REQUIREMENTS FOR SYNCYTIUM FORMATION MEDIATED BY THE FUSION GLYCOPROTEIN OF HUMAN PARAINFLUENZA VIRUS TYPE 3

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Department of Microbiology and Immunology

Faculty of Medicine

By

Sharon Nori Ebata

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Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Recombinant vaccinia viruses VF and VHN were shown to express glycoproteins with molecular weights similar to authentic HPIV3 F and HN proteins that were transported to the cell surface and recognized by monoclonal antibodies specific for HPIV3 F and HN. The HN glycoprotein in VHN-infected cells exhibited both hemagglutinin and neuraminidase activities. Both cleaved and uncleaved forms of the F glycoprotein were immunoprecipitated from VF-infected cell lysates. Fusogenic activity of the F protein was demonstrated only upon coinfection of HeLa $T4^+$ cells with VF + VHN and resulted in syncytium formation and hemolysis.

Recombinant adenoviruses AdF and AdHN, expressed the HPIV3 F and HN proteins on the surface of infected HeLa $T4^+$ cells, respectively. AdHN-infected cells produced HN protein that was glycosylated and exhibited both hemagglutinin and neuraminidase activities. AdF infection led to the synthesis of glycosylated F_0 that was proteolytically cleaved. As was observed for vaccinia virus recombinants, syncytium formation was only observed upon coinfection of HeLa $T4^+$ cells with AdF + AdHN. The F and HN proteins expressed by recombinant adenoviruses were recognized by monoclonal antibodies specific for the HPIV3 F or HN protein and were immunogenic in mice. Sera from AdF- or AdHN-inoculated mice immunoprecipitated appropriate glycoproteins from lysates of HPIV3-infected cells and neutralized HPIV3.

Amino acid substitutions were introduced into three regions of the HPIV3 F protein to identify sequences involved in fusogenic activity. Substitution of uncharged amino acids for positively charged residues within the cytoplasmic tail, or introduction of a charged residue within the transmembrane domain, did not appear to affect fusogenic activity. Several different mutations designed to disrupt the leucine zipper or the heptad repeat motifs resulted in mutant F proteins that not only exhibited decreased fusogenic activity but concomitantly lost the ability to react with monoclonal antibody c128, which recognized an epitope in antigenic site B of the HPIV3 F glycoprotein. An association between the heptad repeat and leucine zipper domains, therefore, appears to be required for the HPIV3 F glycoprotein to adopt a conformation that is recognized by antibody c128 and that appears to be important for fusogenic activity.

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I will be forever grateful for the support of my husband James Haebe and my parents, especially my mother Mrs. Jane Ebata, whose contribution was immeasurable.

DEDICATION

This thesis is dedicated to my husband James Haebe

and our children Laura and Trevor.

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Ad5	adenovirus type 5
BSA	bovine serum albumin
BPIV3	bovine parainfluenza virus type 3
CPE	cytopathic effects
ddH_2O	double distilled water
DTSSP	3,3'-dithiobis(sulfosuccinimidylpropionate)
F	fusion protein
FBS	fetal bovine serum
FFWI	fusion from within
FFWO	fusion from without
G	receptor binding protein
G418	geneticin
GIDH	L-glutamate dehydrogenase
GMEM	growth minimal essential medium
HN	hemagglutinin-neuraminidase protein
HPIV1	human parainfluenza virus type 1
HPIV2	human parainfluenza virus type 2
HPIV3	human parainfluenza virus type 3
HPIV4	human parainfluenza virus type 4
Ig	immunoglobulin

L	large polymerase protein
LB	Luria broth
M	matrix protein
MEM	minimal essential medium
MOI	multiplicity of infection
NA	neuraminidase protein
NDV	Newcastle disease virus
NP	nucleoprotein
NP40	nonidet P40
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFU	plaque forming unit
PMSF	phenylmethyl-sulfonyl fluoride
RBC	red blood cell
RIPA	radioimmunoprecipitation assay
RSV	respiratory syncytial virus
SDS	sodium dodecylsulfate
SV5	simian virus type 5
SV40	simian virus type 40
TBE	tris-borate EDTA
TBS	tris-buffered saline

TEMED	NNN'N'-tetramethylethylene diamine
vRNA	viral RNA
VSV	vesicular stomatis virus
Xgal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside

GENERAL INTRODUCTION

I. General description/properties of HPIV3

Taxonomy and epidemiology

Human parainfluenza virus type 3 (HPIV3) is a member of the family *Paramyxoviridae*, and is characterized by a single-stranded, nonsegmented negative-sense RNA genome enclosed within a helically symmetrical nucleocapsid and surrounded by a lipoprotein envelope (Kingsbury, 1990).

The family *Paramyxoviridae* is divided into subfamilies based upon fundamental differences in capsid morphology and upon nucleotide sequence data; the subfamily *Paramyxovirinae* includes the genera *Paramyxovirus* and *Morbillivirus*, while the *Pneumovirinae* encompasses the genus *Pneumovirus*. The original classification of the *Paramyxoviridae* into three genera was based upon antigenic cross-reactivity between species and biochemical activities of the virus particles such as hemagglutinin and neuraminidase (Kingsbury, 1990).

The human parainfluenza viruses types 1,2,3 and 4 (HPIV1-4) have been placed in the genus *Paramyxovirus*. Additional members of this genus include Sendai virus, which infects mice; simian virus type 5 (SV5), which was originally isolated in a monkey cell line but causes respiratory infection of dogs; Newcastle disease virus (NDV), which infects chickens; and mumps virus, which, like HPIV1-4, is a

human pathogen (Kingsbury, 1990). Members of the genus *Paramyxovirus* exhibit both hemagglutinin and neuraminidase capabilities while measles virus, and other members of the genus *Morbillivirus* possess only hemagglutinin ability. Respiratory syncytial virus (RSV) is the prototype of the genus *Pneumovirus*, and demonstrates neither hemagglutinin nor neuraminidase activity. The nucleocapsid of RSV is also smaller in diameter than the nucleocapsids of other paramyxoviruses. Negatively stained preparations of RSV nucleocapsids have a diameter of 12-15 nm rather than 18 nm (McIntosh and Chanock, 1990).

As a group, the parainfluenza viruses are second only to RSV as an important cause of lower respiratory tract disease in infants and young children. Reinfection with parainfluenza viruses is also common among older children and adults and can produce upper respiratory tract illnesses (Pringle, 1987).

Parainfluenza viruses, like other respiratory agents, are spread through respiratory secretions. The incubation period ranges from 2 to 6 days and virus is shed for approximately one week (White and Fenner, 1994). There is considerable diversity among the parainfluenza viruses with respect to the epidemiology and the clinical presentation of infection. HPIV1 is the leading cause of laryngotracheobronchitis (croup) among children. Infection with HPIV2 gives clinical manifestations similar to HPIV1, however, serious illness arising from HPIV2 infection occurs less frequently. HPIV4 is more difficult to recover from tissue culture than other types, and illnesses associated with HPIV4 infection are usually mild (Glezen et al., 1989).

HPIV3 is one of the leading causes of pneumonia and bronchiolitis in infants younger than 6 months (Glezen, 1989). HPIV3 was originally isolated in 1958, based upon the ability of infected rhesus monkey kidney cell lines to adsorb guinea pig erythrocytes, a function of the hemagglutinin-neuraminidase (HN) glycoprotein (Chanock and McIntosh, 1990). Like all parainfluenza viruses, HPIV3 is spread via direct person-to-person contact or through aerosolized virus, but does not exhibit prolonged survival in the environment and is inactivated by stomach acid or intestinal enzymes (Black, 1991). Typically, the interval between the entry of the virus into the upper respiratory tract and the onset of symptoms is 3 to 6 days, although incubation periods of 2 to 4 days have been observed during outbreaks. HPIV3 is shed from the oropharynx usually for 3 to 10 days during primary infection and during a shorter interval upon reinfection, however, infants and young children may shed virus for as long as 3 to 4 weeks (Chanock and McIntosh, 1990).

Members of the family *Paramyxoviridae* differ significantly in the type of immune response that they elicit in the infected host. While infection by measles or mumps viruses induces a strong, enduring immunity to reinfection, parainfluenza viruses and RSV are disseminated through a population by repeated infections of individuals (Black, 1991).

HPIV3 is considered to be a monotypic virus (Coelingh et al., 1985), although phylogenetic analyses based upon F gene sequences have identified two lineages of HPIV3 circulating in North America (Prinoski et al., 1991). This genetic heterogeneity is characterized by antigenic variation in several epitopes within the

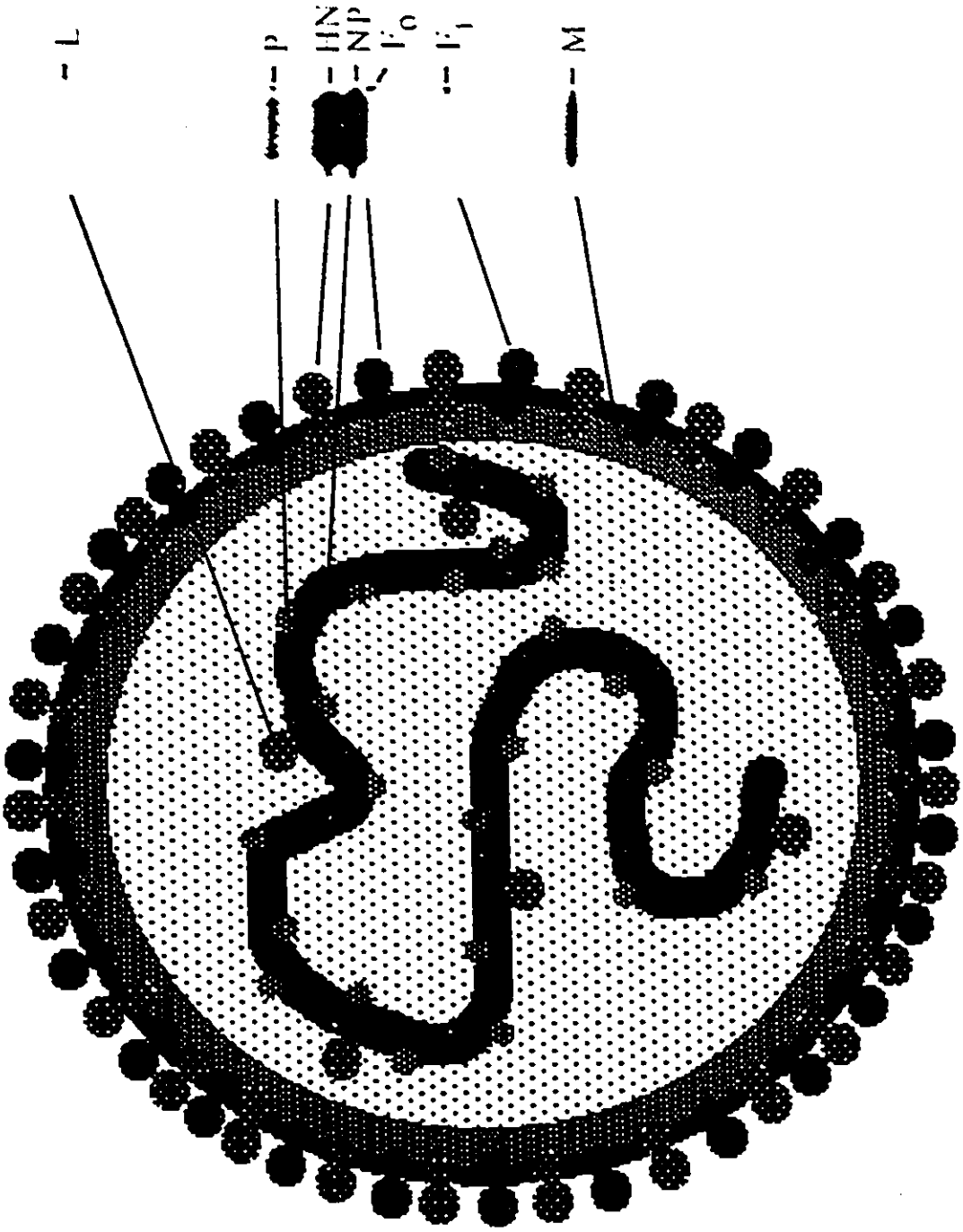
HN or F glycoproteins observed in clinical isolates of HPIV3 (Coelingh et al., 1985; Coelingh, 1991; Coelingh and Winter, 1990). Although prior parainfluenza virus infection results in transient immunity to lower respiratory tract illness upon reinfection by a homotypic virus, it does not prevent reinfection by homotypic or heterotypic parainfluenza viruses (Welliver et al., 1982). In one study, 66% of children had developed antibodies to HPIV3 by the age of 2. However, six months later, 13.8% of these children had experienced reinfection by HPIV3, and 14.6% had been infected with heterotypic parainfluenza viruses (Welliver et al., 1982). The incidence of infection or reinfection by HPIV3 decreases in children older than 4 years of age, and, conversely, the incidence of HPIV3 infection begins to increase slightly in persons older than 65 (Glezen et al., 1984).

Paramyxovirus morphology

The paramyxoviruses are the largest of the RNA viruses (Pringle, 1987). The virion consists of a nucleocapsid that incorporates the genome, enclosed within a pleomorphic envelope that ranges in size from 150 to 300 nm in diameter (Vainionpää and Hyypiä, 1994). Figure 1 is a schematic, representative of the HPIV3 virion.

The helical morphology and structural integrity of the nucleocapsid is conferred by 2200 to 2600 nucleoprotein (NP protein) molecules that are associated with the genome (Portner and Murti, 1986). The nucleocapsid also contains 250-300

Figure 1. Schematic representation of the HPIV3 virion and associated proteins. Proteins are also shown resolved by electrophoresis on a 7.5-15% polyacrylamide gel containing SDS. L indicates the large polymerase protein. P indicates the phosphoprotein. HN indicates the hemagglutinin-neuraminidase protein. NP indicates the nucleoprotein. F_0 indicates the uncleaved fusion protein. F_1 indicates a subunit of the cleaved fusion protein. M indicates the matrix protein. From Galinski and Wechsler (1991).


L
P
HN
NP
F0
F1
M

phosphoprotein (P protein) molecules, and 20-30 large polymerase (L protein) molecules that occur discontinuously as clusters in nucleocapsids extracted from infected cells (Portner et al., 1988; Galinski and Wechsler, 1991; Vainionpää and Hyypiä, 1994).

The viral envelope is derived from the host cell membrane and acts as a protective lipoprotein coating for the RNA genome and associated viral proteins. Glycoproteins on the surface of the envelope are responsible for the preliminary stages of infection of the host cell. The hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins, present as integral membrane proteins on the viral envelope, appear as spikes 8-12 nm in length and are spaced 8-10 nm apart (Galinski and Wechsler, 1991). The F and HN proteins function in the attachment of the virus to the host cell receptor and entry of the nucleocapsid into the cell by fusion of the viral envelope and the host cell membrane. Antibodies directed against either the F or HN proteins can neutralize infectivity by interfering with the adsorption or penetration of the virion (Merz et al., 1980).

The matrix (M) protein plays a crucial role in virion assembly and interacts with both the nucleocapsids and the glycoproteins associated with the plasma membrane. The M protein is characterized by an abundance of basic residues that may be important for the ionic interaction with NP proteins, which are acidic in all paramyxoviruses. The M protein also possesses an abundance of hydrophobic amino acids that are believed to facilitate interactions between the F and HN

glycoproteins as well as the cellular membrane (Galinski and Wechsler, 1991). Assembly of nascent Sendai virus is triggered by the interaction of the M protein with cellular membranes containing the viral glycoproteins. In the absence of these glycoproteins the M protein of Sendai virus does not bind efficiently to cell membranes, however the presence of either the F or HN protein is sufficient to promote binding of the M protein to cellular membranes (Sanderson et al., 1994).

II. Genome organization and replication

Genomic organization of HPIV3

The genome of HPIV3 is a linear, nonsegmented, single stranded RNA molecule of negative polarity 15, 463 nucleotides(nt) in length (Galinski, 1991; Storey et al., 1984). The genome serves as a template for transcription of six individual mRNA molecules that encode 6 structural proteins (NP, P, M, F, HN and L protein), and one (C protein, Luk et al, 1986; Spriggs and Collins, 1986), or possibly two (D protein, Galinski et al., 1986), nonstructural proteins.

The order of the HPIV3 genes was determined from sequencing virion RNA (vRNA) and is as follows: 3'-NP-P+C-M-F-HN-L-5' (Spriggs and Collins, 1986; Dimock et al., 1987). The six structural proteins resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), are shown on the right and the location of each protein in the virion is indicated in Figure 1. The L gene is 6755 nt in length and

encodes a protein of 2233 amino acids (aa) with predicted molecular weight (MW) of 255,800 (255.8K). The P gene is 2009 nt in length and encodes a protein of 602 aa with a MW of 67.5K. The HN gene is 1888 nt in length and encodes a protein of 572 aa, which possesses four potential N-linked glycosylation sites and has a MW of 69K-72K after glycosylation. The NP gene is 1641 nt in length and encodes a protein of 515 aa with a MW of 57.8K. The F gene is 1845 nt in length, and encodes a protein of 539 aa, which also possesses four glycosylation sites. The F protein is synthesized as a glycosylated precursor protein F_0, with a MW of 63K, which is proteolytically cleaved into F_1 (50K) and F_2 (10K) subunits. The M gene is 1150 nt in length and encodes a polypeptide of 353 aa with a MW of 39.5K (Spriggs et al., 1986; Chanock and McIntosh, 1990).

The nonstructural C protein is encoded by independent translation initiation of the C codon that is in a +1 reading frame relative to the initiation codon of the P gene (Galinski et al., 1986). The putative D protein has an internal initiation sequence downstream of the C sequence and the D open reading frame is accessed by transcriptional editing of the P gene by viral proteins. Clones derived from HPIV3 P gene mRNAs had sequences containing variable numbers of G residues (5-14) at the D gene editing site. The addition of nontemplated G residues at this site results in the fusion of the discontinuous P and D cistrons and leads to the translation of the D protein which has been observed *in vitro*. A cryptic V cistron is present in the HPIV3 P gene, the V cistron, which has been identified in mRNA of SV5, mumps virus and HPIV4, encodes a V protein with highly conserved

cysteine residues at the carboxy-terminal domain. However, the V cistron of HPIV3 is not accessed via ribosomal frameshifting (Galinski et al., 1992). All the information necessary to trigger transcriptional editing has been shown for measles virus to be contained within the target RNA sequence (Liston et al., 1995).

The HPIV3 genome has a 55-nucleotide leader region at the 3' terminus and a 44 nucleotide trailer at its 5' terminus (Dimock et al., 1986; Côté et al., 1987). The first 20 nucleotides at the 3' end of the genome are highly conserved among paramyxoviruses, which suggests biological significance with respect to initiation of mRNA transcription and in genome replication (Galinski and Wechsler, 1991). Rescue of synthetic analogs of genomic RNA show that the signals for encapsidation, replication, transcription and packaging of HPIV3 are contained within the 3'-terminal 111 nucleotides and the 5'-terminal 115 nucleotides (Dimock and Collins, 1993). Both the 3' and 5' terminal sequences appear to be sensitive to inactivation by mutagenesis (De and Banerjee, 1993). Each HPIV3 gene, with the exception of the M gene, possesses a consensus sequence that defines gene-start (UCCUNNUUUC) and gene-end (UUNAUA(or U)U(or C)UUUUU) regions, which are believed to act as signals for the polymerase to initiate (or reinitiate) transcription or to terminate transcription. The gene-end sequence of the M gene is characterized by an 'insertion' of eight nucleotides, which appears to result in a high degree of read-through transcription. The intergenic regions are identical for all HPIV3 genes and consist of the trinucleotide 3'-GAA-5' (Spriggs and Collins, 1986, Dimock et al., 1986).

Replication and assembly of HPIV3

Attachment and fusion of the HPIV3 virion with the host cell membrane releases the nucleocapsid into the cell cytoplasm where transcription and replication ensue. Since the genome of HPIV3 consists of a single strand of negative-sense RNA, the HPIV3 genome must first be transcribed into virus-specific mRNA species. An RNA-dependent RNA polymerase is not produced by eucaryotic cells and is carried into the cell by HPIV3 in the form of the active transcriptional apparatus consisting of the genome as well as the NP, P and L proteins (Vainionpää and Hyypiä, 1994).

Primary transcription of HPIV3 RNA is believed to begin at the 3' end of the genome and although it does not require *de novo* viral protein synthesis, it does, however, require host cell factor(s) (De et al., 1990). The polymerase recognizes a single entry site on the genomic RNA and moves along, transcribing each gene, stopping and reinitiating transcription at the gene boundaries without copying the trinucleotide intergenic sequence. Viral mRNA molecules are modified to contain a 5'-methylated cap structure and 3'-polyadenylated tail to enable them to mimic eucaryotic message for translation into proteins by the host cell (Vainionpää and Hyypiä, 1994). *In vitro*, HPIV3 mRNAs are synthesized in a polar fashion from the 3' NP gene to the 5' L gene, and results in NP mRNA as the most abundant species and L mRNA as the least abundant. The amount of M mRNA synthesized in infected cells is lower than the amount of F mRNA, which could be due to the insertion in the M gene-end sequence that encourages read-through of the

transcriptional termination signal at the end of the M gene and leads to synthesis of a bicistronic M/F transcript (De et al., 1990).

The negative-sense genome also serves as a template for the synthesis of antigenomes, which are full length, unmodified, complementary RNAs that are used as intermediates for genome replication. During replication, the RNA-dependent RNA polymerase must ignore the gene-end and gene-start sequences and read through the gene boundaries, which also include the intergenic trinucleotide. This switch from transcription to replication in the family *Paramyxoviridae* is not well understood and characterization of this process has been largely based upon studies carried out using vesicular stomatitis virus (VSV), a member of the family *Rhabdoviridae,* as a model for nonsegmented negative-strand RNA virus replication (Vainionpää and Hyypiä, 1994).

For Sendai virus, the switch from transcription to replication is regulated by the intracellular level of the NP protein, therefore, replication, unlike transcription, requires viral protein synthesis. When isolated by electrophoresis, genomic RNA appears as a ladder of micrococcal nuclease-resistant fragments, indicating that genomic RNA associates with the NP protein during synthesis before it is completed (Galinski and Wechsler, 1991). A high level of NP protein favours replication and the production of full-length antigenomes, conversely, a scarcity of NP protein favours transcription and the production of monocistronic mRNAs (Vidal and Kolakofsky, 1989). The C proteins of Sendai virus, which are a nested set of four carboxy-coterminal proteins, are able to inhibit transcription in the absence or

presence of the NP protein but have little effect on replication. The Sendai virus C proteins interact with the P and L proteins during polymerase formation and it is possible that although these complexes are inactive for transcription, they are still competent for replication of the genome (Curran et al., 1992). On the other hand, the measles virus C protein does not appear to interact with either the P or L proteins, or with any measles virus-specific protein (Liston et al., 1995).

The P and L proteins are assembled together with the NP proteins and the genome to form nucleocapsids. The F and HN proteins are synthesized on the rough endoplasmic reticulum (RER) and are assembled cotranslationally into oligomers (Collins and Mottet, 1991). Nascent proteins are transported through the various membranous compartments where they acquire asparagine-linked oligosaccharide side chains (Schwalbe and Hightower, 1982), and arrive finally at the cell surface. Throughout this migration the viral glycoproteins remain constituents of membranes since they are never detected as soluble proteins (Klenk, 1974). The nucleocapsids make their way to the areas on the cell membrane that have lost their host-cell proteins and have been modified by insertion of the two virus-specific glycoproteins and the associated M protein, leading to budding of the nascent virions from the host cell (Chanock and McIntosh, 1990).

III. Characterization of the glycoproteins of HPIV3

The hemagglutinin-neuraminidase glycoprotein

The HN protein of HPIV3 is one of the two glycoproteins that are integral to the viral envelope. In its native state, the HN glycoprotein forms a spike that is morphologically distinct from the F glycoprotein spike (Scheid et al., 1972). Sequence analysis of the HPIV3 HN gene shows the lack of a typical amino-terminal hydrophobic signal sequence, instead, there is a stretch of 20 hydrophobic amino acids beginning at position 32 from the amino-terminus (Elango et al., 1986; Galinski et al., 1987; Storey et al., 1987). Therefore, like the influenza virus neuraminidase (NA) protein, the HPIV3 HN protein is a type II glycoprotein, such that a sequence near the amino-terminus of the protein serves as both a signal sequence (McGinnes et al., 1987) and as a transmembrane anchor in both the viral envelope and the membranes of infected cells (Hiebert et al., 1985). Chemical cross-linking studies have shown that the mature HPIV3 HN protein is present on the surface of infected cells as a homooligomer, most likely in a tetrameric configuration (Collins and Mottet, 1991).

