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Biological Activity of Paramyxovirus Fusion Proteins: Factors Influencing Formation of Syncytia

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The fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins of the paramyxovirus simian virus 5 (SV5) were expressed individually or coexpressed in CV-1 cells by using SV40-based vectors and recombinant vaccinia viruses. The extent of detectable fusion in a syncytium formation assay was found to be affected by the expression system used. In addition, when HN was coexpressed with F, it was found that the expression vector system influenced the contribution of HN in forming syncytia. The abilities of the SV5, human parainfluenza virus type 3, and Newcastle disease virus F glycoproteins to cause fusion, when expressed alone or coexpressed with HN, were directly compared by using the SV40-based vector system in CV-1 cells. The F proteins exhibited various degrees of fusion activity independent of HN expression, but the formation of syncytia could be enhanced to different extents by the coexpression of the homotypic HN protein.

The paramyxoviruses contain two surface spike glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion protein (F) (reviewed in reference 21). The HN protein mediates attachment of virions to sialic acid-containing receptor molecules and also has receptor-destroying (neuraminidase) activity. The F protein functions in penetration of virus into the host cell by mediating fusion of the virion envelope with the cellular plasma membrane. In addition, in paramyxovirus-infected cells, the F protein is involved in the induction of cell fusion and the formation of syncytia (reviewed in reference 41). However, the ability of a specific paramyxovirus infection to cause syncytia also depends on the lipid composition of the target membrane (3, 10, 14, 29).

The earliest work implicating the F protein in fusion activity came from the finding that the protein is synthesized as an inactive precursor F_0, which has to be activated by cleavage with a host protease to form the biologically active disulfide-linked heterodimer, F_1-F_2 (11, 33–35). To obtain biochemical evidence that the F protein of Sendai virus is the active molecule in fusion, it was reconstituted into lipid vesicles in the absence of HN. It was found that the reconstituted F protein mediated hemolysis of erythrocytes provided that a lectin, wheat germ agglutinin, was added to the vesicles. These findings suggested that the F protein is functional in mediating fusion but that the liposomes and target cell have to be brought into close proximity, with the lectin substituting for the attachment function of HN (13). Further studies on the reconstitution of the Sendai virus F protein into vesicles also indicated that F was biologically active (36), and studies using Sendai virus mutant *ts*271 virions that lack HN indicated that these virions can fuse with cardiolipin liposomes (7).

Perhaps the most direct evidence that a paramyxovirus F protein, in the absence of other viral proteins, can mediate membrane fusion was provided by the finding that expression of the simian virus 5 (SV5) F cDNA in CV-1 cells by using an SV40-derived vector caused syncytium formation, although it was observed that many more cells expressed the SV5 F protein at the cell surface than were involved in syncytium formation (12, 26, 28). Recently it was found that when glycine-to-alanine changes were introduced into the F_1 N-terminal fusion peptide domain of the SV5 F protein, alterations which have the propensity to cause an increase in the α-helical nature of a peptide, a very large increase in fusion activity was observed (12). These findings provide further evidence that the SV5 F protein is biologically active as the fusogen without expression of HN. Infection of CV-1 cells with recombinant SV40 does not cause an obvious cytopathic effect of cell rounding until >70 h postinfection (p.i.), and this is probably an important factor in the successful formation of syncytia. When the SV5 F protein was expressed in cells by using a recombinant vaccinia virus, which causes a severe cytopathic effect in CV-1 cells, fusion could be detected only when the F-expressing cells were overlaid with uninfected cells (27). In addition to finding biological activity with the SV5 F protein, syncytium formation was observed in 293 cells when the measles virus F protein cDNA was expressed by using an adenovirus vector (1).

