl) for 30 min at 4°C, washed thoroughly with P_i/NaCl, and photographed as described above.

RESULTS

Expression of the F and HN DNAs in CV1 Cells. The SV40 F and HN recombinant DNAs together with DNA from an SV40 early-region deletion mutant were introduced into CV1 cells by DEAE-dextran-mediated transfection (27) and virus stocks were made. CV1 cells were infected with first-passage lytic stocks of SV-Fm (mRNA sense) and SV-Fv (viral RNA sense) and labeled at 40–48 hr after infection with either [^{35}S]methionine or [^{3}H]glucosamine. Proteins were immunoprecipitated by using anti-F IgG and analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 2, the SV-Fm vector synthesized F polypeptides (lane 3) indistinguishable in electrophoretic mobility from the uncleaved precursor protein F_0 ($M_r \approx 66{,}000$) and the cleavage products F_1 ($M_r \approx 52{,}000$) and F_2 ($M_r \approx 14{,}000$) detected in SV5-infected cells (lane 2). These polypeptides were not precipitated from either mock-infected (lane 1) or SV-Fv-infected CV1 cells (lane 4). A greater accumulation of cleaved F_1 and F_2 relative to F_0 is observed in the SV-Fm-infected cells compared with SV5-infected cells. This is due to the longer labeling period used for the SV-Fm-infected cells and does not reflect a change in the efficiency of cleavage of F_0. It can be seen in lane 2 that under the conditions used in these experiments the anti-F IgG precipitates some HN from SV5-infected cell lysates, but this does not affect the analysis shown in lane 3 or in the experiments described below.

To determine whether the F protein synthesized in SV-Fm-infected cells is glycosylated, infected cells were labeled with [^{3}H]glucosamine. As shown in Fig. 2, lane 7, the F_0, F_1, and F_2 polypeptides precipitated from SV-Fm-infected cells could not be distinguished on the basis of their electrophoretic mobility from those precipitated from [^{3}H]glucosamine-labeled SV5-infected cells (lane 6), suggesting that the SV-Fm F polypeptides are glycosylated normally. Further evidence that the F protein expressed by the SV-F recombinant virus is correctly glycosylated was obtained by labeling SV-Fm-infected CV1 cells with [^{35}S]methionine in the presence of tunicamycin, an inhibitor of N-linked glycosylation. In the presence of tunicamycin, F_0, F_1, and F_2 could no longer be detected and a new protein of greater electrophoretic mobility than F_0 and F_1 was observed in both SV5- and SV-Fm-infected cells (Fig. 3, lanes 6 and 7). This protein has been identified previously as the unglycosylated F protein (33).