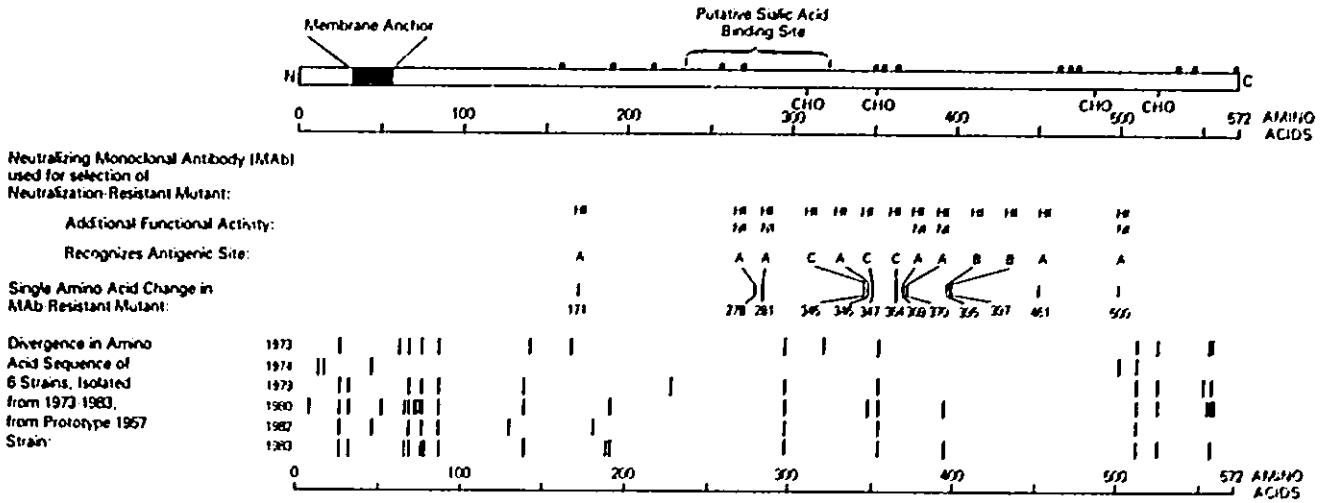
The HN protein is multifunctional, exhibiting both hemagglutinin and neuramindase activities. The hemagglutinin activity of the HN protein mediates the attachment of the virion to host cell receptors that contain sialic acid, while the neuraminidase activity of the protein may provide the opposite function, which is removal of sialic acid from receptor molecules (Scheid and Chopin, 1974). The

hemagglutinin and neuraminidase activities have been localized as two distinct functional sites for several different Paramyxovirus HN molecules, amino acid changes that confer resistance to hemagglutination-inhibition or neuraminidase-inhibition are located throughout the primary sequence of the HN protein and shown in Figure 2 (Portner et al., 1987; Thompson and Portner, 1987; Gorman et al., 1991; Lyn et al., 1991; Mirza et al., 1994; Huberman et al., 1995).

All known influenza neuraminidase (NA) proteins possess 15 charged residues, which are strictly conserved and make up the rim of a pocket that binds sialic acid (Colman et al., 1983). It has been suggested that the predicted secondary structure of the sialic-acid binding site of the HPIV3 HN protein is similar to the known structure of influenza A viral neuraminidase sialic acid binding site that was identified by X-ray crystallography (Varghese et al., 1983), since enzyme active sites could possess some degree of conservation in related molecules (Jorgensen et al., 1987).

The putative sialic-acid binding site of the neuraminidase domain of the HPIV3 HN protein has been localized between amino acids 234 and 323 and is shown in Figure 2. The hexapeptide NRKSCS is the longest linear stretch of residues that is conserved among the HN proteins of all paramyxoviruses and mutations within this region of the NDV HN protein decreased neuraminidase activity (Mirza et al., 1994). HPIV3 variants that were resistant to neutralization by neuraminidase-inhibiting monoclonal antibodies have amino acid changes corresponding to

Figure 2. Structural and antigenic features of the HPIV3 HN protein. The black box represents the amino-terminal membrane anchor. The putative sialic acid binding site refers to the neuraminidase domain. Cysteine residues (●) and potential N-linked carbohydrate acceptor sites (CHO) are indicated. The activity of neutralizing antibodies (Mab) in hemagglutinin-inhibition (HI) and neuraminidase-inhibition (NI) assays are indicated. Positions of amino acid substitutions for Mab-resistant mutants are shown. The divergence of amino acids from the prototype HPIV3 1957 strain is shown for clinical six clinical strains isolated from 1973 to 1983. From Coelingh (1991).


Membrane Anchor
Putative Sialic Acid Binding Site
N
C
CHO
CHO
CHO
CHO
0
100
200
300
400
500
572 AMINO ACIDS
Neutralizing Monoclonal Antibody (MAb) used for selection of Neutralization-Resistant Mutant:
Additional Functional Activity:
Recognizes Antigenic Site:
Single Amino Acid Change in MAb-Resistant Mutant:
171
278
281
345
346
347
354
358
372
396
397
451
500
Divergence in Amino Acid Sequence of 6 Strains, Isolated from 1973-1980, from Prototype 1957 Strain:
1973
1974
1979
1980
1982
1980
0
100
200
300
400
500
572 AMINO ACIDS

surface loop structures within, or adjacent to the putative sialic acid binding site. HPIV3 variants that were resistant to neutralization by hemagglutinin-inhibiting monoclonal antibodies also had amino acid changes located within these surface loops and perhaps suggest that the enzymatic activities are structurally related (Coelingh, 1991).

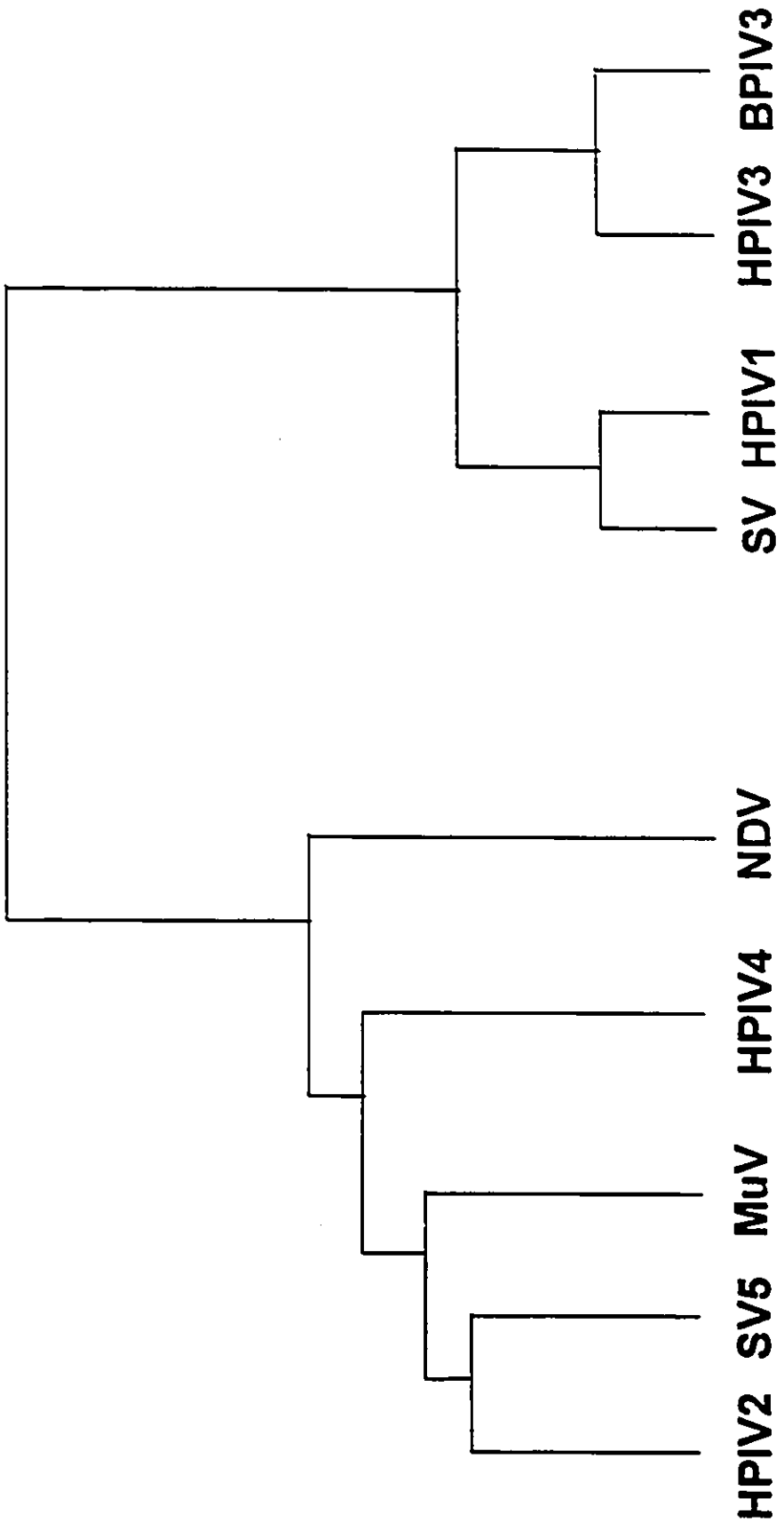
The role of neuramindase in the virus life cycle has not been well defined, however, study of an HPIV3 variant with low neuraminidase activity revealed that neuraminidase activity was essential for the release of nascent virions from the cell surface. Growth properties of the variant virus showed increased fusogenic activity as well as a delay in the release of virus particles into the supernatant, however, the decreased neuraminidase activity had no effect on virus entry, replication or assembly. The neuraminidase activity may modulate the number of available receptors and subsequently determine the degree of cell fusion. The level of neuraminidase activity was also found to determine whether the outcome of HPIV3 infection is cytopathic or persistent in cell culture. Low levels of neuraminidase activity resulted in a greater number of available receptors, increased syncytium formation and a decreased opportunity for persistent infection to develop (Huberman et al., 1995).

Comparisons of the HN protein sequences of several members of the family *Paramyxoviridae* supports the division of this family into three groups based upon hemagglutinin and/or neuraminidase activities. HPIV3 (Elango et al., 1986; Galinski et al., 1987), HPIV1 (Gorman et al., 1990), HPIV2 (Kawano et al., 1990b),

HPIV4 (Bando et al., 1990), NDV (McGinnes et al., 1987), Sendai virus (Shioda et al., 1986), SV5 (Hiebert et al., 1985), mumps virus (Kövamees et al., 1989) and bovine parainfluenza virus type 3 (BPIV3) (Suzu et al., 1987), show alignment of 9 cysteine residues but poor conservation of potential N-glycosylation sites (Morrison, 1988). The conservation of the positions of the cysteine residues may indicate evolutionary relatedness and a phylogenetic tree of paramxyoviruses is shown in Figure 3. HPIV3, BPIV3, HPIV1 and Sendai virus (Storey et al., 1987), make up one branch of the phylogenetic tree, these viruses have 4 additional conserved cysteine residues for a total of 13 of 16. Members of the second branch of the phylogenetic tree are SV5, mumps virus, HPIV2, HPIV4 and NDV, which have 5 additional conserved cysteine residues for a total of 14, HPIV2 and SV5 share an additional 2 residues for a total of 16 and are more closely related phylogenetically (Suzu et al., 1987; Kövamees et al., 1989, Kawano et al., 1990b).

Protein sequence analysis of the hemagglutinin (H) glycoproteins of measles virus (Alkhatib and Briedis, 1986) and rinderpest virus, which causes disease in cattle (Tsukiyama et al., 1987), shows conservation in the positions of all 13 cysteine residues. The attachment glycoprotein (G) of RSV shows no significant amino acid sequence similarity to any of the aforementioned attachment protein sequences (Morrison, 1988). The conservation of cysteine residues indicates the importance of inter- and intramolecular bonds in HN protein conformation for the arrangement of functional hemagglutinin or neuraminidase sites.

Figure 3. Phylogenetic tree of paramyxoviruses. Phylogenetic tree was constructed based upon HN and F protein sequences. HPIV1-4: human parainfluenza viruses types 1-4. BPIV3: bovine parainfluenza virus type 3. SV5: simian virus type 5. MuV: mumps virus. NDV: Newcastle disease virus. SV: Sendai virus. Length of the branches does not represent a time line. Modified from Kawano et al., (1990b) and Komada et al., (1995).


BPIV3
HPIV3
HPIV1
SV
NDV
HPIV4
MuV
SV5
HPIV2

A potential role of the HN protein in fusion has been speculated upon for some time, since monoclonal antibodies specific for the HN protein of Sendai virus bound distinct regions on the HN protein and inhibited membrane fusion and hemolysis, yet did not inhibit either neuraminidase or hemagglutination activities (Miura et al., 1982). In fact, the neuraminidase activity of NDV HN protein was shown to be necessary for fusion of liposomes with cellular membranes (Huang et al., 1980). A variant of HPIV3 with increased fusogenic activity has been shown to have amino acid substitutions in the HN protein but no mutations in the F protein sequence (Moscona and Peluso, 1993). One of the primary objectives of this thesis is to evaluate the role of the HN protein in fusion mediatedby HPIV3.

The fusion glycoprotein

The F glycoprotein of HPIV3 is the second of the two glycoproteins that are integral to the viral envelope and is associated with infectivity, hemolysis and cell-to-cell membrane fusion (Scheid and Choppin, 1974). The cell-to-cell fusing activity of HPIV3 results in the fusion of adjacent cell membranes and allows for the lateral spread of virus in addition to the usual mode of virus dissemination by released virus. Antibodies specific for the F protein can prevent the spread of infection in cells that are sensitive to cell-to-cell fusion as well as in nonfusing cells (Merz et al., 1980). The F protein is a type I glycoprotein that is inserted into the lipid bilayer in the opposite orientation to the HN protein and is anchored by its carboxy-terminal

end. The hydrophobic amino-terminal signal sequence directs the translocation of the protein across the membrane of the endoplasmic reticulum and is cleaved by a signal peptidase. The signal sequence of the type I protein, except for the presence of a cleavage site, has been shown to be structurally and functionally similar to the noncleaved hydrophobic domain of type II membrane proteins that also functions as a signal sequence (Paterson and Lamb, 1990).

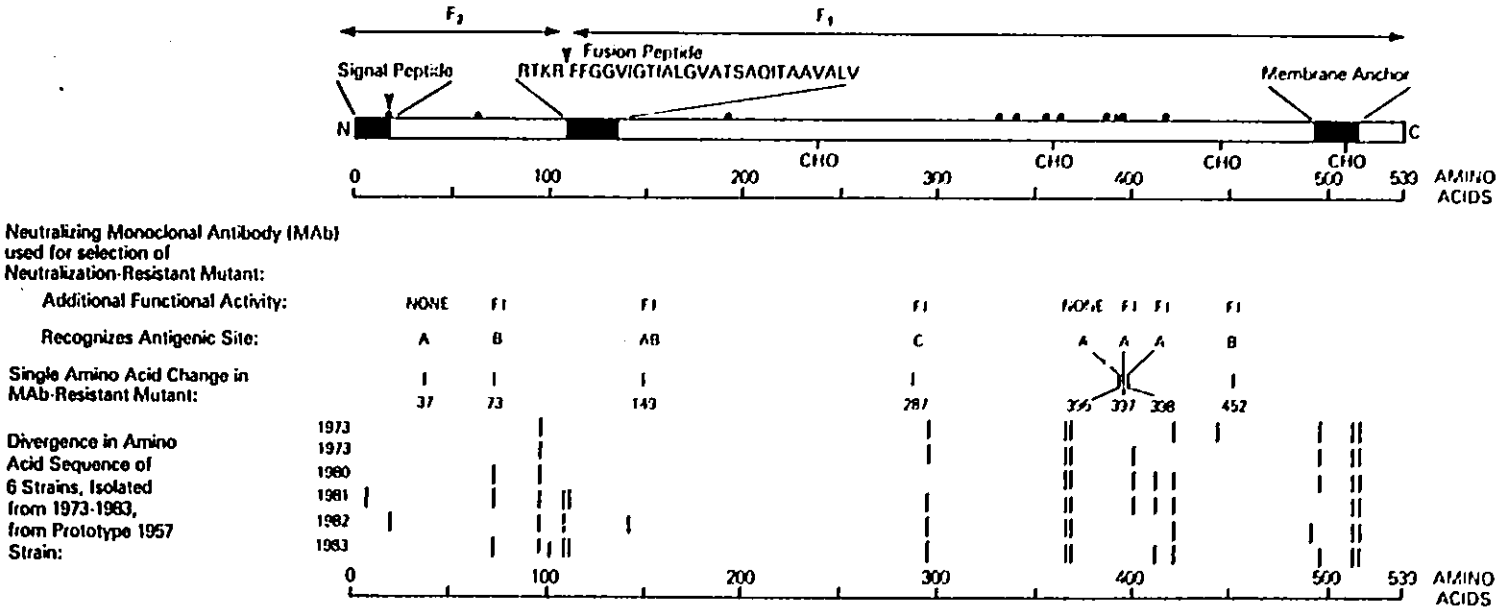
The F glycoprotein can be observed in two forms, a biologically inactive protein (F_0), and a biologically active form (F_1, F_2), which can be identified by a change in the electrophoretic pattern of the polyprotein under reducing conditions of PAGE (Homma and Ohuchi, 1973). The activation of the F_0 protein is the result of internal cleavage of the protein by a host cell enzyme to yield F_1, F_2 and the two polypeptides are linked by a single disulfide bond (Scheid and Choppin, 1977).

Among members of the family *Paramyxoviridae,* the F proteins share many identical structural characteristics. All F proteins have a hydrophobic signal sequence at the amino-terminus and an internal hydrophobic region that becomes the new amino-terminus of the F_1 protein. The F proteins all possess a cysteine-rich domain in the F_1 subunit and a hydrophobic region at the carboxy-terminus that functions as a transmembrane anchor. The two most striking features that are shared among the F proteins of the family *Paramyxoviridae* are the highly conserved amino acid sequence of the internal hydrophobic domain and the conservation of cysteine residues. Figure 4 represents a schematic diagram of the

HPIV3 F protein that shows the position of hydrophobic regions as well as cysteine residues.

The phylogenetic linkages of the *Paramyxovirus* F proteins are identical to those seen previously for the HN protein (Figure 2). The conservation of cysteine residues among F proteins is striking particularly with respect to the F_1 subunit and the strict conservation of cysteine residues could signify the importance of secondary structure for F protein function (Morrison, 1988). HPIV3 (Spriggs et al., 1986; Côté, et al., 1987; Galinski et al., 1987), HPIV1 (Merson et al., 1988), HPIV2 (Hu et al., 1990; Kawano et al., 1990; Varsanyi et al., 1991), HPIV4A, HPIV4B (Komada et al., 1995), NDV (McGinnes and Morrison, 1986), mumps virus (Elango et al., 1989), SV5 (Paterson et al., 1984), Sendai virus (Shioda et al., 1986), BPIV3 (Suzu et al., 1987), measles virus (Richardson et al., 1986; Buckland et al., 1987) and canine distemper virus (Barrett et al., 1987) show alignment of all 9 cysteine residues of the ectodomain of the F_1 subunit. These viruses differ only in the number of cysteines of the F_2 subunit and the number of cysteine residues present in the transmembrane domain. HPIV3, BPIV3 and Sendai virus exhibit the most sequence similarity with respect to the F and HN glycoproteins (Suzu et al., 1987). The F protein of RSV also shares 8 of the 9 cysteine residues found in the other F proteins, however it contains an additional 4 cysteine residues in the cysteine-rich region.

Figure 4. Structural and antigenic features of the HPIV3 F protein. The F protein is synthesized as a single polypeptide that undergoes proteolytic cleavage at two sites (▼). Black boxes represent three hydrophobic sequences characteristic of the HPIV3 F protein. These include (starting from the amino-terminus or left-end of the diagram); the signal peptide that is subsequently cleaved (▼), the 26-residue hydophobic peptide that becomes the new amino terminus upon cleavage (▼) of the precursor F_0 protein into F_1 and F_2 subunits and a hydrophobic sequence near the carboxy-terminus of the F_1 subunit that anchors the F protein into the viral envelope. Cysteine residues (●) and potential N-linked carbohydrate acceptor sites (CHO) are indicated. The activity of neutralizing monoclonal antibodies (Mab), in fusion-inhibition (IF) assays are indicated. Positions of amino acid substitutions for Mab-resistant mutants are shown. The divergence of amino acids from the prototype HPIV3 1957 strain is shown for six clinical strains. From Coelingh (1991).


F2
F1
Fusion Peptide
Signal Peptide
RTKR FFGGVIGTIALGVATSAQITAAVALV
Membrane Anchor
N
C
CHO
CHO
CHO
CHO
0
100
200
300
400
500
533 AMINO ACIDS
Neutralizing Monoclonal Antibody (MAb) used for selection of Neutralization-Resistant Mutant:
Additional Functional Activity:
NONE
F1
F1
F1
NONE
F1
F1
F1
Recognizes Antigenic Site:
A
B
AB
C
A
A
A
B
Single Amino Acid Change in MAb-Resistant Mutant:
37
73
143
281
395
397
398
452
Divergence in Amino Acid Sequence of 6 Strains, Isolated from 1973-1983, from Prototype 1957 Strain:
1973
1973
1980
1981
1982
1983
0
100
200
300
400
500
533 AMINO ACIDS

Cleavage of the F_0 subunit occurs during maturation, probably in the *trans*-Golgi (Morrison et al., 1985). The cleavage sites of members of the family *Paramyxoviridae* are characterized by varying numbers of basic amino acids. The HPIV3 cleavage site has 3 basic amino acids at its cleavage site, which consists of a pair of basic residues separated from a single arginine residue by a threonine residue (Spriggs et al., 1986). BPIV3 has the same motif as HPIV3 (Suzu et al., 1987), however, Sendai virus is characterized by a single basic residue (Shioda et al., 1986).

Sendai virions grown in the allantoic sac of chick embryos possess cleaved, functional F protein, conversely, Sendai virions grown in MDBK cells possess inactive, uncleaved F protein. It was initially thought that the MDBK cells lacked the host enzyme necessary for intracellular cleavage and that the ability of the host cell to cleave the F protein must be considered a factor in host range and tissue tropism (Scheid and Choppin, 1974). However, it has been shown that it is not the cell line that lacks the appropriate cleavage enzyme, but that the single arginine residue at the Sendai virus cleavage site is resistant to the protease(s), which recognizes multibasic motifs.

Using an antibody specific for the F_2 subunit of Sendai virus that inhibited cleavage, Tashiro and coworkers (1993) showed that proteolytic cleavage of Sendai virus occurs extracellularly by factor Xa from the allantoic fluid of chick embryos *in ovo* and by the enzyme Tryptase Clara from rat lungs *in vivo*. Similarly, avirulent strains of NDV have 2 basic residues separated by a non-basic residue

instead of the 2 pairs of basic residues separated by a single non-basic residue observed in virulent strains. The F_0 protein of avirulent strains is cleaved only in certain types of cells such as the endodermal cells of the chorioallantoic membrane of chick embryos, whereas the F_0 proteins of virulent strains of NDV are cleaved in many different cell types *in vivo* and in cell culture (Nagai et al., 1976). Also, cleavage of the F_0 protein from avirulent NDV strains occurs extracellularly, only after the F_0 protein has been transported to the plasma membrane (Sakaguchi et al., 1991). Cleavage of the F_0 precursor protein is not a prerequisite for transport since it is found on the surface of infected cells and is incorporated into virions.

Proteases that are involved in the processing of dibasic or multibasic cleavage sites belong to a family of subtilisin-like eucaryotic enzymes, of which the prototype is the Kex2 protease. Kex2 is a yeast cell protease which activates the precursors of mating factor α and killer toxin. Kex2 is also able to process mammalian proproteins at a basic tetrapeptide motif. Other mammalian proprotein convertases were identified by gene homology, among these enzymes is the subtilisin-like endoprotease furin, which belongs to a group of paired basic amino acid cleavage enzymes (PACE). HPIV3 F proteins can be cleaved by exogenous furin, and to a lesser extent, by Kex2. Cleavage characteristics suggest that the enzyme that processes the HPIV3 F_0 protein in monkey kidney cells such as CV-1 cells, is also furin (Ortmann et al., 1994). As well, furin has been shown to cleave the F protein of pathogenic NDV strains (Gotoh et al., 1992).

The internal hydrophobic region that becomes the new amino terminus upon proteolytic cleavage of the paramyxovirus F protein has been implicated in fusion events mediated by the F protein and has been designated as the 'fusion peptide'. Oligopeptides that resemble the F_1 amino terminus of the measles virus F protein were able to inhibit penetration of the virus into the cell, virus-induced syncytium formation and hemolysis (Richardson et al., 1980). Oligopeptides bound specifically to cell membranes but not to measles virus itself, suggesting that the F_1 amino terminus interacts directly with the lipid bilayer of the target membrane (Richardson and Chopin, 1983).