In contrast to the data discussed above, other reports have indicated that expression of both the F and HN proteins of paramyxoviruses is required for syncytium formation. Several reports using reconstituted systems have indicated that HN is required with the F protein for fusion because, in contrast to the data obtained by Hsu and coworkers (13), when F was reconstituted into vesicles, hemolysis of added erythrocytes was not observed unless HN was coreconstituted, and in these studies it was found that lectins could not substitute for HN (4, 23, 24). It was also found that some monoclonal antibodies to Sendai virus and mumps virus HN, while permitting hemagglutination to occur, inhibited viral fusion activity, but possible complications due to antibody steric hindrance could not be ruled out (19, 29, 39). With mumps virus, neuraminidase activity was found to modulate fusion, as there was an inverse correlation between levels of neuraminidase activity and the ability of the virus to cause fusion, and inhibitors of neuraminidase activity caused an increase in fusion activity (18, 40). In cells persistently infected with human parainfluenza virus type 3 (HPIV3), it

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has been found that although the cells express F and HN, they are resistant to fusion with each other but readily cause fusion when overlayed with uninfected cells. However, when the overlay cells were desialated by neuraminidase treatment, no fusion was detected, and the addition of lectins to agglutinate the desialated cells did not substitute for HN binding to sialic acid (22). The simplest interpretation of these data for mumps virus and HPIV3 is that in the cell lines used, HN binding to sialic acid receptors is necessary for the fusion process to occur.

When the F and HN (H) glycoproteins of HPIV3, bovine parainfluenza virus type 3, measles virus, and canine distemper virus were expressed from cDNAs by using recombinant vaccinia viruses, it was found that expression of F alone did not cause detectable syncytium formation, whereas when F and HN (H) were coexpressed, extensive cell-cell fusion occurred (5, 14a, 31, 38, 42). Interestingly, Morrison and coworkers (20) reported that when the Newcastle disease virus (NDV) F protein was expressed in chicken embryo fibroblasts by using a retrovirus vector, which seemingly causes little cytopathic effect, no fusion occurred unless NDV HN was expressed in the same cells as was the NDV F protein, and that another virus attachment protein, influenza virus hemagglutinin, could not substitute for NDV HN.

It seemed possible that the differing results obtained with expression of the F and HN proteins of paramyxoviruses might reflect differences in the experimental assay systems (e.g., vectors or cell lines) as opposed to involving distinctly different mechanistic requirements for the fusion process. As discussed above, the SV40 and adenovirus vectors do not cause a severe cytopathic effect within the time course of the experiment, whereas vaccinia virus-based expression systems usually lead to rapid cell rounding and cell death. This latter phenomenon might explain the requirements for HN in fusion; i.e., HN may act as an adhesion molecule ensuring appropriate membrane juxtaposition. We have expressed the SV5, HPIV-3, and NDV F and HN proteins from cDNAs, either alone or in matched pairs by using a constant expression system, of CV-1 cells and an SV40-based vector system. In addition, we have expressed the SV5 F and HN proteins alone or together in CV-1 cells by using recombinant vaccinia viruses.

CV-1 cells were grown as described previously (25), and the recombinant SV40 vectors pSV103-F and pSV103-HN and recombinant vaccinia viruses expressing SV5 F (vac-F) and HN (vac-HN) cDNAs were those used previously (26–28). cDNAs encoding HPIV3 F and HN (6) were kindly provided by Mark Galinsky, Cleveland Clinic Foundation, Cleveland, Ohio, and were subcloned for expression into pSV123 (an SV40-based shuttle vector almost identical to pSV103 except for a *Cla*I restriction endonuclease site linking the bacterial sequences to the SV40 sequences) by