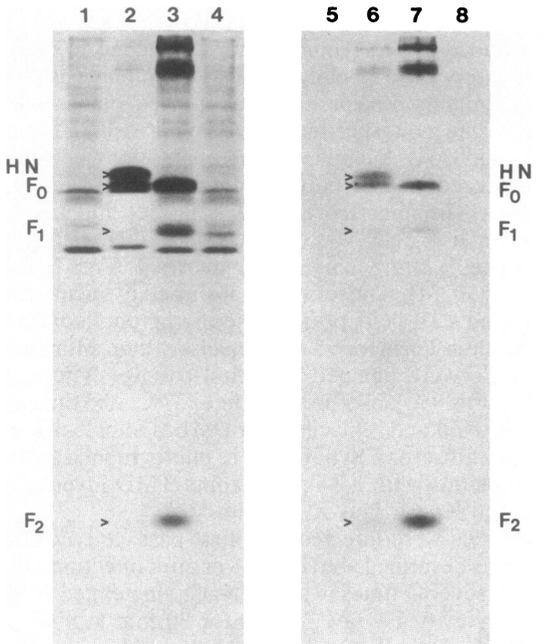

FIG. 2. Comparison of the SV5 F polypeptides synthesized in SV5-infected or SV-F-infected CV1 cells. Cells were infected with the recombinant SV-F virus and SV dl1055 such that more than one in eight cells were productively infected: the SV5 was used at 20 plaque-forming units per cell. Infected cells were labeled with either [^{35}S]methionine (lanes 1–4) or [^{3}H]glucosamine (lanes 5–8), immunoprecipitated with anti-F-specific IgG, and analyzed on 15% polyacrylamide gels. Lanes 1 and 5, uninfected CV1 cells; lanes 2 and 6, SV5-infected cells; lanes 3 and 7, SV-Fm-infected cells; lanes 4 and 8, SV-Fv-infected cells. F_0, F_1, F_2, and HN polypeptides are indicated by arrowheads. The relative levels of F expressed in the vector- and SV5-infected cells have not been rigorously compared. However, the intensity of bands on autoradiographs indicates that during a 3-hr labeling period the accumulation of F in SV-Fm- and SV5-infected cells is approximately equivalent. The anti-F IgG under the conditions used precipitates some HN, as seen in lanes 2 and 6. However, this does not affect the analysis shown here or in Fig. 3, lanes 2 and 6. The F-specific high molecular weight species immunoprecipitated from the SV5- and SV-Fm-infected cells are aggregates of F.

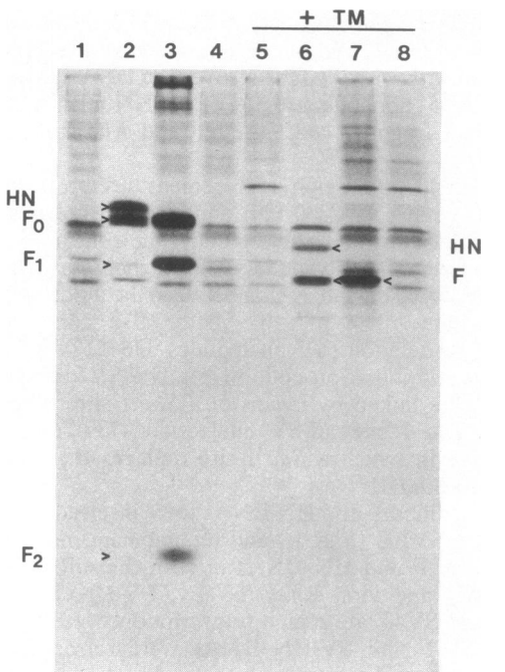

FIG. 3. Immunoprecipitation of F synthesized in the presence of tunicamycin (TM). CV1 cells were infected with SV5 or SV-F, labeled with [^{35}S]methionine, and immunoprecipitated with anti-F IgG. Lanes 1–4 show control samples and lanes 5–8 show polypeptides synthesized in cells treated with 1 μg of tunicamycin per ml. Lanes 1 and 5, uninfected CV1 cells; lanes 2 and 6, SV5-infected cells; lanes 3 and 7, SV-Fm-infected cells; lanes 4 and 8, SV-Fv-infected cells. F_0, F_1, F_2, and HN polypeptides are indicated by arrowheads.

In SV5-infected cells, the F protein was found in the cleaved form (disulfide-linked F_1 and F_2) even in the absence of exogenous proteases (i.e., serum proteases). To test if the vector-synthesized F was cleaved in the absence of serum CV1 cells were infected with SV-Fm. At 40–48 hr after infection the cells were washed extensively with P_i/NaCl and labeled with [^{35}S]methionine, and polypeptides were immunoprecipitated with anti-F IgG. The expressed F protein was found to be cleaved as efficiently in the absence of serum as it is when serum is present (data not shown).

To examine the expression of HN, monolayers of CV1 cells were infected with first-passage lytic stocks of SV-HNm (mRNA sense) and SV-HNv (viral RNA sense) and labeled with [^{35}S]methionine. Proteins were immunoprecipitated with anti-HN IgG (30, 31) and analyzed by polyacrylamide gel electrophoresis (Fig. 4). A major protein ($M_r \approx 70{,}000$) was detected in CV1 cells infected with SV-HNm (lane 5) that was indistinguishable in electrophoretic mobility from the HN synthesized in SV5-infected cells (lane 1) and was not observed in cells infected with SV-HNv where the HN coding sequence is present in the viral RNA orientation (lane 3). The HN proteins synthesized in SV5 and SV-HNm recombinant virus-infected cells were similarly glycosylated, as shown by an identical increase in the electrophoretic mobility of the HN proteins when infected cells were labeled in the presence of tunicamycin (lanes 2 and 6).