In addition to the fusion peptide, other areas of the paramyxovirus F protein have been identified that might also have a role in the fusion. Monoclonal antibodies specific for the F protein of HPIV3 were capable of inhibiting syncytium formation. Antigenic variants of HPIV3 that escaped neutralization by these antibodies were isolated and analysed and are shown in Figure 4 (Coelingh and Tierney, 1989; Coelingh, 1991). Many of the variants had an amino acid change at position 149 in the F_1 protein in antigenic site AB, adjacent to the fusion peptide. However, regions distal to the fusion peptide also exhibited amino acid mutations that resulted in escape from neutralization. Changes also occurred at position 73 in the F_2 subunit and position 452 in antigenic site B; at position 287 in antigenic site C; and positions 397 and 398 in the F_1 subunit at the carboxyl side of the cysteine rich region in antigenic site A. Antigenic sites A and B are composed of amino acids located in both the F_1 and F_2 subunits, which indicates that they are

formed by protein folding, secondary structure appears to be important for fusogenic activity of the F protein.

IV. Membrane fusion

Fusion from without vs. fusion from within

Fusion constitutes an essential element in the infection of cells by enveloped viruses, whether it is for the introduction of the viral nucleocapsid into the host cell or the lateral dissemination of progeny virus. For viruses of the family *Paramyxoviridae*, fusion occurs as a two-step process at neutral pH (Hoekstra et al., 1988). The first step entails the binding of the virion to the host cell receptor and is mediated by the attachment protein, the second step results in membrane fusion, however, the exact requirements for fusion have not been well defined. Paramxyovirus mediated cell fusion is relatively slow, requiring at least 30 minutes before cell fusion or haemolysis is completed. Cell fusion does not require the addition of cations such as Ca^{2+} or Mg^{2+}, nor is it dependent on a specific pH and can occur within the pH range of 5 to 9. The optimal temperature for fusion lies between 30°C and 40°C. Fusogenic activity is relatively insensitive to the composition of the target membrane although fusion is enhanced by the presence of sialic acid containing gangliosides or glycoproteins (White et al., 1983).

Virus-cell fusion is difficult to measure accurately, consequently cell-to-cell fusion and hemolysis have been widely used to assay for fusogenic activity. Hemolysis, or the leakage of hemoglobin from erythrocytes, provides a quantitative assay for the analysis of fusion. Although fusion does not intrinsically result in leakage of cell contents, freeze-thawing or sonication of viral preparations or membranes containing viral glycoproteins will result in preparations with hemolytic activity (Lenard, 1991).

There are two systems with which to study cell-cell fusion. The first is whole virus-induced fusion between cells ('fusion from without' or FFWO), which occurs very soon after the adsorption of infectious or inactivated virus particles to cells at a high multiplicity of infection. The second type of fusion is fusion of confluent cell monolayers induced many hours later by the infection of cells with fewer virus particles and requires the *de novo* synthesis of virally encoded proteins and their transport to the cell surface ('fusion from within' or FFWI) (Hoekstra, 1990, Lenard, 1991). In this thesis, I will be restricting discussion of cell fusion to FFWI, unless otherwise specified.

Requirements for cell-to-cell fusion

The role of the F protein in pH independent fusion was initially demonstrated for Sendai virus F and HN glycoproteins reconstituted into biologically active lipid vesicles (virosomes). Reconstituted particles containing Sendai virus F and HN

proteins in phosphatidylcholine were hemolytic, although the HN protein could be substituted by wheat germ agglutinin, a lectin that binds erythrocytes (Hsu et al., 1978). The role of the Sendai virus HN protein in virosome fusion appeared to be solely as a binding function. However, in later studies virosomes containing both the Sendai virus F and HN proteins were shown to be more fusogenic than virosomes containing the F protein in the presence of wheat germ agglutinin, the HN protein appeared to enhance the function of the F protein (Bagai et al., 1993). Citovsky and coworkers (1986) have also studied hemolysis mediated by virosomes containing Sendai virus F and HN proteins with phosphatidylcholine, these liposomes did not possess receptors for the HN protein. Virosomes containing the F and HN proteins caused hemolysis but those containing only the F protein did not. These studies suggested that the HN protein served some function other than simply receptor binding, and that the HN protein had an active role in fusion. Further studies using a desialylated human hepatoblastoma (Hep-2) cell line (Bagai et al., 1993), showed that virosomes containing the Sendai F protein were fusogenic, but that virosomes containing both the F and HN proteins showed higher levels of fusion.

Various expression systems have been used to express the fusion and receptor-binding glycoproteins and to assess biological activities of these proteins in the absence of other viral proteins. The F and HN proteins of SV5 have been expressed using simian virus 40 (SV40) as a eucaryotic expression vector (Paterson et al., 1985). Glycoproteins expressed by SV40 F and HN recombinant

viruses were anchored in the cell membrane and were biologically active. The expression of the SV5 F protein on the surface of cells resulted in syncytium formation in a monkey kidney (CV-1) cell line. Similarly, expression of the measles virus F protein using recombinant adenovirus type 5 (Ad5) resulted in syncytium formation in two different cell lines (Alkhatib et al., 1990). The measles F protein was fusogenic in both 293 and HeLa cell lines which are derived from human epithelial cells. Although fusion can be induced by measles virus at a multiplicity of infection of 20 for both 293 and HeLa cell lines, recombinant adenoviruses required a multiplicity of infection of 50 and 300, respectively, for cell fusion.

BPIV3 F and HN proteins were expressed on the surface of infected cells by recombinant vaccinia viruses, however, expression of the F protein alone was not sufficient to cause fusion. To avoid problems arising from the severe cytopathic effects of vaccinia virus, Sakai and Shibuta (1989) employed two cell lines, CV-1 cells, a monkey kidney cell line that rapidly exhibited cytopathic effects following vaccinia virus infection, and MDBK cells, a bovine kidney cell line that shows no gross cytopathic effects following infection by vaccinia virus since the infection is abortive. The use of the two cell lines enabled syncytium formation by the BPIV3 proteins to be observed. CV-1 cells were infected with recombinant vaccinia viruses carrying the BPIV3 F or HN genes and then transferred onto confluent, uninfected monolayers of MDBK cells. Coexpression of the BPIV3 HN and F proteins were necessary for syncytium formation in MDBK cells, which could be

inhibited by monoclonal antibodies specific for the BPIV3 HN protein (Sakai and Shibuta, 1989).

V. Statement of objectives

Previous studies indicated that the F proteins of both SV5 and measles virus were fusogenic when expressed in mammalian cells, whereas both the F and HN proteins of BPIV3 were required to produce cell fusion. When the F protein of HPIV3 was expressed in monkey kidney cells using recombinant vaccinia viruses, syncytium formation was not observed, however, cell fusion could have been masked by the gross cytopathic effects that resulted from vaccinia virus infection. These studies began with a search for a cell line that was not susceptible to the rapid cytopathic changes caused by vaccinia virus infection and led to the use of HeLa $T4^+$ cells.

Therefore, the first objective of this thesis was to determine the component or components of HPIV3 required to produce fusion from within. Recombinant vaccinia viruses were used to express the HPIV3 F and HN glycoproteins and the formation of syncytia in HeLa $T4^+$ cell monolayers as well as a hemolysis assay were used to measure fusogenic activity.

In view of the different protein requirements for the production of syncytium formation observed following expression of paramyxovirus glycoproteins in

eucaryotic cells, the second objective of this study was to use recombinant adenoviruses to express the HPIV3 glycoproteins and to compare the requirements for cell fusion using recombinant adenoviruses to those using recombinant vaccinia viruses. Expression of HPIV3 F and HN glycoproteins by recombinant adenoviruses and the ability to elicit an antibody response in mice was also examined as preliminary experiments designed to assess the potential of recombinant adenoviruses as immunogens as a prelude to protection studies in cotton rats.

The final objective of this study was the mutagenesis of the HPIV3 F protein to identify regions on the F protein that were critical for fusogenic activity. The effects of amino acid substitutions within specific domains in the HPIV3 F protein on surface expression and syncytium formation were assessed using a modified vaccinia virus-based expression system. The choice of structural motifs in the HPIV3 F protein as sites for mutagenesis, was based upon the observation that mutation in similar regions of the gp41 transmembrane protein of human immunodeficiency virus type 1 (HIV-1) had been shown to affect fusogenic activity.

MATERIALS AND METHODS

I. Cells, viruses and antibodies

Viruses

Human parainfluenza virus type 3 (HPIV3), strain C-243 (American Type Culture Collection (ATCC), strain 47885) was originally obtained from D.A. McLeod at the Laboratory Centre for Disease Control (LCDC, Ottawa, ONT). Recombinant vaccinia virus Vsc8, carrying the *Escherichia coli* lacZ gene, was provided by D. Hamish (McMaster University, Hamilton, ONT), with the permission of B. Moss (National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD).

Recombinant vaccinia virus vTF7-3, carrying the bacteriophage T7 RNA polymerase gene was obtained from ATCC (Rockville, MD). Recombinant vaccinia viruses VF and VHN, carrying the complete coding sequences of the HPIV3 fusion (F) cDNA, and hemagglutinin-neuraminidase (HN) cDNA, respectively, were constructed by M-J. Côté (Côté, 1989).

Adenovirus type 5 (Ad5) was provided by S.A. Sattar (University of Ottawa). Recombinant adenoviruses AdF and AdHN, carrying the complete coding sequences of the HPIV3 F cDNA and HN cDNA, respectively, were provided by L. Prevec and F.L. Graham (McMaster University) (Prevec et al., 1989).

Cells

The Rhesus monkey kidney cell line, LLC-MK_2, was obtained from Flow Laboratories (Rockville, MD). The African green monkey kidney cell line CV-1 was obtained from ATCC. Human epithelial-like cell lines HeLa, HeLa $T4^+$ and the human embryonic kidney cell line, 293, were obtained from the NIAID AIDS Research and Reference Reagent Program (Rockville, MD). The baby hamster kidney cell line BHK-21, was provided by K.E. Wright (University of Ottawa).

Antibodies

Rabbit antiserum raised against purified HPIV3 virions was provided by D.G. Storey (University of Ottawa). Monospecific rabbit antiserum raised against purified HN protein was provided by R. Ray (Ray and Compans, 1987). Monoclonal antibodies specific for the HPIV3 F glycoprotein (a197, b48, c128, c215, c145, a640, a591, b4, b19 and b69), were provided by B. Murphy and S. Hall (NIAID, NIH) and described by Coelingh and Tierney (1989).

Mouse sera, prepared according to a protocol described previously (Prevec et al., 1989) were provided by L. Prevec (McMaster University). Six to seven week old female Balb/c mice (Charles River Laboratories, St. Constant, Quebec), were immunized by intraperitoneal injection with approximately 10^7 plaque forming units (PFU) of recombinant adenovirus. Sera from mice inoculated with AdF were collected by tail bleed at 16 or 32 days post-inoculation. Sera from mice inoculated

with AdHN were collected at 21 or 42 days post-inoculation. All mouse sera were heat-inactivated at 56°C for 30 minutes. Normal mouse serum was provided by Karen C. Meysick (University of Ottawa).

Propagation of cell lines

All cell lines were grown in autoclavable minimum essential medium containing Earle's salts (MEM, Gibco BRL, Burlington, ONT) supplemented with 0.225% sodium bicarbonate (Gibco BRL), 2 mM L-glutamine (Gibco BRL) and 5% (CV-1 and LLC-MK_2 cell lines) or 10% (HeLa, HeLa $T4^+$ and 293 cell lines) heat inactivated fetal bovine serum (FBS, Gibco BRL). Cell lines were grown in 100 mm polystyrene cell culture dishes and maintained at 37°C in a humidified Shel-Lab incubator (John's Scientific Inc., Toronto, ONT), in an atmosphere containing 5% $C0_2$. All cell lines, with the exception of 293 cells, were passaged by dispersing cells in a Tris-buffered saline solution (TBS: 25 mM Tris-HCl pH 7.2, 137 mM NaCi, 5.6 mM glucose, 5.0 mM KCl and 0.7 mM Na_2HP0_4) containing 0.5% trypsin and 5.3 mM EDTA (Gibco BRL). The 293 cell line was passaged using a saline citrate washing solution (130 mM KCl and 15 mM trisodium citrate). All cell lines were grown in the presence of 50 µg/mL of gentamycin (Roussel Canada, Montreal , PQ). In addition, the HeLa $T4^+$ cell line was grown in the presence of 1 mg/mL geneticin (G418, Sigma, St. Louis, MO).

II. Virological methods

Propagation of viruses

HPIV3 virus was propagated and plaque purified in CV-1 cells and titrated in LLC-MK_2 cells. Confluent cell monolayers were infected with HPIV3 at a multiplicity of infection of 5 in a volume of 1.0 mL of maintenance medium without FBS in 100 mm cell culture dishes. The virus was allowed to adsorb to the monolayer for 1 hour at 37°C, during which, the dishes were rocked periodically to prevent drying of the cells. The inoculum was removed and 5 mL of growth medium (GMEM; MEM supplemented with L-glutamine, sodium bicarbonate and FBS) were added to each monolayer. Cells were monitored for cytopathic effects (CPE) and virus was harvested up to 48 hours post-infection. To harvest HPIV3, cell culture fluids were clarified using the JA10 rotor at 8 K rpm for 15 minutes at 4°C in a Beckman (model J2-21M) centrifuge. Supernatants were aliquoted into polypropylene tubes and frozen at -70°C. For virus titration, serial dilutions of HPIV3 were inoculated onto monolayers of LLC-MK_2 cells and, following infection, monolayers were overlaid with a mixture of 0.8% agarose in GMEM and incubated for 72 hours. To visualize plaques, cells were fixed with a solution of 10% formalin-0.8% NaCl for at least 1 hour, and stained with a solution of 0.1% crystal violet after removal of the agarose overlay in running water.

Vaccinia viruses were propagated, plaque purified and titrated using the CV-1 cell line. Prior to infection, one tenth volume of 0.5% trypsin-5.3 mM EDTA was

added to the inoculum and the inoculum was incubated at 37°C for 15 minutes. For preparation of large virus stocks confluent CV-1 monolayers were infected with vaccinia virus at a multiplicity of infection of 0.1 to 1.0 in 1.0 mL of GMEM that did not contain FBS. The virus was allowed to adsorb to the cells for 1 hour at 37°C, the inoculum was removed and 10 mL of GMEM were added. Cells were monitored for cytopathic effects for up to 72 hours, infected cells were harvested when the monolayers had a 'lace like' appearance. Cells were washed once with TBS and scraped from the cell culture dishes in 3 mL of TBS using a rubber policeman. Cells were pelleted in a Sorvall GLC-2B benchtop centrifuge and resuspended in 0.8 mL per 100 mm cell culture dish of GMEM without FBS. Vaccinia virus was released from the cells by three rounds of freezing at -80°C and thawing at 37°C. The final thaw was carried out in a water bath sonicator (Branson 1200). Virus titration was carried out on CV-1 cell monolayers as described previously for HPIV3. Infected cell monolayers were fixed and stained 48 hours post-infection.

Vaccinia viruses were plaque purified after 3 passages on CV-1 cells to ensure a high proportion of recombinant viruses in the stock. After infection, cell monolayers were overlaid with GMEM containing 0.8% agarose (BioRad, Mississauga, ONT). A second GMEM / agarose overlay containing 300 µg/mL of 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (XGal, Boehringer Mannheim Biochemicals, Laval, PQ), was applied 48 hours post-infection for plaque purification of recombinant vaccinia viruses Vsc8, VF and VHN. For recombinant vaccinia virus vTF7-3, the second overlay contained 0.02% neutral red (Matheson

Coleman & Bell, Rutherford, NJ). Plaques were picked using a sterile glass pipette. The preparation of small stocks of viruses was carried out using the protocol described by Mackett and coworkers (1985).

Adenoviruses Ad5, AdF and AdHN were propagated in the 293 cell line. 293 cell monolayers were infected with adenovirus at a multiplicity of infection of 2 for 1 hour at 37°C, the inoculum was removed and GMEM containing 5% FBS was added to the monolayers. Cells were harvested 84 to 96 hours post-infection following the protocol used for vaccinia viruses, with the exception that each infected dish was resuspended in 0.8 mL of GMEM. Virus was released from cells by 12 cycles of freezing/thawing. Titration of adenoviruses was carried out using the HeLa cell line and infected cell monolayers were incubated for 7 days prior to fixing and staining. Plaque purification of adenoviruses was carried out on HeLa cell monolayers using a second overlay containing neutral red.

Plaque reduction assay for HPIV3 neutralization

Approximately 200 plaque forming units (PFU) of HPIV3 in 50 µL of GMEM without FBS, were incubated with serial dilutions of mouse serum in 50 µL of GMEM without FBS for 30 minutes at room temperature. This inoculum was diluted in 300 µL of GMEM and used to infect duplicate monolayers of LLC-MK_2 cells grown in 60 mm tissue culture dishes for 1 hour at 37°C. The inoculum was removed and an overlay of 0.8% agarose in GMEM was added. After 4 days, the cell monolayers

were fixed in formalin-saline and stained with 0.1% crystal violet to visualize plaques. Dilution of mouse serum was plotted against the number of HPIV3 plaques and the neutralization titre was represented by the reciprocal of the dilution of serum required to neutralize 50% of virus.

III. Hemadsorption and hemolysis

Hemadsorption and hemolysis assays

Human erythrocytes for the hemadsorption and hemolysis assays were very kindly donated by A. Bergeron (University of Ottawa) with the help of E. Wanas (University of Ottawa). Using a heparin-coated syringe, blood was collected and centrifuged at 2 K rpm for 5 minutes in a DuPont Sorvall GLC-2B centrifuge. The buffy coat and the serum were both removed. The erythrocytes were then washed twice in cold TBS, stored at 4°C, and used within 24 hours. For the hemadsorption assay, infected cell monolayers were washed once with TBS at 37°C. Two mL of a 0.5% (v/v) suspension of erythrocytes in cold TBS were added to the monolayer which was then incubated for 20 minutes at 4°C. Cell monolayers were washed four times with cold TBS and photographed using a Leitz Labovert FS microscope and Polaroid type 667 film. To assay for neuraminidase activity, monolayers were then incubated at 37°C to release the erythrocytes.

For the hemolysis assay, infected cell monolayers were trypsinized to obtain a single cell suspension. Cells were pelleted at 2 K rpm for 5 minutes in a DuPont Sorvall GLC-2B centrifuge and then washed once in TBS. Aliquots containing 5 X 10^6 cells resuspended in 800 µl of TBS were subjected to three cycles of freezing and thawing to lyse the cells which increased the hemolytic activity (Fraser and Martin, 1978). 200 µL of a 10% suspension of erythrocytes were added to the cell lysates and samples were incubated at 37°C for 16 hours with constant inversion. The ratio of erythrocytes to HeLa $T4^+$ cells was approximately 100:1. Cell debris and erythrocytes were pelleted at 10 K rpm for 2 minutes in a microfuge and the absorbance of the supernatant at 550 nm was determined in a 1 mL cuvette using a Beckman DU-8B spectrophotometer.

IV. Plasmids and plasmid purification

Recombinant plasmids containing HPIV3 F and HN cDNA

Plasmid pIBIF70 was constructed by M-J. Côté and contained 1851 nucleotides corresponding to the complete HPIV3 F gene, inserted between the *HindIII* and *BamHI* sites of the plasmid vector pIBI31 (IBI, New Haven, CT). Plasmid pIBIF70 also contained 70 nucleotides derived from the M gene, gene-end and intergenic sequences located between the M and F genes, and 40 nucleotides from the 3' poly(A) tail of the F mRNA. Plasmid pIBIF70(-) was derived from plasmid pIBIF70

by digestion of plasmid DNA with the restriction endonuclease *PvuII* (New England Biolabs (NEB), Mississauga, ONT) to release a PvuII fragment that included the HPIV3 sequence as well as the T7 polymerase promoter. The digestion mixture containing the PvuII fragment and linearized plasmid vector were extracted with phenol/chloroform and precipitated using ethanol. The PvuII fragment and linearized vector were religated using T4 DNA ligase. Resulting colonies were screened by restriction endonuclease digestion on an 0.8% agarose gel using *XmnI* (Stratagene, LaJolla, CA) and *HindIII* (Promega, Madison, WI) to identify clones with the PvuII fragment in the opposite orientation. For restriction digestion, the buffers used were those recommended by the supplier. The frequency of clones obtained with the PvuII fragment in the reverse orientation was 50%.

Plasmid pHN1 was originally constructed by T. Binder (University of Ottawa) and consisted of the complete HPIV3 HN gene inserted between the *EcoRI* and *BamHI* sites of plasmid vector pBR322. The HPIV3 HN gene consisted of the gene start sequence, 63 nucleotides of nontranslated sequence and the complete coding region consisting of 1716 nucleotides. The 3' end of the gene consisted of the stop codon followed by 6 nucleotides and a *BamHI* restriction site. The EcoRI/BamHI fragment containing the HPIV3 sequences were sub-cloned into plasmid vector pIBI30 (IBI) by J. Dufresne (University of Ottawa) and designated pIBIHN.

Purification of plasmid DNA

Small scale purification of plasmid DNA (mini-prep) was carried out following the procedure outlined by Sambrook and coworkers (1989). For preparation of DNA for sequencing the mini-prep protocol was modified such that DNA was extracted using only chloroform rather than the phenol:chloroform mixture. Large scale purification of plasmid DNA (midi-prep and maxi-prep) was carried out using the reagents and protocols provided with the Qiagen plasmid kit (Chatsworth, CA). Plasmid DNA that was purified using the Qiagen system was quantified using a Varian DMS 200 spectrophotometer.

V. Site-specific mutagenesis

Site-specific mutagenesis of plasmid DNA

Site-specific mutagenesis of the HPIV3 F cDNA in plasmid pIBIF70(-) was carried out using the method described by Kunkel (1985) and the reagents and protocols provided with the Muta-Gene® Phagemid In Vitro Mutagenesis Kit, Version 2 (Bio-Rad, Mississauga, ONT). Mismatched positive-sense HPIV3 F-specific oligodeoxyribonucleotides were synthesized in the laboratory of P.L. Collins or purchased from the University of Ottawa Biotechnology Research Institute (Ottawa, ONT) and are shown in Appendix 1.

Transformation of various *E. coli* strains was carried out using either chemically-prepared competent cells or by electroporation using the Cell-Porator (Gibco BRL). To prepare chemically-competent cells, *E. coli* strain CJ236 (*dut*, *ung*) was grown overnight at 37°C with rigorous shaking to an optical density at 600 nm of 0.8. Cells were chilled on ice for 15 to 30 minutes and centrifuged in a JA20 rotor at 4°C, 3K for 5 minutes in a Beckman J2-21M centrifuge. The supernatants were removed and the cell pellets were resuspended in 20 mL of ice cold 100 mM $MgCl_2$. Cells were again centrifuged and the cell pellets were gently resuspended in 2 mL of ice cold 100 mM $CaCl_2$. A further 20 mL of 100 mM $CaCl_2$ were added and the suspensions were kept on ice for 30 to 90 minutes. After a final centrifugation the cell pellets were resuspended in 2 mL of 85 mM $CaCl_2$ and 15% glycerol. The suspensions were frozen in a bath of dry ice and ethanol in 300 µL aliquots and stored at -80°C.

For preparation of electrocompetent cells, DH5α cells (ϕ80*lacZ*ΔM15) were grown overnight and diluted 1/100 in LB broth (1% w/v bactotryptone, 0.5% w/v bacto yeast extract, 0.05% w/v NaCl) and grown to an OD_{600} of 0.5 to 1.0. Cells were washed two times with dd$H_2O$and resuspended in 3 mL of 15% ultrapure glycerol.

For transformation, approximately 10 ng of DNA was added to 0.1 mL of competent cells and incubated on ice for 1 hour. Tubes were placed in a 42°C water bath for 3 minutes and reincubated on ice for 5 minutes. Cells were grown for 1 hr at 37 °C with rigorous shaking and plated onto LB (1% w/v bactotryptone,

0.5% w/v bacto yeast extract, 0.05% w/v NaCl and 1.2% w/v bacto agar) plates containing 50 µg/mL of ampicillin. For electroporation, approximately 10 ng of DNA were added to 20 µL of electrocompetent cells and grown as described previously.

Plasmids isolated from individual colonies were screened by sequence analysis and the frequency of mutagenesis was greater than 75%. Bacterial clones containing plasmids with the desired mutations were prepared by Qiagen purification and the F genes were sequenced in their entirety.

VI. DNA sequencing

Sanger sequencing of DNA

DNA sequence analysis was carried out using the chain-termination method originally developed by Sanger and coworkers (1977) using the reagents and protocol provided with the Sequenase® Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). Sequencing products were labeled with [α-^{32}P]dATP(Amersham, Oakville, ONT), and 7-deaza-dGTP (Pharmacia, Baie D'Urfé, PQ) was used to inhibit compressions.

Sequencing was carried out on gels 4 mm thick composed of 6% acrylamide, 0.3% bis-acrylamide and 8M urea in a 1X tris-borate buffer (TBE: 89 mM tris-borate, 89 mM boric acid, 2.5 mM EDTA). Gels were electrophoresed using a gradient of 1X to 1.5 X tris-borate buffer at a constant power of 55 watts using an IBI STS-45

sequencing apparatus. After electrophoresis, the sequencing gels were transferred to Whatman 3MM paper and dried *in-vacuo* at 80°C for 1.5 hours. Dried gels were exposed to X-ray film at room temperature.