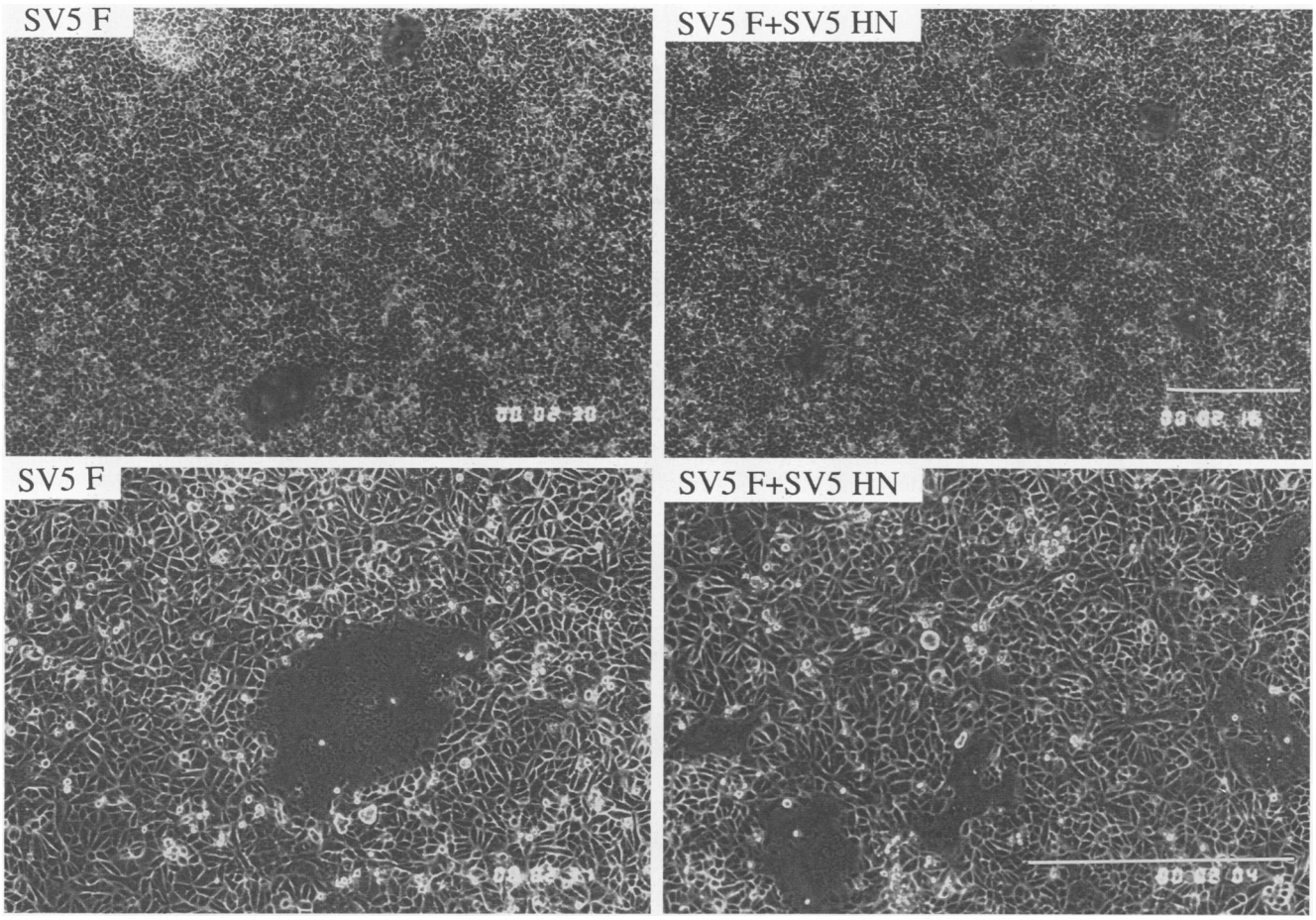

FIG. 1. Expression of the SV5 F and HN proteins in CV-1 cells by using a recombinant SV40-based vector. CV-1 monolayers were transfected with pSV103 F or pSV103 HN DNA or both DNAs. Monolayers were photographed at 5 to 7 days posttransfection. Bar = 500 μm.