The Expressed F and HN Proteins Are Transported to the Cell Surface. To demonstrate that the expressed F and HN proteins are transported to the cell surface, indirect immunofluorescence was performed on live CV1 cells 18 hr after infection with SV5 or 48 hr after infection with the SV40 F and HN recombinant viruses (Fig. 5). Surface fluorescence similar to that observed with SV5-infected cells (Fig. 5 *A* and

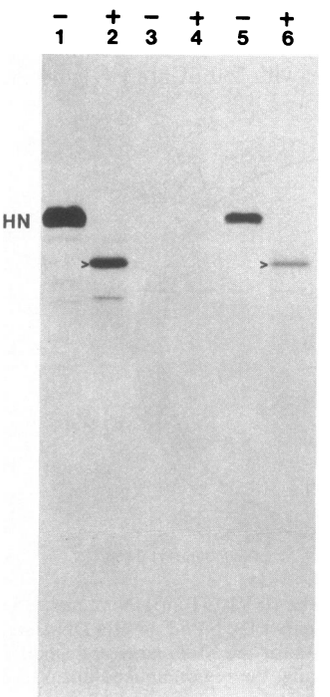

FIG. 4. Synthesis of HN in SV5- and SV-HN-infected CV1 cells in the presence (+) or absence (–) of tunicamycin. Infected cells were labeled with [^{35}S]methionine in the absence or presence of 1 μg of tunicamycin per ml, immunoprecipitated with anti-HN-specific IgG, and analyzed on 15% polyacrylamide gels. Lanes 1 and 2, SV5-infected cells; lanes 3 and 4, SV-HNv-infected cells; lanes 5 and 6, SV-HNm-infected cells. The unglycosylated HN protein synthesized in the presence of tunicamycin is indicated by arrowheads.

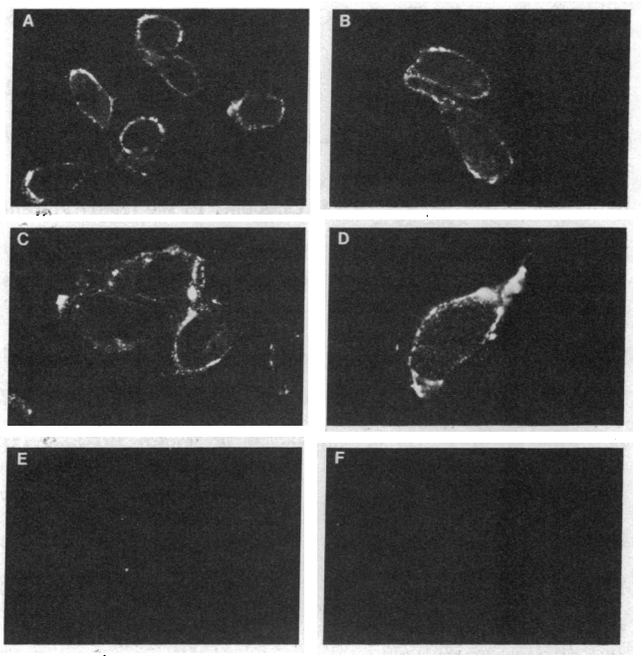

FIG. 5. Indirect immunofluorescent staining of F and HN expressed at the cell surface. Infected CV1 cells were incubated sequentially with P_i/NaCl containing 1% bovine serum albumin, either anti-F- or anti-HN-specific IgG, and fluorescein-conjugated goat anti-rabbit IgG. Immunofluorescence was carried out with a Zeiss Photomicroscope III. (*A*) SV5-infected cells stained with rabbit anti-F-specific IgG. (*B*) SV5-infected cells stained with rabbit anti-HN-specific IgG. (*C*) SV-Fm-infected cells stained with rabbit anti-F IgG. (*D*) SV-HNm-infected cells stained with rabbit anti-HN IgG. (*E*) SV-Fv-infected cells stained with rabbit anti-F IgG. (*F*) SV-HNv-infected cells stained with rabbit anti-HN IgG. Exposure times for *E* and *F* were manually adjusted to be the same as those for *C* and *D*, respectively.