VII. Expression of HPIV3 glycoproteins

Expression of HPIV3 glycoproteins

HPIV3 F and HN glycoproteins were analyzed following infection of cell lines with HPIV3, recombinant vaccinia viruses, recombinant adenoviruses or following transfection of cell monolayers with recombinant plasmids. Infection with HPIV3 was carried out at a multiplicity of infection of 5; infection with recombinant vaccinia viruses VF and VHN at a multiplicity of infection of 5, and infection with recombinant adenoviruses AdF and AdHN at a multiplicity of infection of 20.

The following method was used for the transient expression of HPIV3 F and HN proteins from plasmid DNA. HeLa T4$^+$ cell monolayers were grown to sub-confluency in 60 mm cell culture dishes. Cell monolayers were infected with recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 10 for 1 hour in a volume of 800 µL of GMEM without FBS. During infection, plasmid DNA was diluted in ddH_2O to a final volume of 50 µL in a 5.0 mL polystyrene tube. In a separate tube, 30 µL of Lipofectin® (Gibco BRL) was diluted in ddH_2O to a final volume of 50 µL. The diluted DNA and Lipofectin® were mixed in a single tube and

allowed to stand at room temperature for 15 minutes. The virus inoculum was removed from the cell monolayer and replaced with 3.0 mL of serum-free, MMEM. The DNA-Lipofectin® mixture was added to the cell monolayer dropwise, with slow and gentle mixing after each drop. If the incubation of the monolayer exceeded 10 hours, the medium was replaced with 5 mL of GMEM supplemented with antibiotics.

The amounts of pIBIF70(-) and pIBIHN plasmid DNA that produced the most extensive fusion following transfection of cell monolayers in 60 mm cell culture dishes (approximately 2.0 X 10^6 cells), were 0.5 µg and 5.0 µg, respectively. For radiolabelling and immunoprecipitation, immunofluorescence or flow cytometry, the amount of F or HN plasmid DNA transfected into cell monolayers was 1.0 µg DNA/10^6 cells.

For photography of cell fusion, cell monolayers were fixed and stained. Cells were washed once in TBS, 2 mL of methanol was added to each cell culture well and incubated at room temperature for 7 minutes. Fixed cells were allowed to air dry. 2 mL of Giemsa stain (Sigma) diluted 1:20 in ddH_2O was added to each well and incubated at room temperature for 60 minutes. Cells were washed 5 times with ddH_2O and allowed to air dry. Photographs were taken using a Pentax 35mm camera on a Cambridge PhotoZoom inverted microscope.

VIII. Immunofluorescence

Immunofluorescence

Cells were assayed either by indirect cell surface immunofluorescence, indirect immunofluorescence following acetone fixation or by flow cytometry. The conditions for incubation of cells with the primary and secondary antibody were identical for all three assays. HPIV3 infected cells were assayed 24 hours post-infection, cells infected with recombinant vaccinia viruses were assayed 16 hours post-infection, cells infected with recombinant adenoviruses were assayed 24 to 48 hours post-infection, and transfected cells were assayed 16 to 18 hours post-transfection. Monoclonal antibodies were used at a dilution of 1:200 and monospecific anti-serum was used at a dilution of 1:50 in TBS. Fluorescein-linked sheep anti-mouse immunoglobulin (Ig) (Amersham) and Texas Red®-linked donkey anti-rabbit Ig (Amersham) were used at a dilution of 1:5 in TBS.

For indirect cell surface immunofluorescence, cell monolayers were trypsinized to produce a single cell suspension, washed and resuspended in TBS. Approximately 5.0×10^5 - 1.0×10^6 cells were aliquoted into 5 mL polystyrene tubes, pelleted and resuspended in 20-25 µL of TBS containing primary antibody and incubated on ice for 45 to 90 minutes. Cells were washed three times in TBS supplemented with 2% bovine serum albumin (BSA), resuspended in 20-25 µL of diluted secondary antibody and incubated 45 minutes to 1 hour. Cells were again washed three time with TBS/2%BSA, resuspended in three drops of mounting fluid,

applied to a glass slide and covered with a coverslip. The mounting fluid for fluorescein was 90% glycerol in TBS, pH 9.6. The mounting fluid for Texas Red® was 90% glycerol in 1M Tris, pH 8.6.

For flow cytometric analysis, cells were divided into aliquots prior to incubation with individual monoclonal antibodies. After incubation with both primary and secondary antibodies, cells were resuspended in 1.0 mL of TBS and passed several times through a 22G1 needle (Becton Dickenson) immediately before analysis using a Coulter EPICS XL fluorescence-activated flow cytometer.

For indirect internal immunofluorescence, cells were grown and infected/transfected on glass slides using 8-well Nunc Lab-tek chamber slides (Gibco BRL). Chambers were removed and slides were immersed in cold acetone for 10 minutes at -20°C and then allowed to air dry. Individual monolayers were re-wet using 20 µL of TBS, each of the eight sections could be treated separately due to the surface tension in this volume of TBS. Immunofluorescence was carried out using the procedure described above. Photographs were taken either using a Zeiss Axioplan Universal microscope or a Leitz Laborlux K microscope and Kodak Ektachrome 400 ASA film.

IX. Protein analysis

Radiolabelling of HPIV3 proteins

Following infection in 100 mm tissue culture dishes, cell monolayers were incubated as follows: i) 18 hrs for HPIV3-infected cells; ii) 24 hrs for Ad5 or recombinant adenovirus infected cells; or iii) 3 hrs for recombinant vaccinia virus infected cells. Cells were then starved of leucine, essential amino acids, or sugars for 1 hour by replacing the medium with 3 mL of either leucine-free MEM (Gibco BRL) supplemented with FBS and L-glutamine, GMEM without essential amino acids, or glucose-free GMEM, the latter two were prepared in the laboratory. Following transfections, cells were incubated for 1-2 hours in starvation medium prior to radiolabelling. Monolayers were radiolabelled for 3 hours by the addition of either 30 µCi/mL of [L-3,4,5-^{3}H (*N*)] leucine (Dupont NEN, Mississauga, ONT), [^{3}H] L-amino acids (Dupont NEN), [D-3,4^{3}H(N)] mannose (Dupont, NEN), or [D-6-^{3}H(*N*)]glucosamine hydrochloride (Dupont NEN).

Cells were scraped from the cell culture dishes, pelleted by centrifugation and washed once in TBS. Cells were resuspended in cold RIPA buffer (50 mM Tris-HCl, pH 7.2 and 150 mM NaCl), 500 µL/100 mm cell culture dish, and transferred into 1.0 mL microfuge tube in 500 µL aliquots. HeLa cells, HeLa T4$^+$ cells and 293 cells were placed on ice for 15 minutes. CV-1 cells and LLC-MK$_2$ cells were lysed immediately. An additional 500 µL of RIPA buffer containing 2X detergents (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.2% SDS, 2% sodium

deoxycholate and 2% triton X-100) were added to each tube. Proteinase inhibitors phenylmethyl-sulfonyl fluoride (PMSF) and benzamidine-HCl were added to all RIPA buffers to final concentrations of 2 mM. Cells were immediately centrifuged for 1 minute in a microfuge to remove nuclei and cell debris. Cell lysates were removed from the tubes and stored in 1 mL aliquots at -80°C.

Immunoprecipitation of HPIV3 proteins

Radiolabelled proteins were immunoprecipitated from cell lysates using either HPIV3-specific rabbit antiserum or monoclonal antibodies specific for HPIV3 F or HN proteins. Each immunoprecipitation reaction was composed of approximately 300 µL of cell lysate, antibody, and either protein A Sepharose® CL-4B beads (Pharmacia), or protein G Sepharose® 4 Fast Flow beads (Pharmacia) in a final volume of 500 µL. Protein A Sepharose® beads (20 mg per reaction), were allowed to swell in RIPA buffer for at least one hour and washed twice by centrifugation in RIPA buffer prior to use. Protein G Sepharose® beads were provided as a suspension and the equivalent of 10 mg were used per reaction washed twice by centrifugation in RIPA buffer prior to use. Five µL of monoclonal antibody or 30 µL of rabbit antiserum were used in each reaction. The mixture was incubated overnight at 4°C with constant mixing by rotation. After incubation, the beads were washed 5 times by centrifugation with RIPA buffer containing 1X detergents. Beads were resuspended in 25 µL of 2X sample buffer (62.5 mM Tris-

HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue and 10% β-mercaptoethanol) and heated to 95°C for 5 minutes. The beads were then removed by centrifugation and samples were either stored at -80°C or subjected to electrophoresis immediately on SDS-polyacrylamide gels.

Polyacrylamide gel electrophoresis

Protein analysis was carried out by electrophoresis in 10% polyacrylamide gels containing SDS (SDS-PAGE) under reducing or nonreducing conditions. The resolving gel was composed of 10% acrylamide, 0.27% bis-acrylamide, 0.1% SDS and 750 mM Tris-HCl, pH 8.8. The stacking gel was composed of 5% acrylamide, 0.13% bis-acrylamide, 0.1% SDS and 66 mM Tris-HCl, pH 6.8, both the resolving and stacking gels were polymerized using 0.1% ammonium peroxodisulphate (BDH, Toronto, ONT) and a 1/1000 dilution of TEMED (NNN'N'-tetramethylethylenediamine, BDH). Rainbow™ coloured protein markers (Amersham) were used to estimate molecular mass. SDS-PAGE gels were subjected to electrophoresis in a running buffer solution (25 mM Tris base, 192 mM glycine and 0.1% SDS) at 100 volts for 90 minutes (mini-sized gel) or 45 volts overnight (large-sized gel). After electrophoresis the gels were fixed in 30% methanol and 10% glacial acetic acid and prepared for fluorography using either Amplify (Amersham) or Enhance (Amersham). Gels were dried under vacuum for 1½ hours at 60°C and exposed to X-ray film at -80°C.

Chemical cross-linking of HPIV3 F proteins

Following radiolabelling of cell cultures, medium was removed and cells were washed 2X in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4). Cells were scraped from 100 mm cell culture dishes and divided into 3 aliquots. One aliquot was used for chemical cross-linking prior to cell lysis, using DTSSP (3,3'-dithiobis-(sulfosuccinimidylpropionate), Pierce, Rockford IL), These cells were washed twice by centrifugation in ice-cold PBS and resuspended in 10 µL of PBS. 1 µL of a 100 mm solution of DTSSP was added and the cells were incubated on ice for 30 minutes. Cells were then washed twice by centrifugation in ice-cold PBS and lysed in 300 µL of NP40 lysis buffer (1% NP40, 50 mM Tris-HCl, pH 8.0 and 100 mM NaCl), for 15 minutes on ice. Nuclei and cell debris were removed by centrifugation for 30 seconds at 10 K rpm in a microfuge. PMSF and benzamidine-HCl were both added to final concentrations of 2 mM.

The second fraction of cells was used for cross-linking of proteins at the time of cell lysis. Cells were washed twice by centrifugation with ice-cold PBS and lysed in 300 µL of NP40 lysis buffer immediately before the addition of DTSSP. All subsequent steps were as described above.

The third fraction of cells was lysed using NP40 lysis buffer and proteins were immunoprecipitated without exposure to DTSSP. Cross-linked and immunoprecipitated proteins were separated by electrophoresis under nonreducing

conditions in 3.5% polyacrylamide boric acetate gels according to the protocol published by Gething and coworkers (1989).

Bovine L-glutamate dehydrogenase (GIDH, Boehringer Mannheim), was used as a molecular mass marker. GIDH was resuspended and dialyzed overnight in 0.2M triethanolamine-HCl, pH 8.5, using a Pierce Slide-A-Lyzer 10K dialysis cassette and four changes of buffer before being cross-linked using DTSSP according to the protocol described by Gething and coworkers (1989).

X. Densitometry

Densitometry of radiolabelled proteins

Photoimages of autoradiographs were captured using the Biophotonics Gelprint 2000i (BIO/CAN Scientific, Mississauga, ONT), and densitometry was carried out using the computer software GPTools version 3.01 (Biophotonics). The percentage of cleaved ^{3}H-leucine labelled HPIV3 F protein was estimated from the densitometric values for F_0 and F_1 using the following formula:

$$F_1 (1.3) / \ F_1(1.3) + F_0$$

The factor of 1.3 is the ratio of the number of leucine residues in F_0 compared to F_1 (without signal sequence), which are 46 and 35 respectively.

PART I: REQUIREMENTS FOR CELL FUSION MEDIATED BY HPIV3

INTRODUCTION

Expression of SV5 F and HN envelope glycoproteins by recombinant SV40 vectors demonstrated that the HN protein possessed both hemagglutinin and neuraminidase activities, and that the F protein mediated syncytium formation of infected cell monolayers (Paterson et al., 1985). Similarly, expression of the measles virus F and H proteins by recombinant adenoviruses also demonstrated the fusogenic activity of the F protein (Alkhatib et al., 1990). For BPIV3, however, expression of the F and HN proteins by recombinant vaccinia viruses showed that the F protein was fusogenic only upon coexpression of the HN protein (Sakai and Shibuta, 1989). Therefore, the first objective of this study was to determine whether the HPIV3 F protein was functional on its own, or, whether coexpression of both F and HN proteins was necessary for cell-to-cell fusion.

Vaccinia virus was chosen as the expression vector since it was commonly used and had been well characterized biochemically and genetically. Vaccinia virus, like HPIV3, synthesize mRNA that are capped, methylated and polyadenylated, as well, the entire life cycle of the vaccinia virus occurs in the cytoplasm (Mackett et al., 1985). Construction of recombinant vaccinia viruses carrying the HPIV3 F and HN cDNA was accomplished by M-J. Côté (Côté, 1989). Recombinant vaccinia viruses

that expressed the HPIV3 F and HN proteins had also been used previously as immunogens in a cotton rat model to assay their potential as candidates for live vaccines against HPIV3 (Spriggs et al., 1987).

Vaccinia virus is a well-characterized member of the family *Poxviridae*. The genome of vaccinia virus consists of approximately 180 kb of DNA and has a coding capacity for more than 150 polypeptides. Although vaccinia is a DNA virus, its life cycle, like that of HPIV3, is completed entirely in the cytoplasm. Recombinant vaccinia viruses are created by *in-vivo* homologous recombination between a plasmid carrying foreign DNA and the viral genome. The plasmid pSC11, which was used as a vector for HPIV3 cDNA, contained two vaccinia virus promoters flanked by sequences derived from the vaccinia virus thymidine kinase gene as well as the *E. coli* β-galactosidase gene, which served as a marker during plaque purification (Mackett and Smith, 1986).

The recombinant vaccinia virus expressing the HPIV3 F protein did not induce fusion in CV-1 cells (M-J. Côté, 1989). Also, LLC-MK_2 or CV-1 cells infected with vaccinia virus exhibited extensive cytopathic effects which included cell rounding and shrinkage 6 to 8 hours post-infection, making it difficult to detect syncytia (data not shown). The HeLa $T4^+$ cell line is a transformed HeLa cell line that expresses the $CD4^+$ molecule and was originally created to support infection by human immmunodeficiency viruses (Maddon et al., 1986). Although HeLa cells infected with vaccinia virus exhibit rapid cytopathic effects, the HeLa $T4^+$ cell line does not exhibit these cytopathic changes for up to 24 hours post-infection (Mulligan et al.,

1990). This cell line was therefore selected for its ability to delay cytopathic changes as a result of infection by recombinant vaccinia viruses and to provide a system in which to examine syncytium formation.

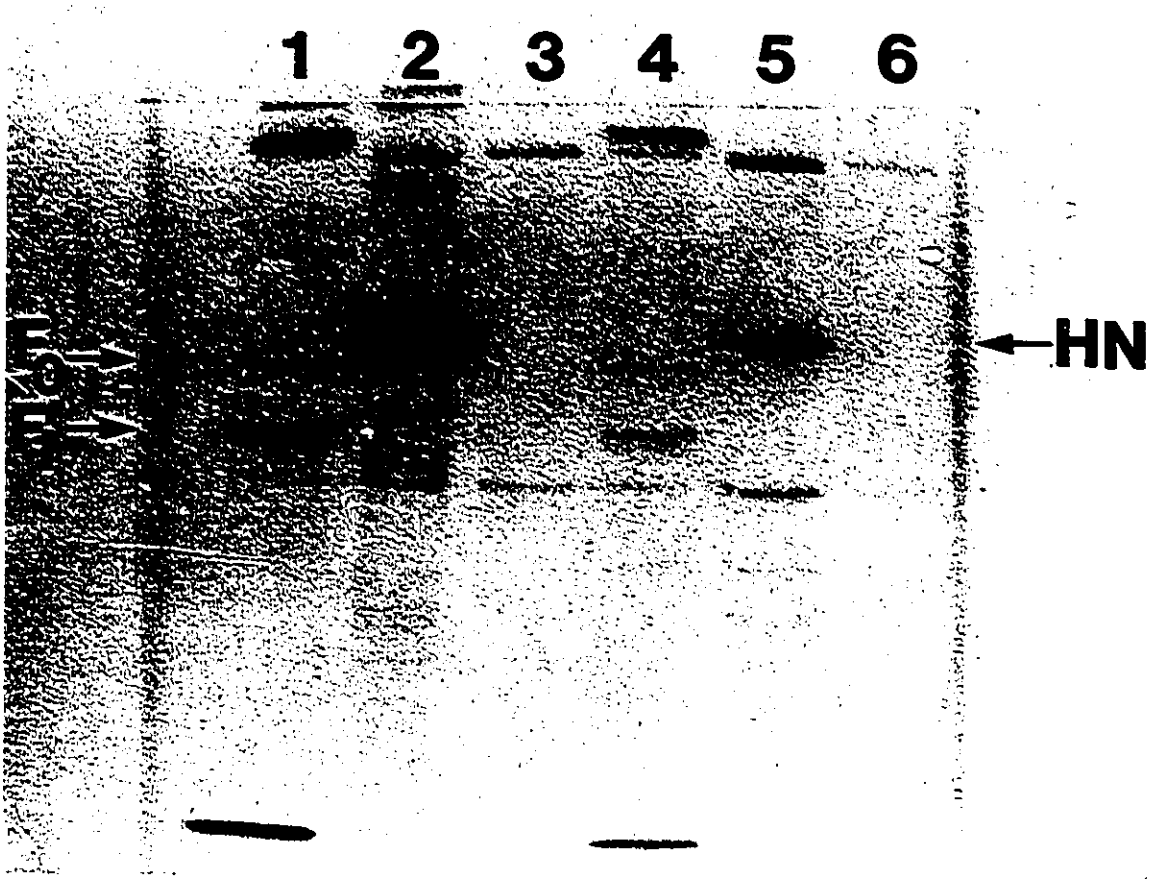
RESULTS

I. Expression of HPIV3 F and HN proteins

The HPIV3 F and HN proteins were expressed in HeLa T4$^+$ cells using recombinant vaccinia virus vectors. To characterize the HPIV3 F and HN glycoproteins expressed by recombinant vaccinia viruses, HeLa T4$^+$ cell monolayers were infected with HPIV3 or recombinant vaccinia viruses VF, VHN or Vsc8 at a multiplicity of infection of 5. Recombinant vaccinia virus Vsc8 carried the β-galactosidase gene and served as a vaccinia virus control. HPIV3-infected cells were incubated for 18 hours and cells infected with recombinant vaccinia viruses were incubated for 3 hours, starved for 1 hour in medium free of essential amino acids and labelled for 3 hours with [^{3}H]-L-amino acids.

HPIV3 proteins were immunoprecipitated from lysates of HPIV3-infected cells using monoclonal antibodies specific for the F or HN proteins (Rydbeck et al., 1986) and analysed by electrophoresis on 10% polyacrylamide gels containing SDS using reducing conditions. Gels were fixed, processed for fluorography and dried. Results are shown in Figure 5. The monoclonal antibody specific for the HPIV3 F protein recognized two proteins in lysates of VF-infected cells (Figure 5, lane 4) with apparent molecular weights similar to the F_0 (63K) and F_1 (50K) proteins immunoprecipitated from HPIV3-infected cell lysates (Figure 5, lane 1). The HPIV3

Figure 5. Immunoprecipitation of HPIV3 F and HN proteins expressed by recombinant vaccinia viruses VF and VHN. HeLa T4$^+$ cells were infected with virus and labelled with [^{3}H]-L amino acids. Cells were lysed and proteins were immunoprecipitated from cell lysates with monoclonal antibodies specific for the HPIV3 F protein (Lanes 1 and 4), the HN protein (Lanes 2 and 5) or with both monoclonal antibodies (Lanes 3 and 6). Proteins were analyzed by electrophoresis on a 10% polyacrylamide gel containing SDS under reducing conditions. The gels was fixed, processed for fluorography, dried and exposed to X-ray film. Lanes 1 and 2, HPIV3-infected cells; Lane 3, Vsc8-infected cells; Lane 4, VF-infected cells; Lane 5, VHN-infected cells; Lane 6, mock-infected cells.


1
2
3
4
5
6
HN

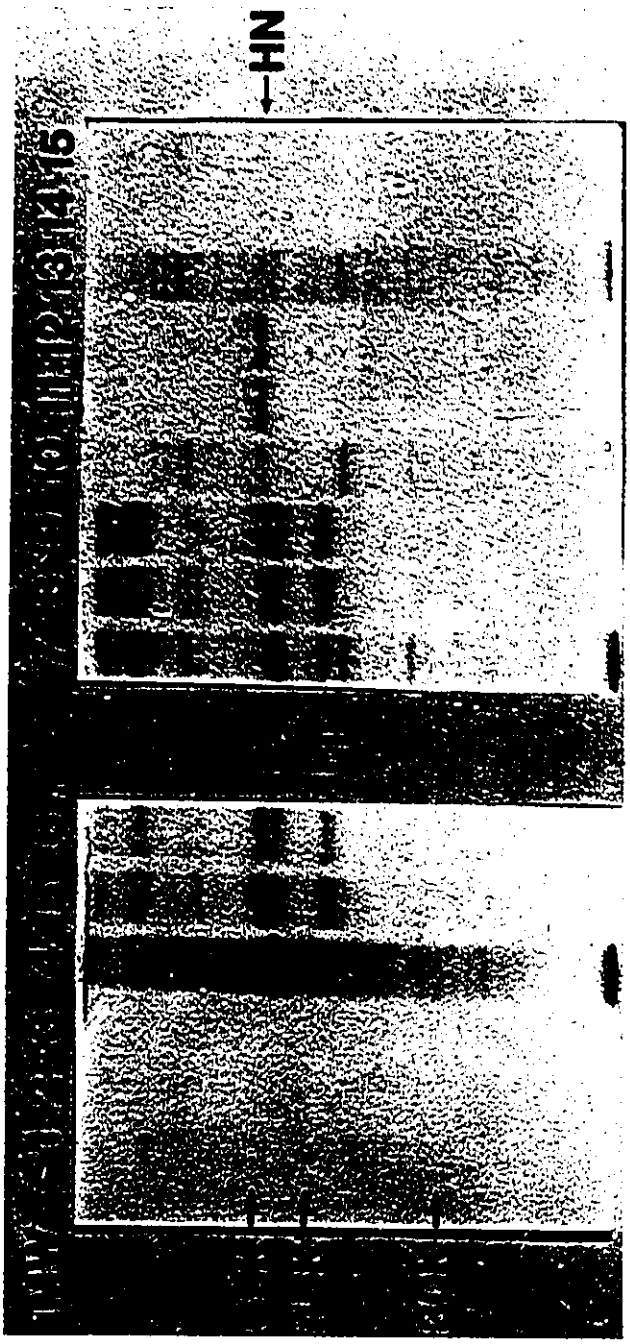
fusion protein expressed by recombinant vaccinia virus VF, therefore, was cleaved into its functional subunits F_1 + F_2, however, the F_2 subunit is not usually observed following electrophoresis on 10% polyacrylamide gels. The monoclonal antibody specific for the HPIV3 HN protein recognized a single protein in lysates of VHN-infected cells (Figure 5, lane 5) that comigrated with the HN protein (69K) immunoprecipitated from lysates of HPIV3-infected cells (Figure 5, lane 2). No corresponding proteins were detected in lysates from Vsc8-infected cells or lysates from mock-infected HeLa $T4^+$ cells (Figure 5, lanes 3 and 6). Therefore, the F and HN proteins expressed by the recombinant vaccinia viruses VF and VHN, possessed electrophoretic and immunologic properties similar to those of authentic HPIV3 glycoproteins.