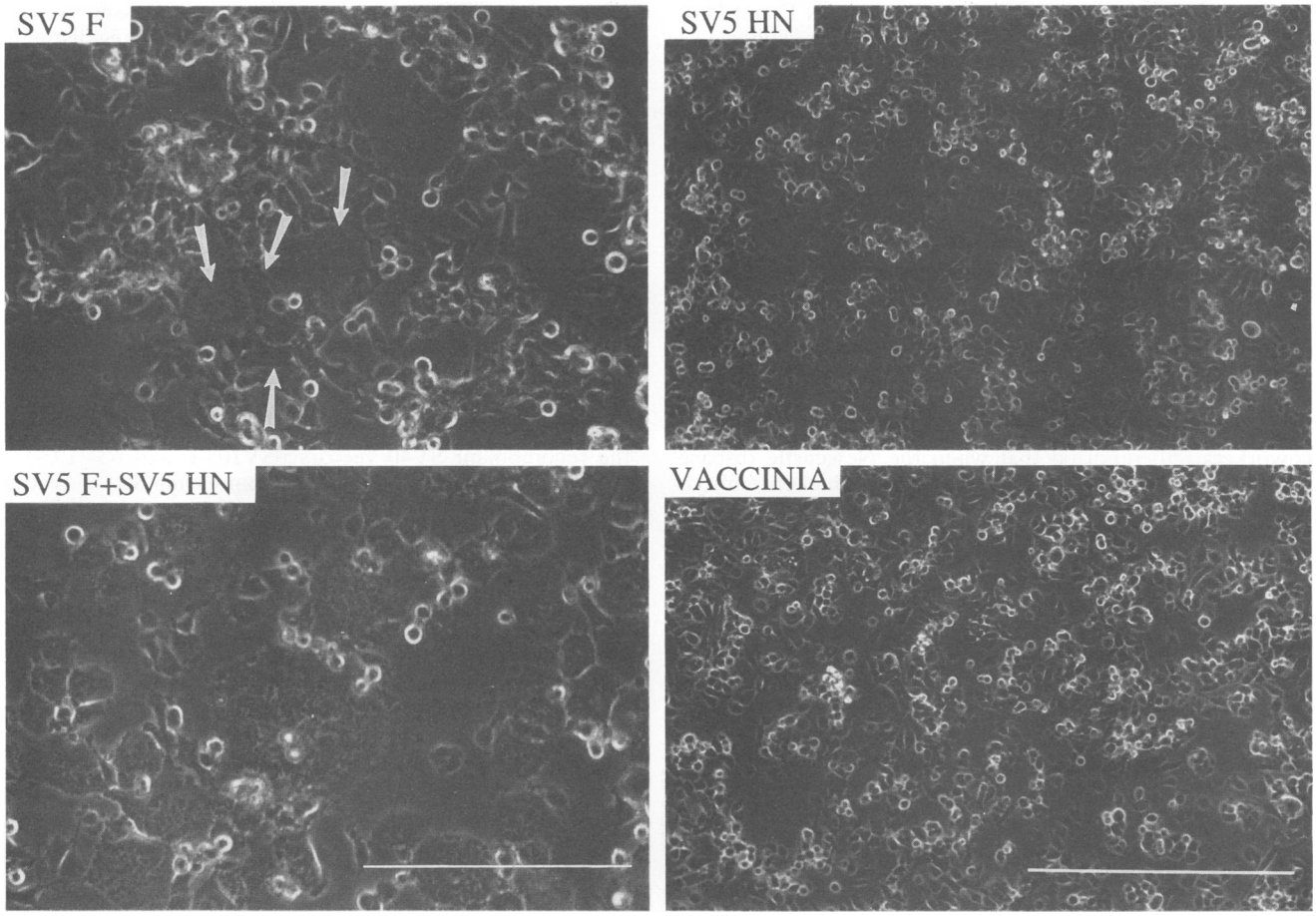

FIG. 2. Expression of the SV5 F and HN proteins in CV-1 cells by using a recombinant vaccinia virus. CV-1 monolayers were infected with recombinant vaccinia virus F (SV5 F) or recombinant vaccinia virus HN (SV5 HN) or were coinfected with both (SV5 F+SV5 HN) viruses. At 8 h p.i., infected cells were overlayed with an equal number of freshly EDTA-dispersed uninfected CV-1 cells. Monolayers were photographed at 16 h p.i. Control cells were infected with wild-type vaccinia virus (vaccinia). For panels, SV5 F and SV5 F+SV5 HN, bar = 250 μm; for panels, SV5 HN and vaccinia, bar = 500 μm. In panel SV5 F, arrows indicate multinucleated cells.