B) was detected with cells infected with either SV-Fm or SV-HNm (Fig. 5 *C* and *D*) but not with SV-Fv- or SV-HNv-infected cells (Fig. 5 *E* and *F*).

The biological activity of the F protein expressed at the cell surface of SV-Fm-infected CV1 cells was determined by examining the cells for syncytium formation. Polykaryons were first detected 2 days after infection and syncytia were photographed after 3–4 days (Fig. 6*A*). Polykaryons continued to increase in size until cell death occurred. Syncytium formation was not observed in cells infected with SV-Fv (viral RNA sense) (Fig. 6*B*). The number of syncytia observed in SV-Fm-infected monolayers was considerably less than the number of cells expressing F on the surface, as detected by immunofluorescence. It is possible that there is a minimum number of F molecules required for fusion to occur, and therefore only cells expressing large amounts of F on their surface can induce fusion.

To examine whether the cell surface-expressed HN protein is biologically active the ability of SV-HN-infected cells to bind fowl erythrocytes to their surface was assayed. As shown in Fig. 6*C*, foci of hemadsorption were observed in monolayers of CV1 cells infected with SV-HNm. Cells infected with SV-HNv were unable to bind erythrocytes (Fig. 6*D*).

DISCUSSION

The SV5 F and HN glycoproteins have been expressed from cDNA under the control of the SV40 late promoter and cannot be distinguished either in terms of their electrophoretic mobility, glycosylation, cell surface expression, or biological activity from the proteins synthesized during a SV5 infection.

The paramyxovirus F protein is synthesized as an inactive precursor (F_0) that has to be cleaved to produce the active form of the protein consisting of the disulfide-linked subunits F_1 and F_2 (3, 6–9). On analysis of the proteins synthesized in CV1 cells infected with a SV40 recombinant virus containing cDNA encoding the F protein, in the mRNA sense with respect to the SV40 late promoter, F_0, F_1, and F_2 are all detected even in the absence of exogenous proteases—i.e, serum protease. This indicates that cleavage of the SV5 F protein is dependent on neither the presence of other SV5-specific proteins nor changes induced in the host cell as a result of infection with SV5 but that cleavage is a function of endogenous host cell proteases and the fusion protein itself. The SV5 F protein cleavage site consists of five arginine