Mannose and glucosamine are present not only in the core oligosaccharide of immature glycoproteins before trimming of sugars, but also in mature glycoproteins (Klenk and Rott, 1980). It was observed that labelling with sugars decreased the background of non-specific proteins immunoprecipitated from cell lysates when compared to [^{3}H]-leucine labelling. Therefore, infected cells were labelled with [^{3}H]-mannose or [^{3}H]-glucosamine and proteins were immunoprecipitated from cell lysates with rabbit antiserum raised against purified HPIV3. Immunoprecipitated proteins were resolved by electrophoresis and gels were prepared as described previously. The F and HN protein products produced by recombinant vaccinia viruses VF and VHN were labelled with glucosamine and mannose. Therefore, both

proteins expressed by recombinant vaccinia viruses were glycosylated. The strong protein band labelled with [^{3}H] leucine and immunoprecipitated by anti-HPIV3 rabbit antiserum from lysates of HPIV3-infected cells, and which comigrated with the F_0 protein, is the HPIV3 NP protein (Figure 6, lane 4). The NP protein masks the presence of the F_0 protein but was not seen when proteins were labelled with sugars or following immunoprecipitation with monospecific or monoclonal antibodies.

A protein with an apparent molecular weight of >200K was immunoprecipitated from lysates of HPIV3- and VF-infected cells using monoclonal antibody specific for HPIV3 F (Figure 5, lanes 1 and 4), or anti-HPIV3 rabbit serum (Figure 6, lanes 4 to 9). This protein or protein complex, was not immunoprecipitated from lysates of HPIV3-infected cells by monoclonal antibody specific for the HPIV3 HN protein nor from lysates of mock-infected or Vsc8-infected cells with monoclonal antibody specific for the F protein or anti-HPIV3 rabbit serum. Since this large protein (or protein complex) (>200K) was radiolabelled with sugars and immunoprecipitated from lysates of HPIV3- or VF-infected cells using HPIV3-specific rabbit serum, it likely includes at least one glycoprotein.

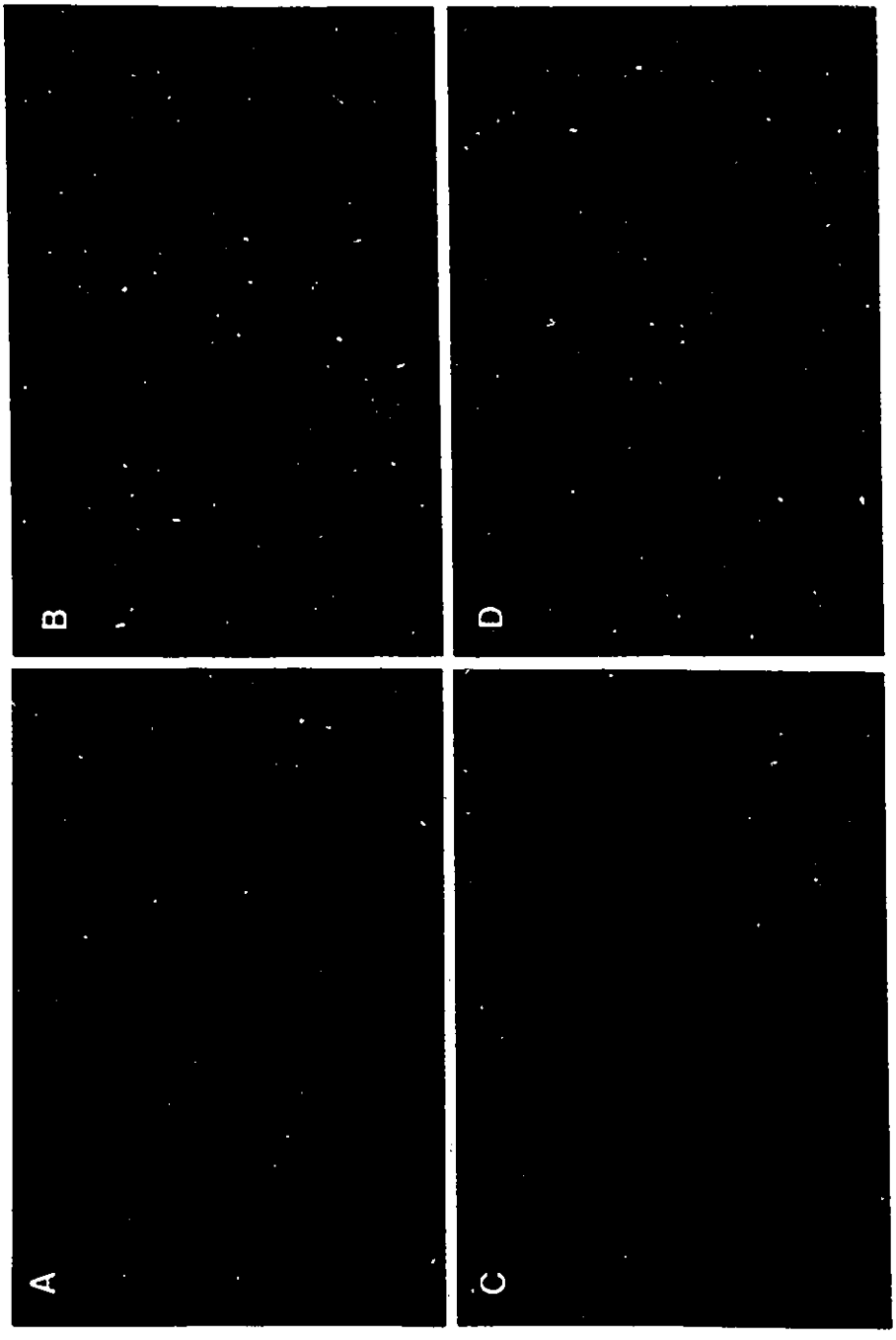
Figure 6. Immunoprecipitation of sugar-labelled glycoproteins expressed by recombinant vaccinia viruses VF and VHN. HeLa T4$^+$ cells were infected with virus and labelled with [^{3}H]-leucine (Lanes 1, 4, 7, 10 and 13), [^{3}H]-mannose (Lanes 2, 5, 8, 11 and 14) or [^{3}H]-glucosamine (Lanes 3, 6, 8, 12 and 15). Cells were lysed and proteins were immunoprecipitated from cell lysates with anti-HPIV3 rabbit antiserum specific for HPIV3 and resolved by electrophoresis on a 10% polyacrylamide gel containing SDS under reducing conditions. Gels were fixed, processed for fluorography, dried and exposed to X-ray film. Lanes 1, 2 and 3, mock-infected cells. Lanes 4, 5 and 6, HPIV3-infected cells. Lanes 7, 8 and 9, VF-infected cells. Lanes 10, 11 and 12, VHN-infected cells. Lanes 13, 14 and 15, Vsc8-infected cells.


HN
HHPR(3)1415

II. Cell surface expression of F and HN proteins

Transport to the cell surface is crucial to the function of the HPIV3 F and HN glycoproteins. To determine whether the F and HN proteins expressed by recombinant vaccinia viruses were transported to the cell surface, infected HeLa $T4^+$ cells were examined by indirect immunofluorescence. Cells infected with recombinant vaccinia viruses VF, VHN or Vsc8, were trypsinized 18 hours post-infection, washed, and incubated with primary antibodies. Primary antibodies included a mouse monoclonal antibody specific for the HPIV3 F protein and rabbit antiserum specific for the HPIV3 HN protein. Secondary antibodies included fluorescein(FITC)-conjugated sheep anti-mouse immunoglobulin and Texas Red-conjugated donkey anti-rabbit immunoglobulin. Primary and secondary antibodies were tested alone or as two mixtures. The emission spectra of FITC and Texas Red chromophores do not overlap and give independent signals when viewed either through an FITC or a rhodamine filter (Parks et al., 1989). The same microscopic fields were photographed first through an FITC filter and then through a rhodamine filter. F and HN proteins were both present on the surface of HeLa $T4^+$ cells coinfected with VF and VHN (Figure 7, panels C and D), and appeared to be distributed in a diffuse pattern. Vsc8-infected cells were negative for surface immunofluorescence (Figure 7, panel A).

Figure 7. HPIV3 glycoproteins are present on the surface of HeLa T4$^+$ cells infected with recombinant vaccinia viruses VF and VHN. HeLa T4$^+$ cells were infected at a multiplicity of infection of 5 and incubated for 18 hours. Cells were trypsinized, washed and resuspended in a mixture of primary antibody consisting of a 1:200 dilution of a mouse monoclonal antibody specific for the HPIV3 F protein and a 1:50 dilution of rabbit antiserum specific for the HPIV3 HN protein. Cells were washed and resuspended in a solution of secondary antibody consisting of 1:5 dilutions of FITC-conjugated sheep anti-mouse and Texas Red-conjugated donkey anti-rabbit immunoglobulin. Cells were mounted on a microscope slide and photographed using a Leitz Labovert FS microscope. Panel A, Vsc8-infected cells photographed through an FITC filter. Panel B, same field as A, photographed using bright field microscopy. Panel C, VF- and VHN-infected cells photographed through an FITC filter. Panel D, same field as C, photographed using a rhodamine filter.


B
D
A
C

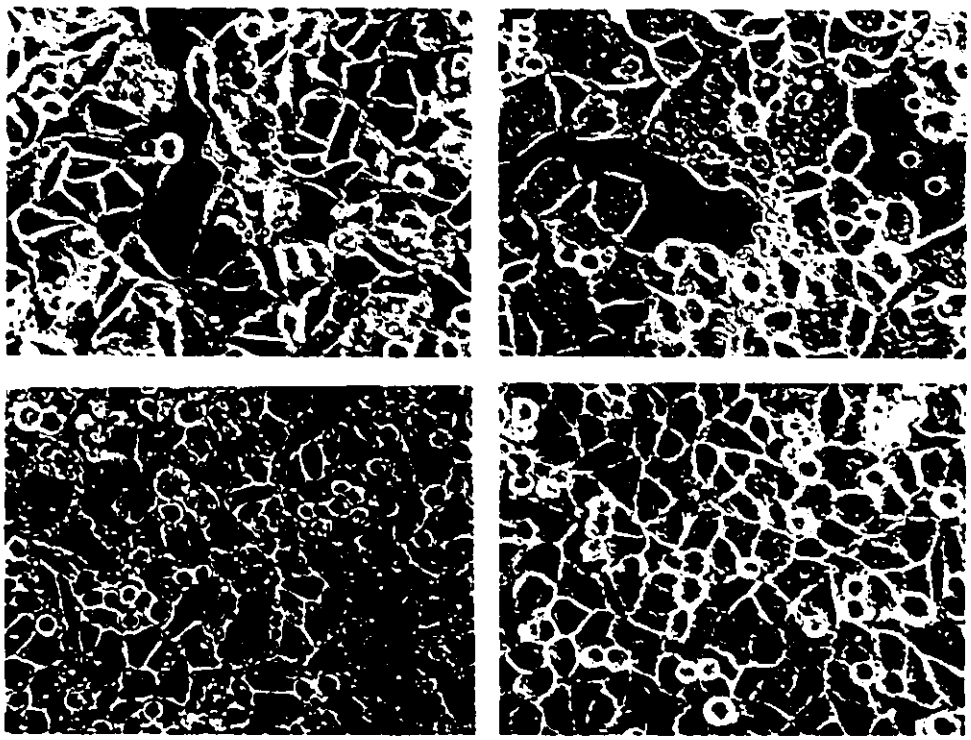
III. Biological activities of F and HN proteins

Hemagglutinin and neuraminidase activities

The HN glycoprotein of HPIV3 possesses two biological activities, the hemagglutinin activity is responsible for adsorption of virions to host cell receptors (Scheid and Choppin, 1974), and the neuraminidase activity is likely responsible for the release of newly assembled virus particles from the cell surface (Huang et al., 1980). To determine whether the HN protein expressed by recombinant vaccinia virus possessed both of these activities, VHN-infected cells were assayed for their ability to bind and release human erythrocytes by removal of sialic acid from receptor molecules (Huberman et al., 1995), which indicated functional hemagglutinin and neuraminidase activities.

HeLa T4$^+$ cells were infected with either HPIV3 (Figure 8, panel C), VHN (Figure 8, panel A), Vsc8 (Figure 8, panel D), or coinfected with VF + VHN (Figure 8, panel B), at a multiplicity of infection 5 for 18 hours for HPIV3-infected cells and 8 hours for vaccinia virus-infected cells. Cells were overlaid with a 0.5% suspension of human erythrocytes for 1 hour at 4°C. Both HPIV3- and VHN-infected cells adsorbed human erythrocytes, demonstrating functional hemagglutinin activity. Cells coinfected with VF + VHN virus also bound human erythrocytes, whereas Vsc8 infected cells did not. Shifting the incubation temperature from 4°C to 37°C resulted in the release of human erythrocytes from monolayers

Figure 8. Hemadsorption of human erythrocytes to infected cell monolayers. HeLa $T4^+$ cell monolayers were infected with either HPIV3 or recombinant vaccinia viruses at a multiplicity of infection 5 and overlaid with a 0.5% suspension of human erythrocytes at 18 hours post-infection for HPIV3-infected cells and 8 hours for vaccinia virus-infected cells. Monolayers were washed vigorously 3 times and photographed. Panel A, VHN-infected cells. Panel B, VF- and VHN-infected cells. Panel C, HPIV3-infected cells. Panel D, Vsc8-infected cells.



infected with HPIV3, VHN or VHN + VF, and demonstrated functional neuraminidase activity (data not shown).

Fusion activity

At the time that these experiments were initiated, the fusion proteins of measles virus and SV5 had been shown to be fusogenic in the absence of additional viral proteins (Paterson et al., 1985; Alkhatib et al., 1990). However, the fusion protein of BPIV3 required coexpression of the BPIV3 HN protein to mediate syncytium formation (Sakai and Shibuta, 1989). The HPIV3 F protein expressed by recombinant vaccinia virus VF was glycosylated, cleaved into functional subunits and transported to the cell surface, yet fusion of CV-1 cells following infection with VF could not be demonstrated. The lack of visible cell fusion following infection by VF could have been attributed to the absence of another component of HPIV3 that was required for fusion, however, it was also possible that the gross cytopathic effects caused by vaccinia virus infection masked cell fusion.

Several other cell lines were infected with Vsc8 or VF, these included: another monkey cell line (LLC-MK_2), two human cell lines (HeLa and 293), and a hamster cell line (BHK-21), which exhibited gross cytopathic effects (data not shown). Extensive cell fusion of HeLa $T4^+$ cells was observed following HPIV3 infection, but these cells did not exhibit gross cytopathic effects up to 24 hours following infection with recombinant vaccinia virus Vsc8 (data not shown). Cell fusion was not,

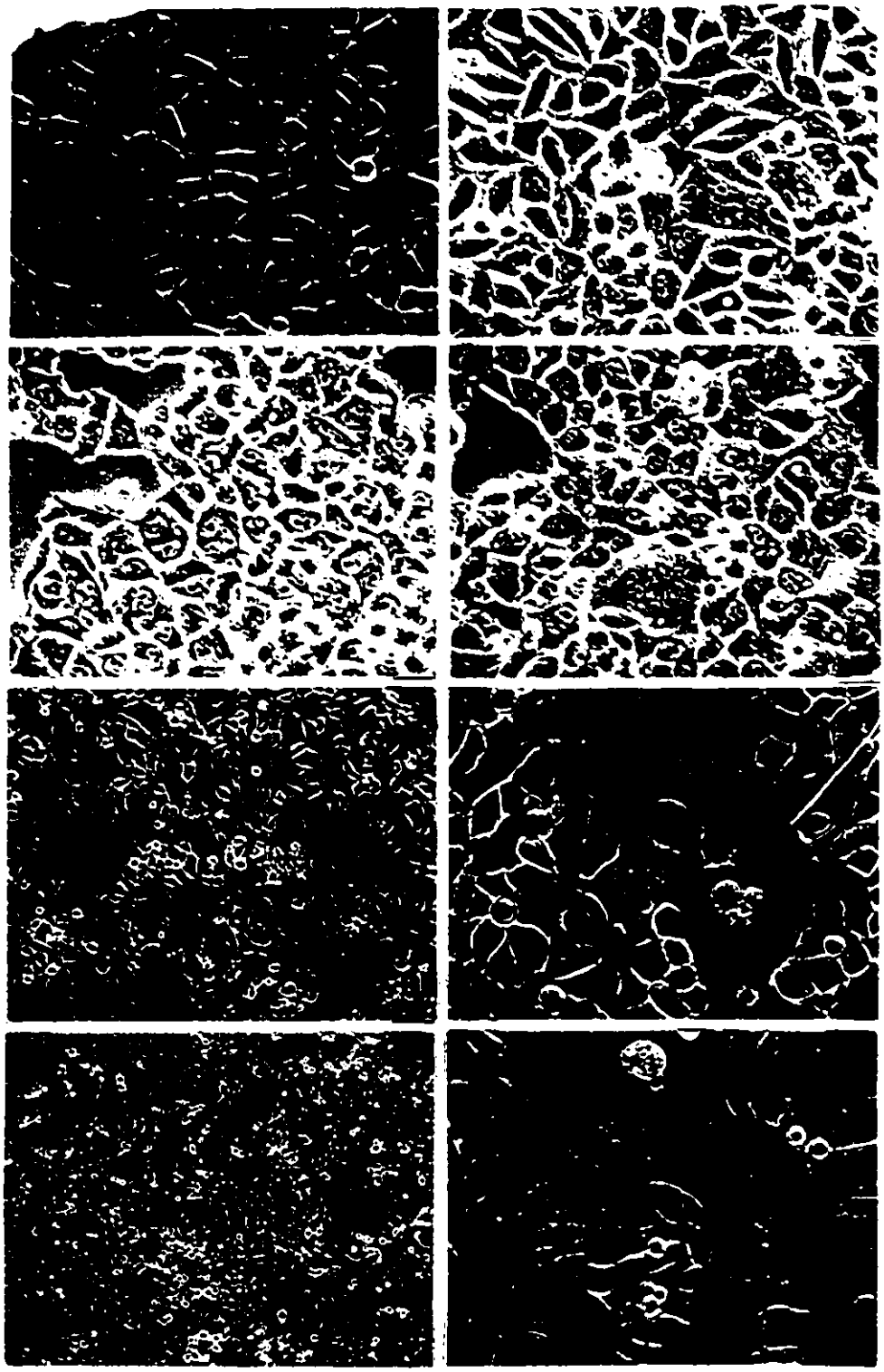
however, observed following infection of HeLa T4$^+$ cells with VF up to 24 hours post-infection (Figure 9, panel D).

To test the possibility that the HPIV3 HN protein was necessary for fusogenic activity, HeLa T4$^+$ cells were infected with HPIV3 or coinfected with recombinant vaccinia viruses at a multiplicity of infection of 5. Monolayers were examined for up to 24 hours post-infection. Following infection with HPIV3, HeLa T4$^+$ cells fused readily and began to form syncytia within 24 hours post-infection (Figure 9, panel H). The syncytia resulting from HPIV3 infection were extensive and were composed of up to 200 nuclei and, at their peak, these large multinucleated cells fused to involve greater than 75% of the infected cell monolayer (Figure 9, panel G).

Mock-infected cells (Figure 9, Panel A), and Vsc8-infected cells (Figure 9, Panel B) remained as confluent monolayers and the cellular membranes and nuclear membranes were clearly visible. Cells containing several nuclei were observed in VF-infected cells (Figure 9, panel D), but this was not considered to be the result of F protein mediated cell fusion. Polykaryocytes containing 6 nuclei or fewer were commonly observed in HeLa T4$^+$ monolayers (Hemingway et al., 1994). Similar multinucleated cells were seen in Vsc8-infected cells and VHN-infected cells (Figure 9, panel C).

Coinfection of cells with VF + VHN (Figure 9, panel G) produced syncytia similar to those seen in HPIV3-infected monolayers. Fusion of VF + VHN coinfected cells began 7 hours post-infection and eventually involved greater than 75% of the

Figure 9. Fusion of HeLa T4$^+$ cells expressing the HPIV3 F and HN glycoproteins. HeLa T4$^+$ cells were infected and examined for the formation of syncytia for up to 24 hours post-infection. Photographs were taken soon after fusion was initially observed, at 24 hours for HPIV3-infected cells and at 8 hours post-infection for recombinant vaccinia virus-infected cells. Panel A, mock-infected cells. Panel B, Vsc8-infected cells. Panel C, VHN-infected cells. Panel D, VF-infected cells. Panel E, VF- and VHN-infected cells (low magnification). Panel F, VF- and VHN-infected cells. Panel G, HPIV3-infected cells (low magnification). Panel H, HPIV3-infected cells.



infected monolayer (Figure 9, panel E). Culture medium did not become acidic during the incubation period, therefore the fusion (Gong et al., 1990) and hemagglutination (Oie et al., 1990) activities of vaccinia virus, which function below a pH of 5.2, were assumed to be inactive.

Human erythrocytes have been used to provide target membranes to study virus-induced membrane fusion (Homma and Ohuchi, 1973). The hemolytic activity of cells infected with recombinant vaccinia viruses expressing the HPIV3 glycoproteins was examined. The hemolysis assay is based on the ability of cell membranes from virus-infected cells to bind and fuse with human erythrocytes. The subsequent release of hemoglobin is a measure of fusion. Infected cell lysates were prepared by 3 cycles of freezing and thawing and incubated with a 0.5% suspension of human erythrocytes for 20 minutes at 4°C. The hemolysis assay was carried out several times and representative data from one of these experiments are shown in Table 1.

Hemolysis occurred when lysates of HPIV3-infected cells or lysates of VF + VHN-infected cells were incubated with human erythrocytes. No significant hemolysis was observed using lysates of VF-, VHN- or Vsc8-infected cells, indicating that expression of both F and HN was required for hemolysis, as was observed for membrane fusion. HPIV3-infected cells that were not subjected to several cycles of freezing and thawing were weakly hemolytic, and VF + VHN-infected cells had no hemolytic activity (data not shown). Although syncytium formation resulting from infection by HPIV3, or by VF + VHN coinfection of HeLa

$T4^+$ cells were indistinguishable, the hemolytic activity of HPIV3 cell lysates was typically 3 to 4 times greater than that of lysates from cells coinfected with VF + VHN viruses. Cell lysates were hemolytic only during the same period of time as infected cells exhibit syncytium formation, this created a window of time during which both fusion and hemolysis were optimal. For example, lysates from VF + VHN coinfected cells 7 or 24 hours post-infection were poorly hemolytic (data not shown). Hemolytic activity was optimal 8 to 9 hours post-infection, the same period of time during which cells coinfected with recombinant vaccinia viruses reached their peak of syncytium formation.

Previous experiments using Sendai virus glycoproteins inserted into liposomes suggested that the function of the Sendai virus HN protein could be replaced using lectins as agglutinins (Hsu et al., 1978). The addition of the lectin, wheat germ agglutinin to VF-infected cell lysates did not result in hemolysis (data not shown). Therefore, the HN protein of HPIV3 appears to provide more than a simple agglutinin function during the fusion process. A mixture of VF-infected cell lysates and VHN-infected cell lysates also did not result in hemolysis, suggesting that the HPIV3 F and HN glycoproteins must be on the same membrane for fusogenic activity (data not shown).

TABLE 1

HEMOLYTIC ACTIVITY OF HELA T4$^+$ CELL LYSATES

VIRUS[1]	A_{540}[2]
None	--
Vsc8	0.006
VF	0.087
VHN	0.003
VF + VHN	0.859
HPIV3	2.97

[1] HeLa T4+ cells were infected at a multiplicity of infection of 5 and incubated for 24 hours for HPIV3-infected cells and 8 hours for vaccinia virus-infected cells at 4°C. Aliquots of 5 X 10^6 cells were frozen and thawed three times and incubated with human erythrocytes. The ratio of human erythrocytes to HeLa T4$^+$ cells was approximately 100:1.

[2] The value for mock-infected cells (0.271), was subtracted from the other absorbance readings.

DISCUSSION

Infection of HeLa T4$^+$ cells with recombinant vaccinia viruses carrying HPIV3 F or HN cDNA led to the expression of proteins that were electrophoretically and antigenically similar to their authentic HPIV3 counterparts. The F and HN proteins expressed by recombinant vaccinia viruses were also glycosylated and transported to the surface of infected cells. The F protein expressed by recombinant vaccinia virus VF was cleaved as evidenced by the presence of the F_1 subunit in infected cell lysates, but was not fusogenic in any of several cell lines that were tested.

Fusion from within, measured by either syncytium formation or hemolysis, required the expression of both the HPIV3 F and HN glycoproteins and the expression of these two glycoproteins was sufficient to mediate membrane fusion. It appeared that both the HPIV3 F and HN proteins must be expressed on the same membrane for syncytium formation to occur and that the contribution of the HPIV3 HN protein to the fusion reaction was more than as a simple agglutinin since it could not be replaced by the lectin, wheat germ agglutinin.

These results imply that there is a functional interaction between the HPIV3 F and HN glycoproteins and suggests that the two glycoproteins may physically interact, either between the cytoplasmic tails of the two proteins or between their ectodomains. It has been proposed that the interaction between the HPIV3 F and HN proteins leads to activation of the F protein resulting in a fusogenic conformation (Lamb, 1993).