standard recombinant DNA procedures (32) to create SV40-HPIV3 F and SV40-HPIV3 HN. For the synthesis of cDNAs to NDV F and HN, CV-1 cells were infected with NDV (strain Australia-Victoria, kindly provided by Ron Iorio, University of Massachusetts, Worcester), and mRNAs were isolated as described previously (25). First-strand DNA synthesis was done by using avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Tampa, Fla.) and priming on mRNA with F- or HN-specific oligonucleotides based on the NDV F and HN nucleotide sequences (16, 17). This procedure was followed by a polymerase chain reaction amplification of the F- and HN-specific cDNAs as described previously (18), using additional F- and HN-specific oligonucleotides. cDNAs for NDV F and HN were cloned and isolated by standard procedures (32). The nucleotide sequence of the F gene was obtained, and the deduced F protein sequence matched that reported previously (16) with the exception of finding an encoded asparagine at residue 162 in place of an isoleucine residue. The corresponding nucleotide difference of a U to an A at position 531 in the F mRNA sequence (16) was confirmed by direct nucleotide sequencing of mRNA isolated from NDV-infected CV-1 cells (data not shown). The NDV F and HN cDNAs were subcloned for expression into an SV40-based vector. Transfection of SV40 vector DNAs and the preparation of recombinant SV40 stocks were done as described previously (37). The HPIV3 and NDV F proteins expressed from the SV40-based vectors were immunoprecipitated by F-specific monoclonal and polyclonal antibodies, respectively; these proteins were cleaved to F_1 and F_2 subunits and could be detected at the cell surface by indirect immunofluorescence (data not shown). The HPIV3 and NDV HN proteins expressed from the SV40 vectors were immunoprecipitated by HN-specific monoclonal and polyclonal antibodies, respectively, and could be detected at the cell surface by both indirect immunofluorescence and hemadsorption assays (data not shown). Antibodies to HPIV3 F and HN were kindly provided by Brian Murphy, Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Md., and antibodies to NDV F and HN were kindly provided by Mark Peeples, Rush Medical School, Chicago, Ill.

To investigate the effect of coexpressing the SV5 F and HN proteins on syncytium formation by using the SV40 vectors, CV-1 cells were transfected with SV40-F or SV40-HN DNAs individually or together. Approximately 15% of the cells expressed F or HN at the cell surface, as determined by indirect immunofluorescence staining. Cell monolayers were examined by microscopy for syncytium