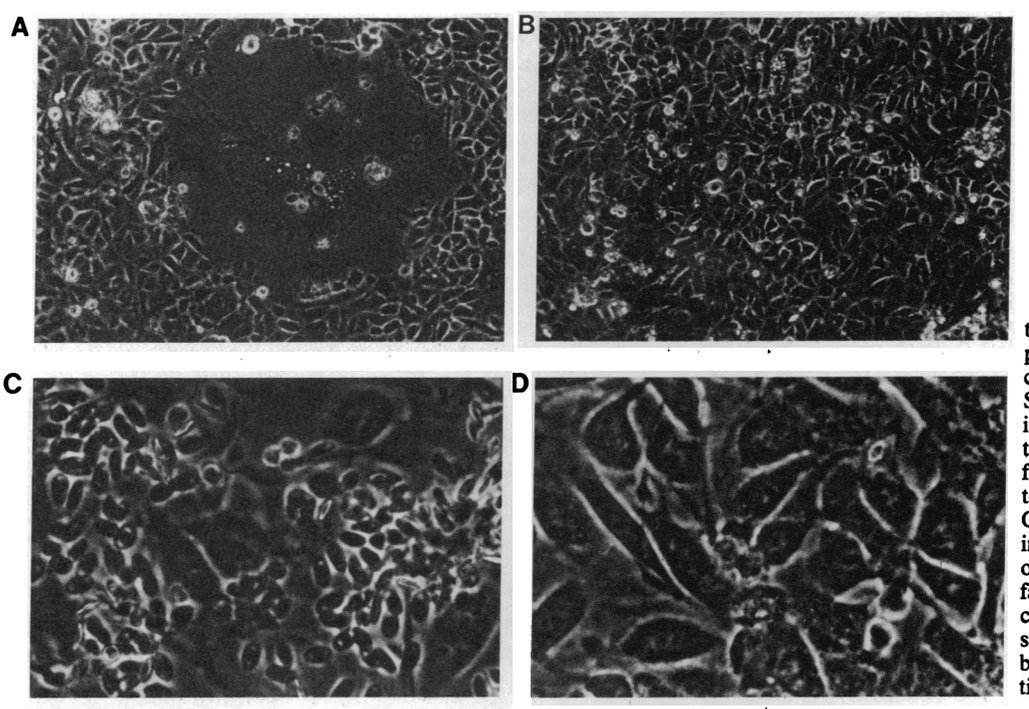

FIG. 6. Functional assays for the biological activity of the expressed F and HN proteins. CV1 cells were infected with SV-F and SV-HN and the biological activity of the surface-expressed proteins was assayed. (*A*) Cell–cell fusion induced by the SV5 F protein expressed in SV-Fm-infected CV1 cells. (*B*) Control CV1 cells infected with SV-Fv. (*C*) Binding of fowl erythrocytes to the surface of SV-HNm-infected CV1 cells. (*D*) SV-HNv-infected cells showing the lack of erythrocyte binding in the absence of functional HN protein.

residues (15) and this long basic connecting peptide is thought to be responsible for the observation that the SV5 F protein is "highly cleavable"—i.e., it is always cleaved when SV5 is grown in tissue culture. The possibility that the five-arginine connecting peptide is important for the cleavage of the SV5 F protein is supported by two lines of evidence. Studies involving the influenza A virus hemagglutinin have shown that highly cleavable forms of the protein have a long basic connecting peptide at the cleavage site, whereas hemagglutinins that require the addition of exogenous trypsin in order to be cleaved are found to have only a single arginine or lysine residue (34, 35). Also, the Sendai virus F protein, which is rarely found in the cleaved form when the virus is grown in tissue culture cells in the absence of serum, recently has been shown to have a single arginine residue at the cleavage site (17, 36).

The vector-expressed F and HN proteins are transported to the cell surface, as shown by live cell indirect immunofluorescence and by the assays for biological activity—i.e., cell fusion and hemadsorption. Whereas F expressed by the vector-infected cells can mediate the fusion of adjacent plasma membranes, reconstituted vesicles containing F only cause cell fusion if a means of attachment of the vesicles to the cell is provided—e.g., the viral HN protein or a lectin such as wheat germ agglutinin (5). Previously it has been suggested, from studies on the cell surface expression of the Sendai virus HN protein in persistently infected cells and cells infected with mixtures of wild-type virus and defective interfering particles, that the paramyxovirus M protein is required for the anchorage of the HN protein in the plasma membrane (37, 38). Our observation that SV-HN expresses HN at the cell surface in a biologically active form capable of causing hemadsorption would indicate that the M protein is not a prerequisite for SV5 HN surface expression.

Membrane fusion is of major importance in cell biology as it is involved in processes such as endocytosis, secretion, myogenesis, and fertilization. However, the mechanisms involved are not understood. Several viral glycoproteins can mediate membrane fusion but only the paramyxovirus F protein is capable of causing cell fusion at neutral pH. The rhabdovirus G protein, influenza hemagglutinin, and the togavirus E_1 and E_2 proteins all require acid pH for fusion activity *in vitro* (reviewed in ref. 39). Direct evidence for the involvement of the influenza virus, togavirus, and rhabdovirus glycoproteins in membrane fusion has been obtained by the expression in eukaryotic cells of cDNAs encoding the proteins (40–43). In each case the glycoprotein promoted membrane fusion if exposed to acid pH. We have shown that the SV5 F protein expressed from cloned cDNA can also cause cell–cell fusion, which occurs without exposure to acid pH. Therefore, this system will provide a means of studying the events involved in protein-mediated plasma membrane fusion at physiological pH.

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