Syncytia resulting from infection by HPIV3 or coinfection by VF + VHN viruses were indistinguishable. The number of nuclei per giant cell did not vary greatly, nor did the amount of the monolayers involved in fusion. On the other hand, differences in the hemolytic activity of membranes from HPIV3-infected cells and VF + VHN-infected cells were observed. What could account for these differences? Theoretically, at a multiplicity of 5, 99% of the cells should be infected with HPIV3 or doubly infected with VF and VHN. Hemolysis is secondary to fusion and a substantial number of cell membrane 'fragments' must fuse with the erythrocyte before lysis occurs (Szoka et al., 1988). It is possible that lysis of vaccinia virus infected cells released a vaccinia virus factor that had an inhibitory effect on cell membranes and resulted in decreased fusion of the membrane fragments with erythrocytes. However, it is most likely that vaccinia virus-infected cell membranes are aggregated after cell lysis, resulting in decreased hemolysis, since clumping was observed in lysates of vaccinia virus-infected cells and not in lysates of HPIV3-infected cells.

I. Comparisons of fusion requirements

When these studies were initiated, it had been demonstrated that expression of the F proteins of SV5 or measles virus in mammalian cells resulted in cell fusion. However, expression of both F and HN proteins of BPIV3 was necessary to mediate

syncytium formation. Like the BPIV3 F protein, the HPIV3 F protein required the HN protein for cell fusion.

Since these studies were completed, the requirement for both the HPIV3 F and HN proteins in syncytium formation has been observed by this researcher (Ebata et al., 1992), and by others (Hu et al., 1992; Hemingway et al., 1994). In addition, the fusion requirements for several other members of the family *Paramyxoviridae* have been examined. Viruses that have been shown to require both the F and HN (or H) proteins for cell fusion include: NDV (Morrison et al., 1991; Horvath et al., 1992), HPIV2 (Hu et al., 1992), measles virus (Wild et al., 1991), mumps virus (Tanabayashi et al., 1992), HPIV1 (Bousse et al., 1994), HPIV4A (Nishio et al., 1994), Sendai virus (Bousse et al., 1994; Taira et al., 1995) and SV5 (Bagai and Lamb, 1995).

The requirement for the receptor binding protein (HN or H) for cell fusion contradicts previous results for SV5 and measles virus. SV5 F protein appeared to be fusogenic when expressed using SV40 as a vector, however, coexpression of the SV5 F and HN proteins increased the number of syncytia in infected monolayers but not the size of each multinucleated cell (Horvath et al., 1992). Similarly, the HPIV3 F protein, while not fusogenic when expressed by vaccinia viruses, was shown to induce a small amount of fusion in the absence of the HPIV3 HN protein when expressed by an SV40-based vector (Horvath et al., 1992). When the SV5 proteins (Bagai and Lamb, 1995), or the HPIV3 proteins (Hu et al., 1992; Hemingway et al., 1994) were transiently expressed in transfected cells from

recombinant plasmids, both the F and HN proteins were required for cell fusion.

The measles virus F protein was fusogenic when expressed by recombinant adenoviruses (Alkhatib et al., 1990) but required the presence of the measles virus H protein when expressed by recombinant vaccinia viruses (Wild et al., 1991). A second recombinant vaccinia virus that expressed measles virus F protein was fusogenic in a human lung large cell carcinoma cell line, however, syncytium formation was enhanced upon coexpression of the measles virus H protein (Alkhatib et al., 1994). It appears that not only the choice of expression system but also the cell line influence the requirements for cell fusion mediated by paramyxovirus glycoproteins.

Unlike all of the other members of the family *Paramyxoviridae* that have been studied, transient expression of the RSV F protein and its receptor binding protein (G) expressed from recombinant plasmid did not mediate syncytium formation (Hemingway et al., 1994b). The attachment protein or G protein of pneumoviruses possesses neither hemagglutinin nor neuraminidase activities. In addition to the F and G glycoproteins, RSV has two other proteins that are associated with the membranes of infected cells, the SH (small hydrophobic) protein and the M2 (second matrix) protein. The SH protein is a type II glycoprotein with a strongly hydrophobic core of 28 amino acids and is found on the surface of infected cells but not in viral envelopes (McIntosh and Chanock, 1990). The M2 protein is the most basic RSV protein (Collins and Wertz, 1985), it is a component of the viral envelope

that is expressed on the surface of infected cells late in the infectious cycle (McIntosh and Chanock, 1990).

Transfection of a plasmid containing the SH coding sequence along with plasmids carrying the F and G coding sequences produced significant amounts of fusion in comparison to F and G plasmids alone. Interestingly, the combination of the RSV F and SH proteins also induced syncytium formation, although to a slightly lesser extent than cells cotransfected with RSV F, G and SH plasmids. Coexpression of the RSV F and G proteins resulted in the formation of several small syncytia but at a concentration of transfected plasmid DNA that was tenfold greater than the concentration required to produce cell fusion with F and SH plasmids. Coexpression of the M2 protein had neither a fusion enhancing nor a fusion inhibiting effect. Functions associated with the paramyxovirus HN protein, such as fusion promotion and receptor-binding activities, therefore appear to be located on separate membrane associated proteins in RSV (Hemingway et al., 1994b).

Like RSV, an SH protein has been described for SV5 (Chanock and McIntosh, 1990), although primary sequences of the RSV SH protein and the SV5 SH protein are poorly conserved. Location of the SH genes before the receptor-binding protein gene in the SV5 and RSV genomes, and the similarity of the physical properties of the SH proteins (small and hydrophobic protein) suggests that these proteins are evolutionarily related (Hemingway et al., 1994b). The SV5 SH protein did not, however, enhance the formation of syncytia when coexpressed with the SV5 F

protein, nor did the SV5 SH protein have any effect when coexpressed with the RSV F protein. Unlike the fusion requirements of RSV, SV5 fusion appears to be independent of the SH protein.

II. Homotypic and heterotypic interaction of glycoproteins

Among members of the family *Paramyxoviridae,* heterotypic interaction, or interaction of glycoproteins from different family members that results in fusogenic activity is limited. The HPIV1 HN protein was shown to promote cell fusion following coexpression with the Sendai virus F protein (Bousse et al., 1994). However, cotransfection of heterologous F and HN genes from HPIV2, HPIV3 and SV5 did not duplicate cell fusion resulting from coexpression of homologous proteins (Hemingway et al., 1994).

The HN protein of HPIV4A, although most similar to the mumps virus HN protein, could not function cooperatively with the mumps virus F protein to elicit fusion (Tanabayashi et al., 1992). Similarly, heterologous combinations of HPIV2 and HPIV3 F and HN proteins did not produce fusion but also did not inhibit the ability of the homologous glycoproteins to induce syncytium formation (Hu et al., 1992). Coexpression of the NDV F protein and the SV5 HN protein using an SV40-based vector in a mammalian expression system did not result in syncytium formation (Horvath et al., 1992). Furthermore, the contribution of the NDV HN protein to

fusion could not be substituted by either ALV (avian leukemia virus) envelope glycoprotein or the HA protein of influenza virus. These studies emphasize the requirement for a specific, homotypic interaction between paramyxovirus envelope glycoproteins (Morrison et al., 1991).

A quantitative dequenching assay involving lipid mixing (Hoekstra, 1991), was used to determine the amount of fusion produced by combinations of paramyxovirus glycoproteins (Bagai and Lamb, 1995). The dequenching assay is very sensitive and can detect small amounts of fusion. The SV5 F protein was fusogenic when coexpressed with heterotypic HN proteins from HPIV3 (10% fusion in comparison to SV5 HN), NDV (15% fusion) and the influenza HA protein (20% fusion). The SV5 F protein was not fusogenic in the presence of lectins, such as wheat germ agglutinin, concanavalin A and phytohemagglutinin. Unlike most other F proteins, the SV5 protein appears to be less stringent in its requirements for fusion, and is able to interact functionally with receptor binding proteins not only from other paramyxoviruses but also with the hemagglutinin protein from another family of RNA viruses.

III. Are the F and HN glycoproteins required to be on the same membrane ?

Mixing lysates from VF-infected and VHN-infected HeLa $T4^+$ cells together did not result in hemolysis and indicated that the HPIV3 F and HN proteins must be

contained within the same membrane to mediate syncytium formation. The necessity for the glycoproteins to reside on the same membrane has been observed for NDV glycoproteins expressed by recombinant retroviruses (Morrison et al., 1991) and has also been confirmed for HPIV3 glycoproteins transiently expressed from recombinant plasmids (Hemingway et al., 1994).

Circular dichroism studies on Sendai virus glycoproteins showed that the conformation of the F + HN glycoproteins in reconstituted viral envelopes or in vesicles was different from the conformation adopted by either F-containing or HN-containing vesicles, or a mixture of these two. The F and HN proteins interacted with each other only when they were contained within the same membrane which resulted in fusogenic conformations of these proteins (Citovsky et al., 1986).

The F and HN proteins of HPIV2 have been reported to be fusogenic when cells transiently expressing HPIV2 F protein from recombinant plasmid were mixed with cells transiently expressing HPIV2 HN protein (Hu et al., 1992). It is possible in these experiments, however, that despite washing of transfected cells, recombinant plasmids carrying HPIV2 F and HN genes were still present in cell cultures. Fusion was not observed immediately after mixing of cells transfected with plasmids carrying either the HPIV2 F or HN gene. Sufficient time had elapsed for a second round of transfection of plasmids and subsequent coexpression of the F and HN proteins on the same membrane to have occurred (Hemingway et al., 1994).

IV. The role of the receptor binding protein in fusion

The receptor-binding proteins of all members of the family *Paramyxoviridae* that were studied, have been shown to play a role in the promotion of fusogenic activity. These included F proteins transiently expressed in mammalian cells by recombinant plasmids for viruses such as: SV5, NDV (Deng et al.,1995; Bagai and Lamb, 1995), HPIV1 (Bousse et al., 1994), HPIV2 (Hu et al., 1992), HPIV3 (Hu et al., 1992; Hemingway et al., 1994; Bagai and Lamb, 1995; Deng et al., 1995), Sendai virus (Taira et al., 1995), mumps virus (Tanabayashi et al., 1992). Similarly, constitutive expression of the F protein of HPIV4A (Nishio et al., 1994), required transient coexpression of the HPIV4A HN protein to mediate cell fusion. Measles virus (Wild et al., 1991) and BPIV3 (Sakai and Shibuta, 1989) F proteins expressed by recombinant vaccinia viruses required the coexpression of their receptor binding proteins for fusogenic activity.

The receptor binding proteins therefore, are essential membrane components for the production of cell-to-cell fusion and have confirmed the studies presented here which suggested that the HPIV3 HN protein is crucial for syncytium formation and could not be replaced by the presence of the lectin, wheat germ agglutinin.

Cells persistently infected with HPIV3 have shown that virus-cell fusion and cell-to-cell fusion differ in their requirements for the sialic acid-containing receptors. Cells persistently infected with HPIV3 do not exhibit cytopathic effects or fuse with each other, although they will fuse with uninfected cells (Moscona and Peluso,

1992). Persistently infected cells become fusion resistant due to the neuraminidase activity of the HPIV3 HN protein which removes sialic acid from HPIV3 receptors on the cell surface. A fusion resistant cell line also resulted from constitutive expression of the HN protein of HPIV4A, cells remained fusion resistant even following infection by HPIV4A (Nishio et al., 1994).

Fusion resistant cell monolayers could also be created by infection with HPIV3 at a low multiplicity of infection and treatment with bacterial neuraminidase. When HPIV3 receptors were present on the cell surface at low levels, HPIV3-cell fusion occurred, and the virus was disseminated in the culture by release of progeny virus. This indicated that there were two thresholds for sialic acid-containing receptors, one sufficient for HPIV3 mediated FFWI and one for virus-cell fusion. When there were virtually no sialic acid-containing receptors remaining on the surface of the cell, HPIV3 could no longer enter the cell to establish infection (Moscona and Peluso, 1992). The interaction of the HPIV3 HN protein with sialic acid-containing receptors is critical for fusion, and it appears that the formation of syncytia requires a greater density of receptors and is more dependent on the receptor-binding protein than virus-cell fusion.

The role of the neuraminidase activity in fusion was also examined in studies using Sendai virus and HPIV1. Sendai virus and HPIV1 are closely related and share a high degree of sequence similarity (Bousse et al., 1994). In fact, coexpression of the HPIV1 HN protein with the Sendai virus F protein resulted in more extensive fusion than seen with homologous Sendai virus F and HN proteins.

The converse pairing of the HPIV1 F protein with the Sendai virus HN protein was not fusogenic. It was suggested that the differences between the abilities of the HPIV1 and Sendai virus HN proteins to complement the Sendai virus F protein were due to the lower neuraminidase activity of HPIV1 HN protein (HPIV1 HN has 15% of the activity of Sendai HN). Decreased neuraminidase activity would result in more sialic acid remaining on the cell surface leading to increased cell fusion. The fusogenic activity of BPIV3 was also shown to be inversely proportional to neuraminidase activity (Shibuta et al., 1983), however, this was not shown to be the case for NDV (Sergel et al., 1993). A variant of HPIV3 with increased fusogenic activity possessed single amino acid changes in the HN protein, however, the neuraminidase activity of the variant was not significantly different from the wild-type isolate (Moscona and Peluso, 1993). Increased fusogenic activity appeared to result from increased affinity of the variant HN protein for the sialic acid-containing receptor, which allowed the HN protein to spend more time bound to available receptors and resulted in increased syncytium formation.

Information obtained to date indicates that the receptor binding protein has a role in cell fusion. The studies presented here showed that a functional interaction between the HPIV3 F and HN proteins within the same membrane was required for fusogenic activity. However, although all the F proteins of the members of the family *Paramyxoviridae* that have been studied require the receptor binding protein to mediate syncytium formation, this requirement appears to be dependent upon the expression system chosen. Some F proteins, like the NDV F protein, are not

functional without the HN protein, and others, like the SV5 or measles virus F proteins have been shown to be less dependent on the HN protein for fusogenic activity.

PART II : EXPRESSION AND IMMUNOGENICITY OF HPIV3 F AND HN PROTEINS EXPRESSED BY RECOMBINANT ADENOVIRUSES

INTRODUCTION

Previous studies have shown that infection of 293 or HeLa cell monolayers with a recombinant adenovirus type 5 expressing the measles virus F protein (Ad5MVF) resulted in cell fusion and hemolysis of monkey erythrocytes. Coinfection of cells with recombinant adenovirus expressing the measles virus H protein (Ad5MV/HA2) did not enhance fusogenic activity of the measles virus F protein expressed by Ad5MVF. Coinfection of Ad5MVF and Ad5MV/HA2 also resulted in decreased hemolytic activity by comparison with cells infected with Ad5MVF recombinant adenovirus expressing the measles virus F protein (Alkhatib et al., 1990). In contrast to results obtained using recombinant adenoviruses, cell fusion mediated by the measles virus F protein expressed by recombinant vaccinia virus required coexpression of the measles virus H protein (Wild et al , 1991). These studies describe conflicting reports concerning the paramyxovirus glycoproteins required for cell fusion.

The second objective of this study was to express the HPIV3 F and HN proteins using recombinant adenoviruses. The reasons for constructing recombinant adenoviruses were twofold: 1) to determine whether the use of an alternative

expression system such as adenovirus type 5, altered the requirement for both the HPIV3 F and HN glycoproteins for cell fusion; and 2) to begin studies on the immunogenicity of the HPIV3 glycoproteins expressed by recombinant adenovirus vectors in a small animal model.

Adenovirus type 5 (Ad5) is a nonenveloped icosahedral virus with a genome consisting of double-stranded, linear DNA approximately 36,000 base pairs in length (Haj-Ahmad and Graham, 1986). Over 40 types of adenoviruses have been isolated, many of which have greater potential to become oncogenic. Ad5 belongs to the adenovirus subgroup C, and is considered to have little or no oncogenic potential (Horwitz, 1990). Previous studies have indicated that Ad5 is safe for use as a vector in humans vaccines (Hsu et al., 1992) and has distinct advantages over vaccinia virus as a vaccine vector for respiratory diseases. Ad5, like HPIV3, is itself a respiratory virus, it can be administered either intranasally or orally and effects an asymptomatic infection which can produce both mucosal and systemic immunity (Pacini et al., 1984; Berkner, 1988). Ad5 can therefore mimic the route of natural infection by a respiratory virus such as HPIV3. Another advantage to the adenovirus constructs is that the cotton rat (*Sigmodon* sp.) is susceptible to both adenovirus infection and HPIV3 infection (Murphy et al., 1988; Pacini et al., 1984). Infection of cotton rats with HPIV3 resulted in lower respiratory tract infections such as bronchiolitis (*Sigmodon hispidus*) or interstitial pneumonia (*Sigmodon fulviventer*) (Porter et al., 1991), similar to what is observed in young children.

Ad5 has been widely studied, it has been well characterized genetically and is particularly useful for the expression of foreign genes since it can be grown in large quantities and has a broad host range *in-vivo* and *in-vitro* (Haj-Ahmad and Graham, 1986). The adenovirus replicative cycle is loosely divided into early (E) and late (L) phases. The early phase of transcription involves seven noncontinuous regions of the viral DNA and transcription is initiated from six promoters (E1A, E1B, E2A, E2B, E3 and E4). An active E1A gene product is produced first, shortly after infection, and is necessary for the transcription and regulation of the other early gene products (Horwitz, 1990). The E3 region is not required for viral replication in cultured cells and deletions in this region have resulted in nonconditional helper-independent vectors with a capacity to accommodate 4.5 to 4.7 kilobases of foreign DNA.

Recombinant Ad5 with deletion of the E3 sequence has been successfully used to express the hepatitis B surface antigen, which was capable of replicating in hamster lungs and elicited an antibody response (Morin et al., 1989). Other viral proteins such as polyoma virus tumour antigens (Mansour et al., 1985); vesicular stomatitis virus glycoprotein (Schneider et al., 1989); herpes simplex virus glycoprotein B (McDermott et al., 1989); rabies virus glycoprotein (Prevec et al., 1990) as well as measles virus glycoproteins (Alkhatib et al., 1990) have been expressed using adenovirus vectors.

The F and HN glycoproteins figure prominently in the immunobiology of HPIV3 infection. Antibodies directed against either the HN or F proteins can neutralize the

infectivity of HPIV3, however, only antibodies specific for the HPIV3 F protein can prevent the lateral spread of virus *in-vitro* (Merz et al., 1980).

Following primary infection the antibody response to the HPIV3 F protein in humans and nonhuman primates is relatively restricted in comparison to the response to the HPIV3 HN protein (Coelingh et al., 1990). The absence of antibodies to the HPIV3 F protein has been associated with the susceptibility of infants and young children to symptomatic reinfection (Kasel et al., 1984). Expression of the HPIV3 F and HN glycoprotein using recombinant vaccinia viruses resulted in vaccinia-HN producing a protective response substantially greater than vaccinia-F in cotton rats (*Sigmodon fulviventer*). Cotton rats inoculated with vaccinia-HN showed a reduction of HPIV3 replication that was >500 fold greater than cotton rats inoculated with vaccinia-F. Inoculation of cotton rats with both viruses produced complete resistance to HPIV3 in the lower respiratory tract (Spriggs et al., 1987). Similarly, immunization of hamsters with recombinant baculovirus expressing the HPIV3 F protein elicited a protective response although it was not proteolytically cleaved into functional subunits F_1 and F_2 (Ray et al., 1989).

Complete protection against HPIV3 challenge resulted from immunization of hamsters with a mixture of affinity-purified F and HN proteins. HPIV3 glycoproteins solubilized in detergent and used in intranasal challenge elicited a specific IgA response that was implicated in resistance to infection upon HPIV3 challenge (Ray et al., 1988b). Higher levels of antibodies to both HPIV3 F and HN proteins were

detected in bronchial lavage samples from hamsters inoculated with both glycoproteins compared to antibody levels from hamsters inoculated with either the F or the HN protein alone. These results suggested that the HPIV3 F and HN proteins may act synergistically to induce a protective response against HPIV3 challenge (Ray et al., 1988).

RESULTS

I. Immunoprecipitation of HPIV3 proteins

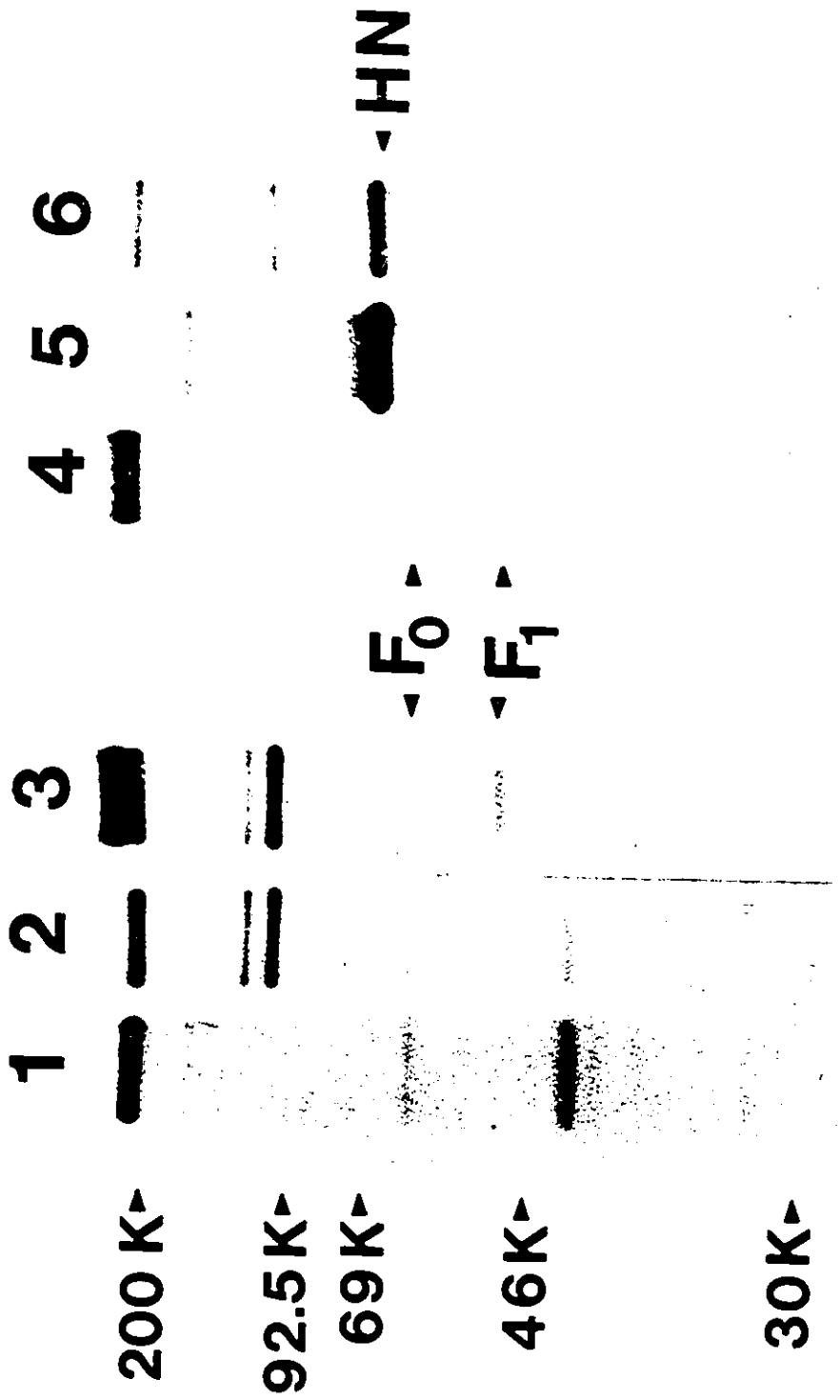
Recombinant adenoviruses were constructed in the laboratory of L. Prevec and a brief description follows. The HPIV3 F and HN cDNAs were inserted into the shuttle vector pFGdX1 which contained the rightward 30 map units of Ad5 DNA with a deletion in the E3 region. These constructs were cotransfected into 293 cells with the plasmid pFG173, which supplied the leftward region of the Ad5 genome. Individual adenovirus plaques were selected 8 to 10 days post-transfection and recombinant adenoviruses were identified by restriction analysis (Hanke et al., 1991).

The ability of recombinant adenoviruses AdF and AdHN to express HPIV3 F and HN glycoproteins was examined by immunoprecipitation of F and HN from lysates of infected cells. HeLa $T4^+$ cells were infected with recombinant adenoviruses AdF or AdHN at a multiplicity of infection of 20, or infected with HPIV3 at a multiplicity of infection of 5 and incubated for 24 hours before labelling with [^{3}H]-leucine. Radiolabelled proteins were immunoprecipitated from cell lysates using monoclonal antibodies specific for the HPIV3 F and HN proteins (Rydbeck et al., 1986) and analyzed on 10% polyacrylamide gels containing SDS under reducing conditions. Incubation of lysates from AdF-infected cells with monoclonal antibody specific for the F protein resulted in the immunoprecipitation of two proteins, F_0 and its

cleavage product F_1 (Figure 10, lane 3), which migrated to the same positions as the corresponding proteins in HPIV3-infected cells (Figure 10, lane 4). Similarly, the HN protein immunoprecipitated from lysates of AdHN-infected HeLa T4$^+$ cells by a monoclonal antibody specific for the HPIV3 HN protein (Figure 10, lane 6) had the same apparent molecular weight as the HN protein immunoprecipitated from HPIV3-infected cell lysates (Figure 10, lane 5). The F and HN proteins expressed by recombinant adenoviruses were also labelled with [^{3}H]-glucosamine and [^{3}H]-mannose (data not shown). While the amounts of the F protein in HPIV3- and AdF-infected cells appeared to be similar, the amount of HN protein immunoprecipitated from AdHN-infected cell lysates appeared to be several fold lower than the amount of HN protein in HPIV3-infected cell lysates.