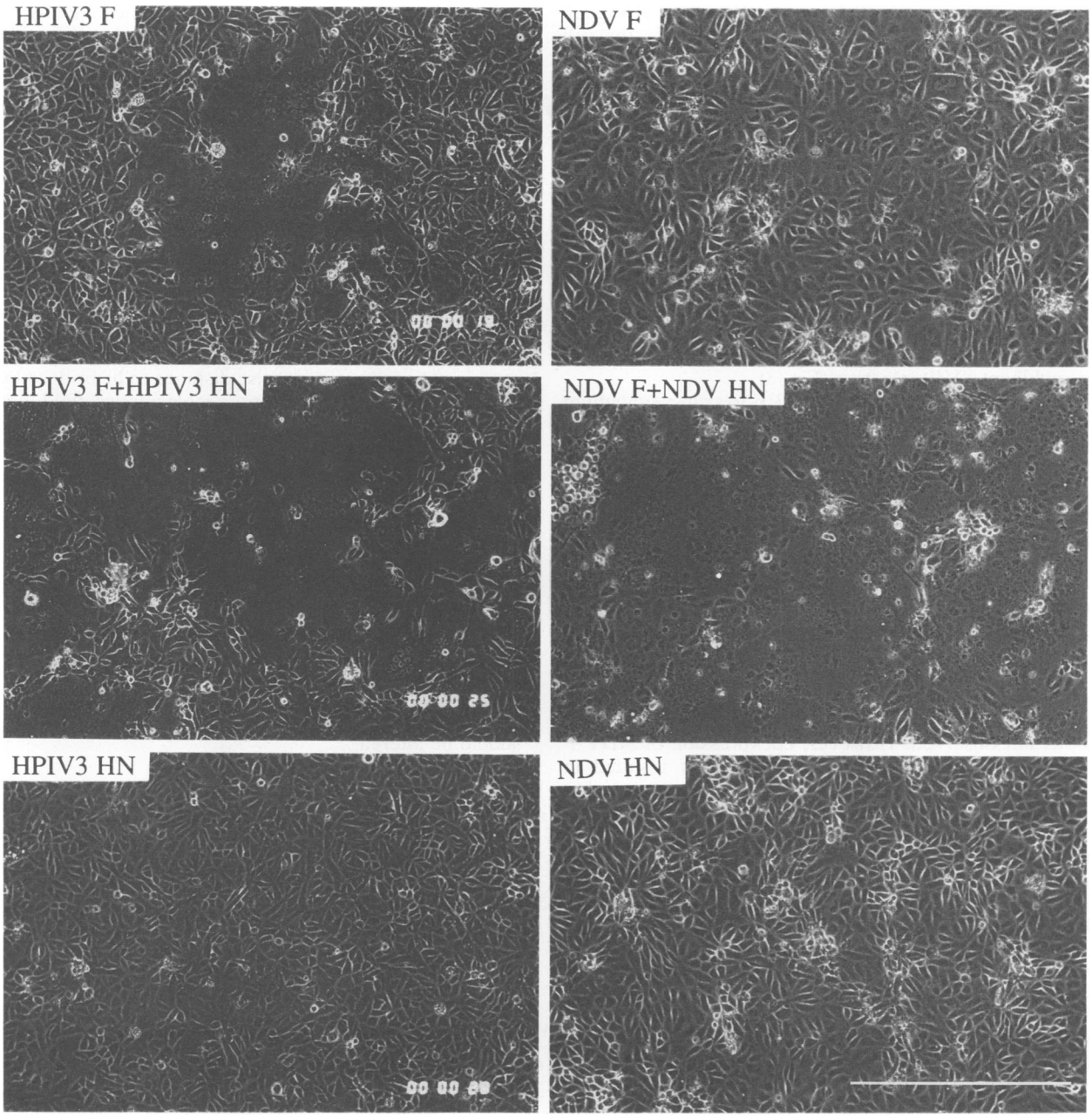

FIG. 3. Expression of the HPIV3 and NDV glycoproteins in CV-1 cells. CV-1 cells were transfected with pSV123 HPIV3 F or pSV123 HPIV3 HN DNA or both DNAs and photographed at 4 days posttransfection (HPIV3 F or HPIV3 HN) or 3 days posttransfection (HPIV3 F+HPIV3 HN). For expression of the NDV F or HN protein, CV-1 cells were infected with recombinant SV40 stocks expressing NDV F, NDV HN, or both recombinant viruses. Cells were photographed at 48 h p.i. Bar = 500 μm.

formation and were photographed 5 to 7 days posttransfection. No syncytia could be observed in cells expressing SV5 HN (data not shown), but syncytia could be readily detected in cells expressing SV5 F (Fig. 1). As observed previously, the number of cells expressing F greatly exceeded the number of syncytia observed (26). Coexpression of SV5 F and HN proteins increased the number but not the size of the syncytia two- to threefold over that observed with expression of F alone (Fig. 1). Two photographic magnifications of the monolayers are shown in Fig. 1, the lower-power micrograph to illustrate the frequency of syncytium formation and the higher-power micrograph to show the extent of individual syncytia. When recombinant SV40-F and SV40-HN stocks were produced and used to infect cells, even though 80 to 90% of cells expressed the F and HN glycoproteins at the cell surface, results very similar to that shown in Fig. 1 were obtained (data not shown). Thus, transfection of the SV40 vector DNA or infection with the higher-efficiency lytic virus system yielded similar results.

To examine the effect of coexpressing the SV5 HN protein and the SV5 F protein on syncytium formation by using the recombinant vaccinia viruses, CV-1 cells were infected with

vac-F or vac-HN or coinfected with vac-F and vac-HN, each at a multiplicity of 10 PFU per cell. At 8 h p.i., the cell monolayers were overlayed with an equivalent number of EDTA-dispersed uninfected CV-1 cells, as it has been observed previously that vac-F does not cause syncytia in CV-1 cells unless the cells are overlayed with uninfected cells (27). As shown in Fig. 2, neither cells infected with wild-type vaccinia virus (panel vaccinia) nor those infected with vac-HN (panel SV5 HN) exhibited detectable syncytium formation, but in cells expressing vac-F (panel SV5 F), small areas of syncytium formation could be observed. However, when vac-F and vac-HN were coexpressed (panel SV5 F+SV5 HN), a major increase in the area of the monolayer involved in cell-cell fusion was observed. The cytopathic effect of vaccinia virus infection on CV-1 cells can be readily detected (Fig. 2), as compared with the effect of recombinant SV40 infection (Fig. 3).