As described in the previous chapter, a large (>200 ka) glycoprotein band was evident in immunoprecipitates from lysates of HPIV3-infected or AdF-infected cells using a monoclonal antibody specific for the HPIV3 F protein. This protein was not immunoprecipitated from lysates of AdHN-, mock- or Ad5-infected cells, nor was it immunoprecipitated from lysates of HPIV3-infected cells with the monoclonal antibody specific for the HN protein.

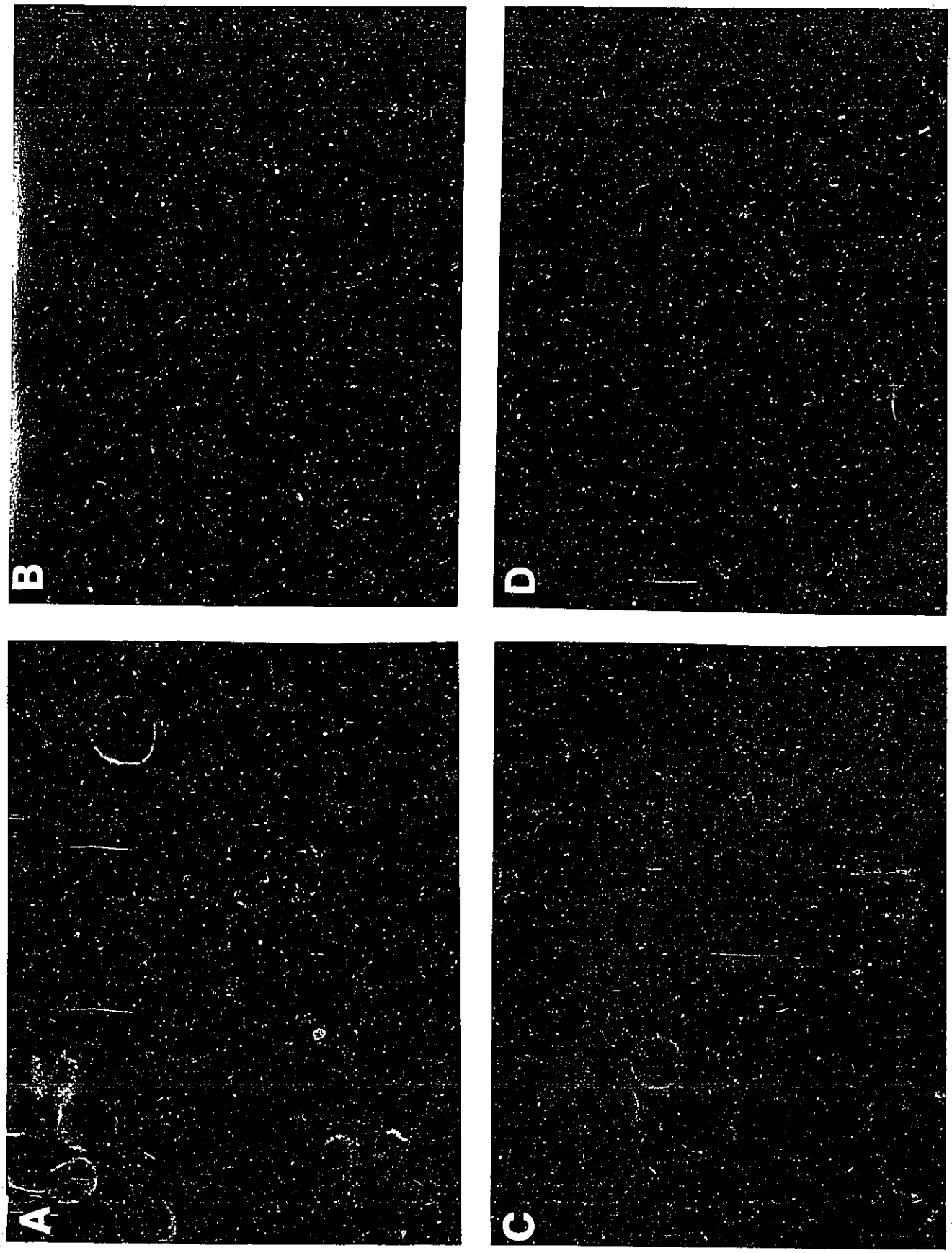
Figure 10 : Immunoprecipitation of F and HN proteins from cell lysates following infection with recombinant adenoviruses. Infected and uninfected HeLa T4$^+$ cells were labelled with [^{3}H]-leucine. Proteins were immunoprecipitated from cell lysates with monoclonal antibodies specific for the HPIV3 F protein (Lanes 3 and 4), the HPIV3 HN protein (Lanes 5 and 6), or with both antibodies (Lanes 1 and 2), and resolved by electrophoresis on a 10% polyacrylamide gel containing SDS under reducing conditions. Lane 1, mock-infected cells. Lane 2, Ad5-infected cells. Lane 3, AdF-infected cells. Lanes 4 and 5, HPIV3-infected cells. Lane 6, AdHN-infected cells.


1
2
3
4
5
6
HN
F0
F1
200 K
92.5 K
69 K
46 K
30 K

II. Cell surface expression of HPIV3 glycoproteins

It can be reiterated that transport of HPIV3 glycoproteins to the cell surface is crucial for their function. Expression of the F and HN glycoproteins on the surface of cells following infection with recombinant adenoviruses was examined by indirect immunofluorescence of live, unfixed cells. HeLa $T4^+$ cells were infected with AdF or AdHN at a multiplicity of infection of 20 and incubated for 24 hours before trypsinization and suspension in a 1:200 mixture of 6 monoclonal antibodies specific for the HPIV3 F protein (Coelingh and Tierney, 1989) or a monoclonal antibody specific for the HPIV3 HN protein (Rydbeck et al., 1986). The secondary antibodies consisted of a 1:5 dilution of FITC-conjugated sheep anti-mouse immunoglobulin. AdF-infected cells incubated with monoclonal antibodies specific for the HPIV3 F protein (Figure 11, panel C), or AdHN-infected cells incubated with monoclonal antibody specific for the HPIV3 HN protein (Figure 11, panel A) showed homogeneous distribution of glycoprotein on the infected cell surface. Ad5-infected cells (Figure 11, panels B and D) exhibited minimal fluorescence.

Figure 11 : Cell surface expression of F and HN glycoproteins following infection of HeLa T4$^+$ cells with recombinant adenoviruses. HeLa T4$^+$ cells were infected with adenoviruses at a multiplicity of infection of 20 and incubated for 48 hours. Cells were trypsinized and incubated first with monoclonal antibodies specific for the HPIV3 HN protein (Panels A and B), or for the HPIV3 F protein (Panels C and D) and then FITC conjugated sheep anti-mouse immunoglobulin. Panel A, AdHN-infected cells. Panel B and D, Ad5-infected cells. Panel C, AdF-infected cells.

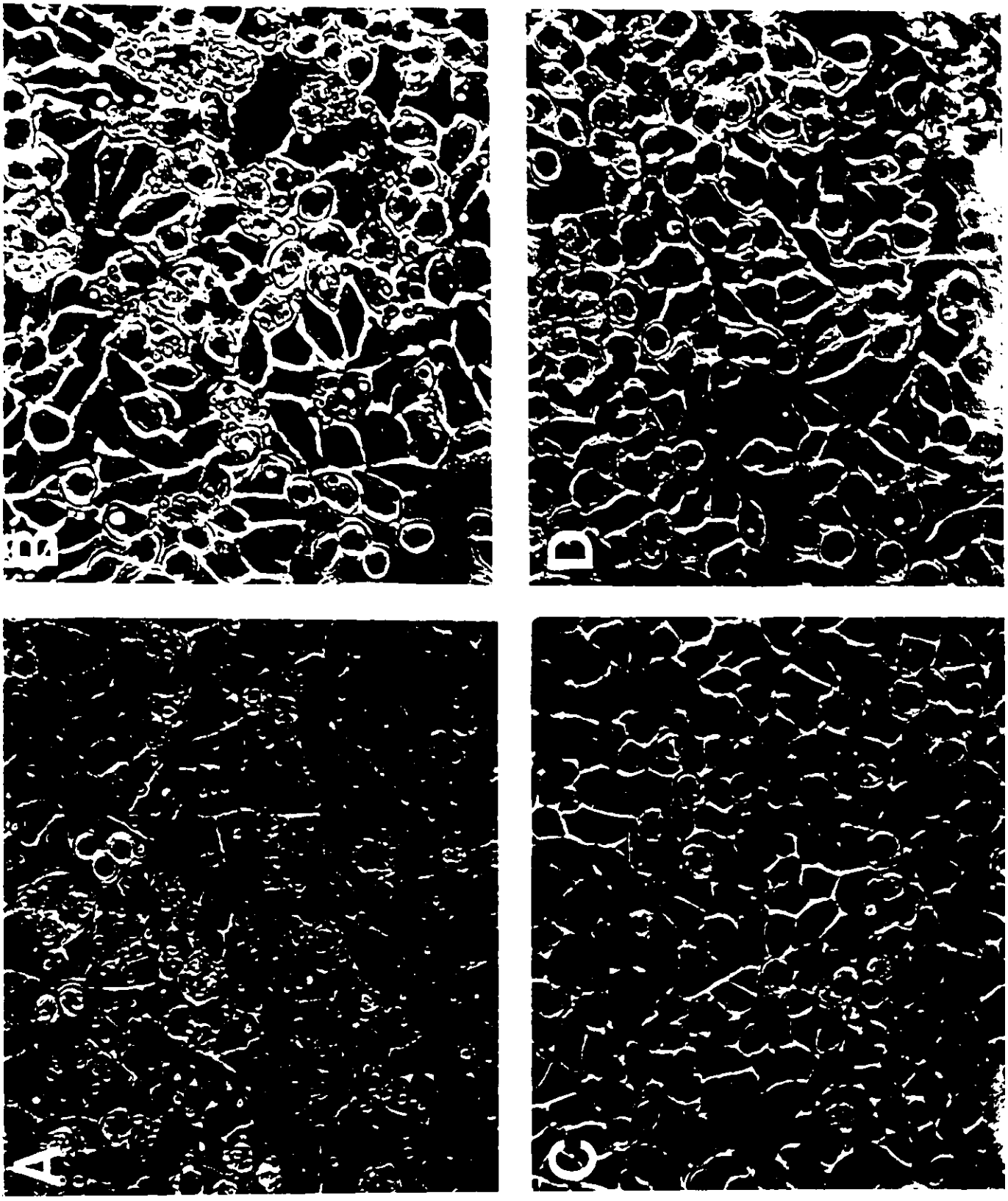

B
D
A
C

III. Biological activities of the HPIV3 F and HN glycoproteins

Hemagglutinin and neuraminidase activities

The biological activities of the HN protein are important factors in the life cycle of HPIV3, and function during binding of virus to cellular receptors and release of progeny virus from infected cells (Scheid and Choppin, 1974). The adsorption and release of human erythrocytes is indicative of functional hemagglutinin and neuraminidase activities of the HPIV3 HN protein. Therefore, the HPIV3 HN protein expressed by recombinant adenovirus was tested for biological activity. HeLa $T4^+$ cells were infected with HPIV3 at a multiplicity of infection of 5, or with recombinant adenoviruses at a multiplicity of infection of 10 and incubated for 24 hours. Infected cells were overlaid with a suspension of 0.5% human erythrocytes and washed vigorously. Only monolayers infected with HPIV3 (Figure 12, panel A) or AdHN (Figure 12, panel B) were capable of adsorbing human erythrocytes, neither mock-infected cells (Figure 12, panel C) nor Ad5-infected cells (Figure 12, panel D) exhibited hemagglutination. When the temperature of incubation was shifted to 37°C, bound erythrocytes were released, indicating functional neuraminidase activity (data not shown).

Figure 12 : Hemadsorption of human erythrocytes to infected HeLa $T4^+$ cell monolayers. HeLa $T4^+$ cells were infected with HPIV3 at a multiplicity of infection of 5 and incubated for 24 hours. HeLa $T4^+$ cells were infected with either Ad5 or recombinant adenoviruses at a multiplicity of infection of 10 and incubated for 24 hours. Cultures were assayed for hemadsorption and photographed with a Leitz Labovert microscope. Panel A, HPIV3-infected cells. Panel B, AdHN-infected cells. Panel C, Mock-infected cells. Panel D, Ad5-infected cells.

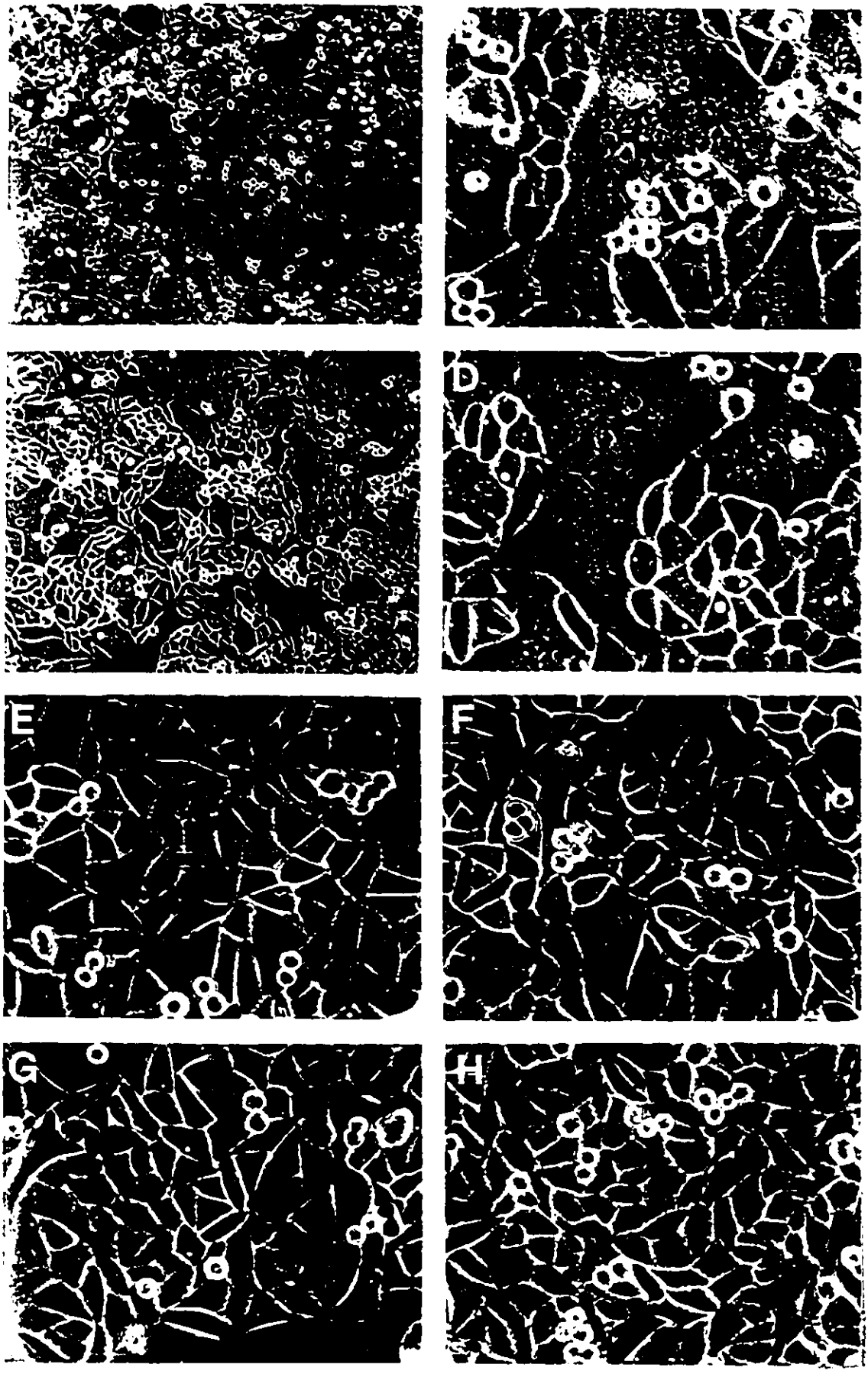

B
D
A
C

Fusion activity

It had been previously established that both the HPIV3 F and HN proteins were necessary for syncytium formation and hemolysis when expressed by recombinant vaccinia viruses. However, since expression of the measles virus F protein by recombinant adenovirus resulted in fusion of 293 cells and HeLa cells, it was important to identify the requirements for fusion when HPIV3 F and HN proteins were expressed in an alternative expression system by recombinant adenoviruses. HeLa $T4^+$ cells were used to assay fusion activity following infection with HPIV3 or recombinant adenoviruses, although preliminary experiments demonstrated that HeLa cells could also form syncytia albeit to a lesser extent (data not shown). HeLa $T4^+$ cells were infected with HPIV3 at a multiplicity of infection of 5 and syncytia were observed 24 hours post-infection (Figure 13, panels A and B).

Infection of HeLa $T4^+$ cells with recombinant adenovirus AdF at a multiplicity of infection of 10 did not result in fusion for up to 24 hours post-infection (Figure 13, panel F). Coinfection of cells with AdF + AdHN using multiplicities of infection of either 5, 10 and 20 resulted in syncytium formation (data not shown). Following coinfection of AdF + AdHN at a multiplicity of 10 (Figure 13, panels C and D) syncytia became evident 12 hours post-infection and were indistinguishable from syncytia in HPIV3-infected cell monolayers. Fused cells contained large numbers of nuclei and involved greater than 75% of the infected monolayer. No fusion was

Figure 13. Syncytium formation in HeLa $T4^+$ monolayers infected with recombinant adenovirus expressing the HPIV3 F and HN glycoproteins. Cells were infected with HPIV3 and incubated for 24 hours, or with recombinant adenoviruses and incubated for 15 hours and then photographed. Panel A, HPIV3-infected cells (low magnification). Panel B, HPIV3-infected cells. Panel C, AdF + AdHN-infected cells (low magnification). Panel D, AdF + AdHN-infected cells. Panel E, AdHN-infected cells. Panel F, AdF-infected cells. Panel G, mock-infected cells. Panel H, Ad5-infected cells.


D
E
F
G
H

observed in monolayers infected with AdHN (Figure 13, panel E) or Ad5 (Figure 13, panel H). A hemolysis assay was attempted several times, however cell lysates infected with Ad5 continually caused lysis of erythrocytes making this assay uninformative.

IV. AdF and AdHN elicit an antibody response in mice

The HPIV3 F and HN proteins expressed by recombinant adenoviruses were transported to the surface of infected cells and were biologically active. A second objective of these studies was to begin the assessment of recombinant adenoviruses expressing the HPIV3 F and HN proteins as immunogens. The ability of AdF and AdHN to elicit an antibody response in mice was tested. Mice were chosen because they are less expensive and develop antibody responses more quickly than rabbits.

Mouse sera were provided by L. Prevec and a brief description follows. Six to seven week old female Balb/c mice were injected intraperitoneally with 10^7 PFU of recombinant adenoviruses AdF or AdHN. Sera were collected at 16 and 32 days post-injection for AdF-inoculated mice and at 21 and 42 days for AdHN-inoculated mice. The reactivities of mouse sera with HPIV3 F and HN glycoproteins were assayed by immunoprecipitation. HPIV3-infected cells were radiolabelled with [^{3}H]-glucosamine and proteins were immunoprecipitated with mouse sera from lysates

of infected cells. Both the F_0 precursor protein (Figure 14, closed circle) and F_1 subunit (Figure 14, open circle) were immunoprecipitated from lysates of HPIV3-infected cells using sera collected from mice inoculated with AdF 16 days and 32 days post-inoculation (Figure 14, lanes 3 and 4). Similarly, sera from AdHN-injected mice reacted specifically with the HPIV3 HN glycoprotein (Figure 14, lanes 6 and 7). The HPIV3 F and HN glycoproteins were not immunoprecipitated with normal mouse serum (Figure 14, lane 2).

A large (>200 ka) protein band was immunoprecipitated from HPIV3-infected cell lysates by sera from mice inoculated with AdF (Figure 14, lanes 3 and 4), but not by sera from mice inoculated with AdHN or with normal mouse serum.

V. Neutralization of HPIV3 using mouse sera

Since sera from mice inoculated with recombinant viruses AdF or AdHN were able to recognize the F and HN proteins in HPIV3-infected cells, they were tested for their ability to neutralize HPIV3 virions. HPIV3 was incubated with dilutions of heat inactivated mouse sera and the neutralization titres of the sera were assessed using a plaque reduction assay. The results of neutralization assays are shown in Table 2. The numbers of HPIV3 plaques counted for each dilution of mouse serum were graphed to obtain the reciprocal of the dilutions of mouse sera from AdF-inoculated mice or AdHN-inoculated mice that were able to neutralize HPIV3 by

50% in a plaque reduction assay. Neutralization titres for AdF- and AdHN-inoculated mice were 520 and 455 respectively. Normal mouse serum did not neutralize HPIV3 even at a dilution of 1:10.

Figure 14. Immunoprecipitation of HPIV3 proteins with sera from mice inoculated with recombinant adenoviruses. HeLa T4$^+$ cells were infected with HPIV3 at an MOI of 5 and incubated for 24 hours. Cells were labelled with [^{3}H]-glucosamine hydrochloride and proteins were immunoprecipitated from cell lysates with sera from control mice or from mice that had been inoculated with recombinant adenoviruses. Proteins were immunoprecipitated from cell lysates and analyzed by SDS-PAGE. Proteins from lysates of HPIV3-infected cells are shown in lanes 2,3,4,6 and 7, and proteins from lysates of mock-infected cell lysates are shown in lanes 1,5 and 8. Lanes 1 and 2, normal mouse serum. Lanes 3 and 5, serum from mouse inoculated with AdF , 32 days post-infection. Lane 4, serum from mouse inoculated with AdF, 16 days post-inoculation. Lanes 6 and 8, serum from mouse inoculated with AdHN, 42 days post-inoculation. Lane 7, serum from mouse inoculated with AdHN, 21 days post-inoculation. The open circle marks the F_1 protein, the closed circle marks the F_0 protein and the square marks the HN protein.

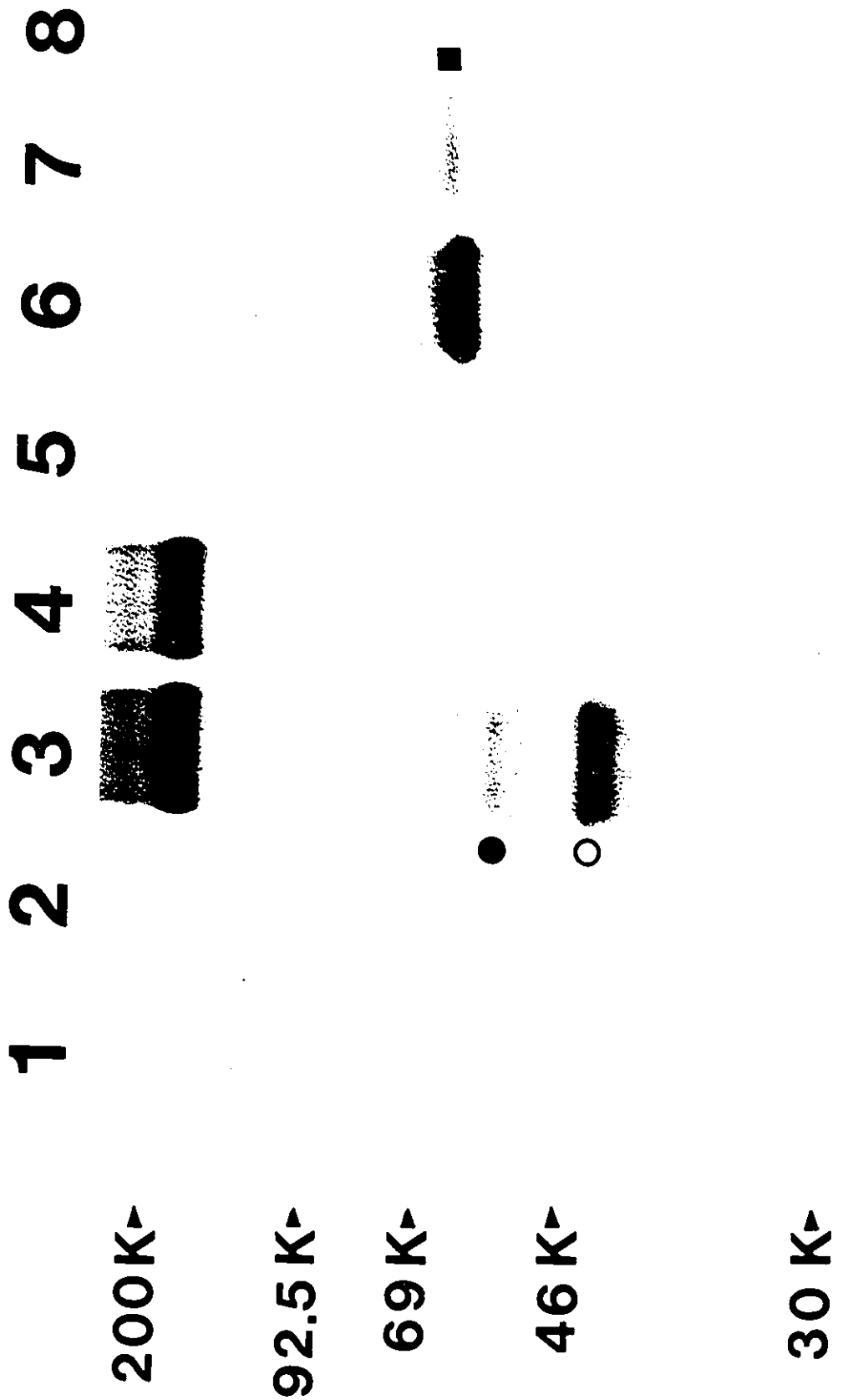

1
2
3
4
5
6
7
8
200K
92.5K
69K
46K
30K

TABLE 2

TITRES OF NEUTRALIZING ANTIBODIES IN MICE

SERUM	DAYS POST-INOCULATION	NEUTRALIZATION TITRE [1]
Mouse / AdF	32	520
Mouse / AdHN	42	455
Normal Mouse	-	NN [2]

[1] The neutralization titre equals the reciprocal of the dilution of serum required to neutralize 50% of virus and was determined by plaque reduction assay.