To determine the ability of HPIV3 F to cause syncytium formation in CV-1 cells when expressed by using the SV40 vector, cells were transfected with SV40-HPIV3 F, SV40-HPIV3 HN, or both DNAs, and cells were photographed 2 to 4 days posttransfection. When HPIV3 F was expressed, syncytia could be detected (Fig. 3), and they occurred at a frequency similar to that found with expression of SV5 F. These data indicate that HPIV3 F is biologically active in the absence of HPIV3 HN expression. However, when HPIV3 F and HN were coexpressed, there was a great increase in the number of syncytia observed such that >50% of the area of a 10-cm monolayer formed syncytia. No cell-to-cell fusion was observed with expression of HPIV3 HN (Fig. 3).

When NDV F or NDV HN was expressed by using recombinant SV40 infection of CV-1 cells, syncytium formation was not observed. However, coexpression of NDV F and HN by using recombinant SV40 stocks resulted in extensive syncytium formation, similar in extent to that observed with coexpression of HPIV3 F and HN (Fig. 3). Thus, as reported previously with use of a different expression system and different host cells (20), fusion was not detected when NDV F was expressed in the absence of NDV HN. When cells separately infected with SV40-NDV F and SV40-NDV HN were removed from the dish, mixed, and replated, syncytium formation was observed but not to the extent observed when NDV F and NDV HN were coexpressed (data not shown). This latter finding is in contrast to findings made when a similar experiment was performed by using chicken embryo fibroblasts and the retrovirus vector, as fusion was not detected (20). When NDV F and SV5 HN were coexpressed by using recombinant SV40, no fusion was observed, suggesting that only the homotypic HN will function with NDV F (data not shown).

The data presented here indicate that when the SV40 vector system in CV-1 cells was used, both the SV5 and HPIV3 F proteins mediated cell-to-cell fusion but that the NDV F protein did not cause detectable syncytium formation. Thus, there is a difference in the detectable fusion activity with the different F proteins with use of a single vector system and reporter cell line. Coexpression of the homologous HN protein only marginally augmented the number of syncytia observed with expression of the SV5 F protein by using the SV40-based vector but caused extensive syncytium formation with expression of the F proteins of HPIV3 and NDV. From a biochemical standpoint, it is important to know whether an activity resides with one polypeptide or more than one, but it should not be forgotten that in a natural paramyxovirus infection, F and HN are both present in virions and at the cell surface. One interpretation of the data presented here is that fusion mediated by SV5 F is mechanistically different from that mediated by HPIV3 and NDV F. However, this view ignores the observations that HPIV3 F is biologically active without HN expression (although it is not as efficient as with HN) and that fusion mediated by the SV5 F protein expressed by the vaccinia virus vector is greatly enhanced by HN expression. A priori, for cell-to-cell fusion to occur, the cells must be in contact. Thus, the simple interpretation of all of these data that we prefer is that depending on the particular F protein (and the effect of the vector when used), an interaction of HN with sialic acid present on a neighboring cell is required to draw the cells into appropriate juxtaposition for fusion to occur. In these cases, we suggest that the inability to replace the homotypic HN with either a heterotypic HN protein or lectin is due to the fact that the molecular architecture of the F protein requires a very precise distance to be bridged by the homotypic HN protein to form a molecular scaffold, permitting perturbation of the recipient cell membrane by the F fusion peptide. In this regard, Henis and coworkers (2, 9, 15) have provided data indicating that F and HN have similar requirements for lateral mobility in the plane of the membrane and suggest that F and HN may form a fusion complex. In future studies, it will be necessary to understand better the qualitative differences observed in fusion activity by developing assays to quantitate the expression levels of biologically active F within a single cell and to quantitate fusion activity by a means that detects all fusion events in addition to those that are observed by the formation of multinucleated cells. However, the evidence that has been reported to date does not indicate that the conclusion that the F protein is the active polypeptide of the fusion process should be altered.

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