[2] Non-neutralizing

DISCUSSION

Recombinant adenoviruses carrying the HPIV3 F and HN coding sequences expressed glycosylated polypeptides that were electrophoretically similar to authentic HPIV3 glycoproteins and were recognized by monoclonal antibodies specific for the HPIV3 F or HN protein. The F protein expressed by recombinant adenovirus AdF was cleaved into functional subunits and both AdF and AdHN expressed products that were transported to the cell surface of infected HeLa $T4^+$ cells.

Unlike the measles virus F protein, the HPIV3 F protein was not fusogenic when expressed by a recombinant adenovirus vector, although infection at high multiplicities, such as those used previously for recombinant adenovirus expressing the measles virus F protein (Alkhatib et al., 1990), were not attempted for the recombinant adenovirus expressing the HPIV3 F protein. Fusion observed following coinfection of HeLa $T4^+$ cells with recombinant adenoviruses AdF + AdHN was similar to fusion seen in HPIV3-infected cells or in cells infected with recombinant vaccinia viruses expressing HPIV3 F + HN proteins.

The HN protein expressed by AdHN possessed both hemagglutinin and neuraminidase activities along with the ability to promote syncytium formation. Syncytium formation was also observed in HeLa cells following infection by HPIV3 or recombinant adenoviruses but was less extensive than cell fusion seen in HeLa $T4^+$ cells. Multinucleated HeLa cells contained fewer nuclei and a smaller

percentage of the monolayer was involved in syncytium formation. These results highlight the sensitivity of the HeLa T4$^+$ cell line to fusion and also that the choice of the indicator cell line is an important factor when evaluating the capability of fusion proteins.

I. Differences in fusion requirements due to assay systems

It would appear that the choice of expression system or the cell line have an impact on the paramyxovirus glycoproteins necessary for fusion. As previously mentioned, a recombinant adenovirus expressing the F protein of measles virus was able to produce syncytia in human epithelial cell lines (293 and HeLa) (Alkhatib et al., 1990), whereas recombinant vaccinia virus expressing the measles F protein was unable to produce syncytium formation in HeLa cells (Wild et al., 1991). In HeLa cells, expression of both measles virus F and H proteins was fusogenic, but cell lines that were permissive for vaccinia virus infection but nonpermissive for measles virus infection did not form syncytia. It is possible that the fusogenic activity of the measles virus F protein expressed by a recombinant adenovirus resulted from high levels of F protein produced, due perhaps to the high multiplicity of infection. It is also possible that adenovirus infection alters host cell membranes or results in the expression of a gene product that in some way permits the measles virus F protein and not the HPIV3 F protein to mediate cell fusion.

The role of the receptor binding protein in fusion promotion appears to be dependent upon the choice of expression system. In an SV40-based expression system the F protein of HPIV3 was shown to be capable of causing a small amount of fusion in the absence of the HPIV3 HN protein, in a monkey kidney (CV-1) cell line (Horvath et al., 1992). Fusion by HPIV3 F was, however, enhanced by coexpression of the HN protein. Unlike the HPIV3 and SV5 F proteins, the NDV F protein was not fusogenic on its own when expressed using the same SV-40 based vector and required coexpression of the NDV HN protein.

Using a vaccinia virus driven expression system, cell fusion required cotransfection of F and HN plasmids for HPIV3 (Hu et al., 1992; Hemingway et al., 1994; Bagai and Lamb, 1995), NDV and SV5 (Bagai and Lamb, 1995). These results again emphasize the dependence of fusion requirements on the choice of expression system. It appears that SV40-based expression allows the SV5 and HPIV3 F proteins to mediate fusion in the absence of HN, in a way that is not possible in the vaccinia-based expression system. The NDV F protein , however, could not respond to the SV40 influence and could not function without the HN protein. Perhaps the SV40 provided a component that replaced the HPIV3 and SV5 HN proteins or it is also possible that the SV40 virus altered cells membranes, making them more sensitive to cell fusion.

II. Antibody response to HPIV3 glycoproteins in mice

Mice injected intraperitoneally with either AdF or AdHN produced antibodies that were able to neutralize HPIV3. Although Ad5 is known to replicate poorly in mouse cells in tissue culture (Prevec et al., 1989) nonetheless, both AdF and AdHN were capable of eliciting an immune response in mice. Production of neutralizing antibodies suggests that there was sufficient expression of the HPIV3 F and HN glycoproteins by recombinant adenoviruses to stimulate the immune system.

Subsequent experiments have shown that intranasal inoculation of cotton rats (*S. hispidus*) with recombinant adenoviruses resulted in the production of antiserum with the ability to neutralize HPIV3. Inoculation with AdF, AdHN or a combination of both adenoviruses elicited a neutralizing antibody response and resulted in reduced amounts of virus recovered from lungs after HPIV3 challenge (J. Dufresne and K. Dimock, unpublished results). Similarly, intranasal or enteric immunization of cotton rats with a recombinant adenovirus carrying the RSV F protein gene induced neutralizing antibodies and reduced RSV replication in lung tissue after challenge with RSV (Hsu et al., 1992). Generally, intranasal immunization with live vaccines mimics the route of natural infection and can result in the development of an effective serum response as well as the induction of a local mucosal response that protects against reinfection (Hsu et al., 1992).

Recent studies using recombinant adenovirus that expressed rabies virus glycoprotein (Xiang et al., 1996) have shown that preimmunity to Ad5 does not

impair the ability of recombinant adenoviruses to elicit a protective immune response in mice. Unfortunately, like recombinant RSV-vaccinia viruses, recombinant adenoviruses (Ad4, Ad5 or Ad7), that expressed the F glycoprotein of RSV were poorly immunogenic in seronegative chimpanzees despite multiple immunizations (Murphy et al., 1994).

Protective immunity to primary infection or reinfection with HPIV3 is most probably a result of both serum neutralizing antibodies and other immune responses such as cellular immunity and the induction of secretory IgA and non-neutralizing antibodies (Spriggs et al., 1987; Ray et al., 1988). Recombinant adenoviruses that express the HPIV3 F and HN proteins may have future potential as an HPIV3 vaccine, but provide a means, nonetheless, to study synthesis, processing and function of the HPIV3 glycoproteins in the absence of other HPIV3 proteins.

PART III : MUTAGENESIS OF THE HPIV3 F GLYCOPROTEIN

INTRODUCTION

The final objective of this study was to introduce mutations into the F protein of HPIV3 that would perturb the local structure of the protein and abolish fusogenic activity without interfering with the processes of cleavage, oligomerization or transport. Once transported to the surface of the infected cell, these altered F proteins could possibly be defective in their ability to interact with the HN protein or some cellular component, or be unable to form the correct conformation to be fusogenic. Mutagenesis could provide information concerning regions of the HPIV3 F protein that are critical for its function in cell fusion.

The construction of recombinant vaccinia viruses carrying mutant HPIV3 F genes was considered to be too time consuming so a third expression system was adopted. This system used a recombinant vaccinia virus (vTF7-3) that expresses the bacteriophage T7 RNA polymerase (Fuerst et al., 1986). Following infection of cells with vTF7-3, protein expression resulting from transfection of plasmid containing a target gene under the control of the T7 promoter sequence is driven by T7 RNA polymerase. This expression system eliminated the arduous task of creating recombinant vaccinia viruses containing mutant F genes.

In choosing sites for mutagenesis, sequence comparisons were made among the F proteins of members of the family *Paramyxoviridae* to identify conserved

sequences. One such region is the hydrophobic sequence adjacent to the cleavage site. This domain becomes the amino terminus of the F_1 subunit upon cleavage and is referred to as the fusion peptide. The fusion peptide was not chosen as a region for mutagenesis since it was shown previously that this region of the SV5 F protein could accommodate both conservative and nonconservative amino acid substitutions without loss of fusogenic activity. Mutant SV5 F proteins that did not function were not transported to the cell surface. F proteins that were transported exhibited fusogenic activity equal to or greater than the wild-type protein (Horvath and Lamb, 1992).

The paramyxovirus F proteins share additional domains that may be critical for F protein conformation and function. There is a marked conservation in the position of cysteine residues among the F proteins. Although the number of amino acid residues between each cysteine is similar, there is little conservation in the actual amino acid sequence. A cluster of 8 cysteine residues is present in the F_1 subunit of all F proteins. Previous work in this laboratory showed that substitution of serine for each cysteine in this cluster resulted in mutant F proteins that were neither cleaved into functional subunits nor transported to the cell surface (Côté, 1989). This was most probably due to improper folding, and retention of the altered F proteins within an intracellular compartment. Therefore, this cysteine rich region was also excluded as a candidate area for mutagenesis.

Heptad repeats are amino acid sequences predicted to adopt an α-helical structure and consist of seven amino acid repeats occurring in sequence periodicity

(*a b c d e f g*), where the side chains of the amino acids in positions *a* and *d* are predominantly bulky, hydrophobic or neutral (Cohen and Parry, 1986). Heptad repeats are also characterized by regions of the protein that are devoid of helix-breaking residues (Chambers et al., 1990). When position *a* of the heptad repeat is a leucine or isoleucine residue, the structure is referred to as a leucine zipper and is characteristic of many DNA-binding proteins (Buckland and Wild, 1989). Heptad repeats have the potential to form α-helical coiled-coils. An α-helical coiled coil was first proposed by F.H.C. Crick in 1953 and is composed of two or more α-helices that are associated lengthwise along their hydrophobic faces to maintain contact, resulting in the α-helices coiling around each other. Coiled-coils are common features of membrane proteins and are believed to stabilize the helix (Cohen and Parry, 1986). Coiled-coils have also been implicated in the formation of protein oligomers (Dubay et al., 1992; Yu et al., 1994) and have the potential to produce both inter- and intra-molecular associations (Chambers et al., 1990).

The HPIV3 F protein and F proteins of other paramyxoviruses contain two heptad repeat motifs located next to the two hydrophobic domains of the F_1 subunit (Buckland and Wild, 1989; Chambers et al., 1990). One motif will be referred to simply as the heptad repeat domain and is contiguous with the amino terminal fusion peptide of the F_1 subunit. The second domain is located on the amino-terminal side of the transmembrane region and will be referred to as the leucine zipper. A leucine zipper domain has been identified in the F proteins of all

members of the family *Paramyxoviridae* (Buckland and Wild, 1989) and several retroviruses (Dubay et al., 1992)

Based on amino acid sequence, similarities have been identified between the envelope glycoprotein *(env)* of the human immunodeficiency virus type 1 (HIV-1) and the F protein of the members of the family *Paramyxoviridae* (Gonzalez-Scarano et al., 1987). HIV-1 infection, like paramyxovirus infection, results in syncytium formation in infected $CD4^+$ cell monolayers. Also similar to the paramyxovirus F subunits, two HIV-1 proteins, gp120 and a transmembrane protein gp41, are produced as a result of proteolytic processing of a glycoprotein precursor gp160. These two proteins mediate binding of the virion to the CD4 cellular receptor and fusion of host and viral membranes, respectively. The tripeptide Phe-X-Gly, which is present at the F_1 amino terminus of all members of the family *Paramyxoviridae*, is also present seven amino acids downstream of the cleavage site of gp160 (Gallaher, 1987; Gonzalez-Scarano et al., 1987).

A leucine zipper motif has also been identified in the HIV-1 gp41 protein, certain nonconservative amino acid substitutions in position *a* of the third heptad repeat affected fusogenic activity (Dubay et al., 1992).

Positively charged amino acids, such as arginine and lysine, are often found on the cytoplasmic side of the transmembrane domain and may be a major determinant of the topology of integral membrane proteins (von Heijne, 1989). Changes in the distribution of positively charged amino acids in the transmembrane domain and cytoplasmic tail of HIV-1 gp41 protein were shown to abrogate fusion and affect

virus entry and/or syncytium formation (Helseth et al., 1990). The F protein of HPIV3, like HIV-1 gp41, has four positively charged amino acids on the cytoplasmic side of the membrane anchor.

In view of these results with the HIV-1 gp41 protein, both α-helical regions, the heptad repeat and leucine zipper domains as well as the transmembrane anchor and the cytoplasmic tail of the HPIV3 F protein were selected for mutagenesis.

RESULTS

I. Site-specific mutagenesis of HPIV3 F cDNA

Three regions of the HPIV3 F protein were chosen for mutagenesis. The heptad repeat region adjacent to the fusion peptide, the leucine zipper motif on the amino terminal side of the transmembrane anchor and the transmembrane anchor and cytoplasmic tail region of the HPIV3 F protein. Amino acid substitutions in the heptad repeat and leucine zipper domains were intended to alter the α-helical structure of the HPIV3 F protein without inhibiting proper folding and transport of the F protein to the host cell surface. Mutations in the transmembrane and cytoplasmic tail region were intended to alter the charge distribution in this region of the F protein.

Recombinant plasmid pIBIF70, which carried the HPIV3 F cDNA inserted in plasmid pIBI31 under the control of the T7 RNA polymerase promoter, was previously constructed by M-J. Côté (Côté, 1989). Recombinant plasmid pIBIHN which carried the HPIV3 HN cDNA, was also under the control of the T7 polymerase promoter and was transferred to a similar vector pIBI30 by J. Dufresne (University of Ottawa).

Site-specific mutagenesis, using the method developed by Kunkel (1985), was carried out directly on HPIV3 F sequences in single stranded DNA of pIBIF70(-). Plasmid pIBIF70(-) was constructed by inverting a Pvu II fragment of pIBIF70 that

contained the HPIV3 cDNA as well as the T7 RNA polymerase promoter. Nucleotide substitutions were introduced into the HPIV3 F coding sequence of pIBIF70(-) by site-directed mutagenesis using mismatched positive-sense HPIV3 F-specific oligodeoxyribonucleotides. Fourteen sites within three domains of the F protein were selected for mutagenesis. The positions of the amino acid substitutions and the residues that were introduced are shown in Table 3 and the sequences of the oligonucleotides are shown in Appendix A.

Mutations in the HPIV3 F protein were designated by amino acid position and type of substitution. There were three substitutions in the heptad repeat region: 137/PRO (GCC to CCC); 160/PRO (ACA to AAA); and 184/SER (CCA to TCA). There were six substitutions at the isoleucine at position 474 in the third repeat of the leucine zipper motif : 474/GLY (ATA to GGA); 474/SER (ATA to TCA); 474/ASP (ATA to GAC); 474/GLU (ATA to GAG); 474/VAL (ATA to GTA); and 474/ALA (ATA to AAA). Lysine was substituted for isoleucine at position 506 in the hydrophobic membrane spanning region of the F_1 subunit to produce mutant protein 506/LYS (ATA to AAA). Four positively charged amino acids in the cytoplasmic tail of the F_1 subunit were replaced by uncharged residues: 517/THR (AAG to ACG); 520/ILE (AGA to ATA); 523/THR (AAG to ACG); and 524/ILE (AGA to ATA). Fusion proteins produced by mutant 137/PRO had an additional substitution of alanine for VAL^{132} within the fusion peptide due to a mismatched base pair in the oligonucleotide.

TABLE 3

SITES OF AMINO ACID SUBSTITUTIONS

AA #	AMINO ACID	MUTATION
137	ALA	→ PRO
160	SER	→ PRO
185	PRO	→ SER
474	ILE	→ GLY
		→ SER
		→ ASP
		→ GLU
		→ VAL
		→ ALA
506	ILE	→ LYS
517	LYS	→ THR
520	ARG	→ ILE
523	LYS	→ THR
524	ARG	→ ILE

II. Fusogenic properties of HPIV3 F glycoproteins

Fusion following transfection with pIBIF70(-) + pIBIHN

Conditions were established to produce the maximum amount of fusion following transfection of vTF7-infected HeLa T4$^+$ cells with plasmids pIBIF70 + pIBIHN, as shown in Table 4. The smallest amounts of pIBIF70 + pIBIHN plasmid that produced the most extensive cell fusion were 0.5 µg and 5.0 µg of DNA, respectively, for each 60 mm tissue culture dish.

Typically, syncytia began to appear in vTF7-3 infected cells transfected with pIBIHN + pIBIF70 (or pIBIF70(-)), 4-6 hours after transfection. Syncytium formation was most extensive approximately 3 hours later (Figure 15, panel A) and was indistinguishable from fusion observed in HPIV3-infected HeLa T4$^+$ cell monolayers (Figure 15, panel C). At its peak, fusion involved >70% of the cell monolayer and typically, syncytia contained more than 30 nuclei, and in large syncytia, nuclei were too numerous to count. Syncytia were not observed in cultures infected with vTF7-3 (Figure 15, panel D), or in vTF7-3-infected cells singly transfected with pIBIF70 (Figure 15, panel B) or pIBIHN (Figure 15, panel E). Multinucleated cells containing 6 nuclei or fewer, which were seen in uninfected HeLa T4$^+$ monolayers as well as transfected and nontransfected HeLa T4$^+$ cells infected with vaccinia virus, were not considered to be the result of F protein mediated cell fusion and have also been observed by others (Hemingway et al., 1994).

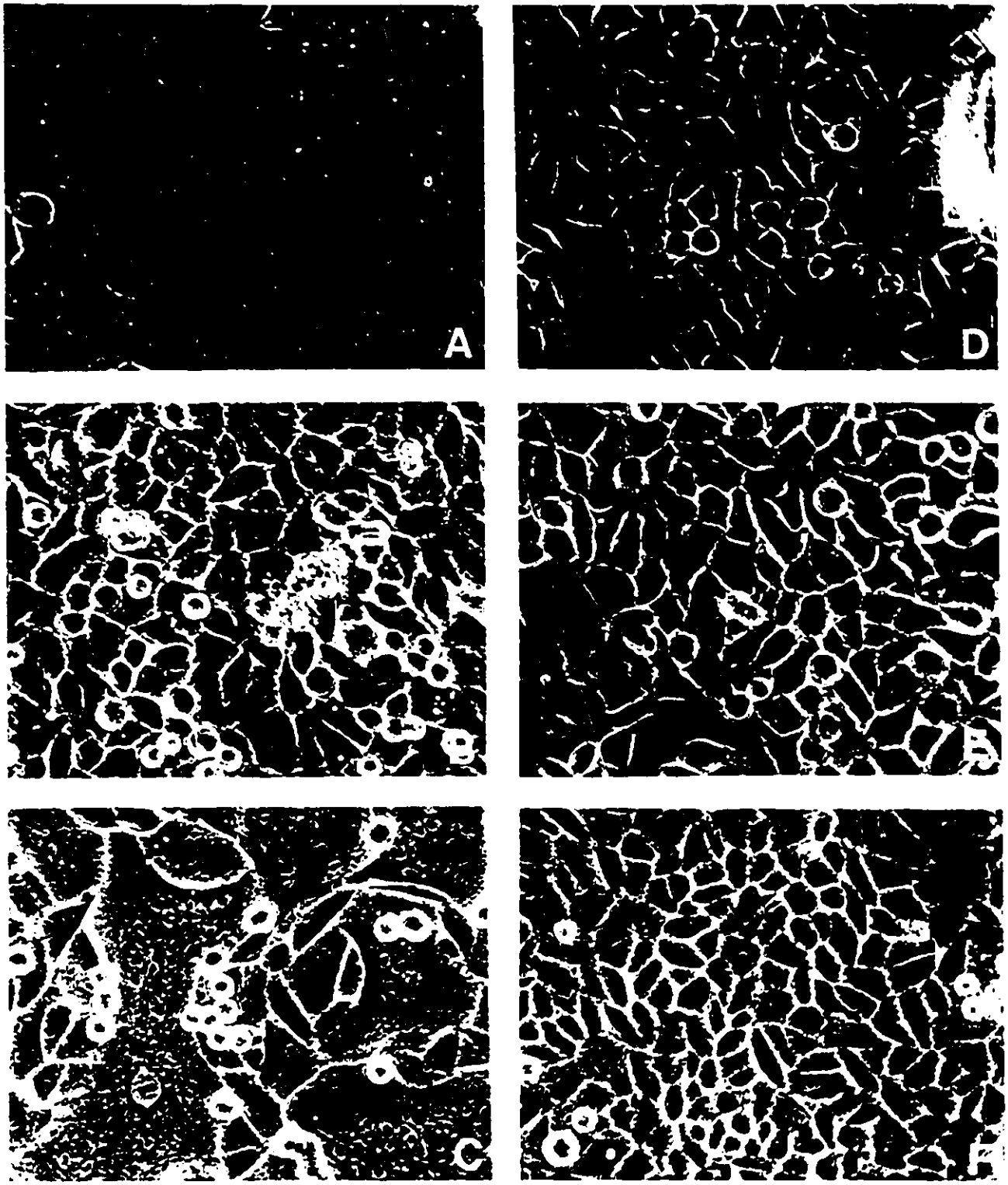
TABLE 4

FUSOGENIC ACTIVITY OF VARIABLE AMOUNTS OF INPUT DNA[1]

pIBIF70 (µg)	pIBIHN (µg)	% Monolayer Fused
0.05	10.0	75-90 %
0.10	10.0	75-90 %
0.20	10.0	75-90+%
0.50	10.0	>90 %
1.0	10.0	>90 %
2.0	10.0	75-90 %
5.0	10.0	25-50 %
7.5	10.0	25-90 %
10.0	10.0	<25 %
10.0	5.0	<25 %
10.0	2.0	<25 %
10.0	1.0	<25 %
0.50	10.0	>90 %
0.50	7.5	>90 %
0.50	5.0	>90 %
0.50	2.0	50-75 %
0.50	1.0	25-50 %
0.50	0.50	25-50 %

[1] Amounts of input DNA used for each 60 mm tissue culture dish.

Figure 15. Syncytium formation in HeLa T4$^+$ cell monolayers infected with HPIV3 or transfected with plasmids pIBIF70 and/or pIBIHN. HeLa T4$^+$ cells were infected with either HPIV3 at a multiplicity of infection of 5 or with the recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 10 for 1 hour. Cells infected with vTF7-3 were then transfected singly or in combination with pIBIF70 and/or pIBIHN. Cultures were photographed 8 hours post-transfection. Panel A, pIBIF70 +pIBIHN. Panel B, pIBIF70. Panel C, HPIV3. Panel D, vTF7-3. Panel E, pIBIHN. Panel F, mock-infected.


A
D
B
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F

Fusion properties of mutant F glycoproteins

To test the fusogenic properties of mutant F glycoproteins, vTF7-3 infected HeLa T4$^+$ cells were cotransfected with pIBIHN and plasmids that contained the altered F genes. Cell monolayers were transfected with a constant amount of pIBIHN (5.0 µg) and the amounts of transfected mutant F plasmid ranged from 0.1 to 5.0 µg of DNA per 60 mm tissue culture dishes. Varying the amounts of transfected DNA accounted for possible differences in the purity of the DNA preparations, which might have affected transfection efficiency. The optimum amount of input DNA for all F plasmids, including pIBIF70(-), that resulted in syncytium formation was 0.5 µg of DNA, as was observed for pIBIF70. No differences were detected in the period of time between transfection and the initial appearance of syncytia for any of the altered F plasmids as compared to the pIBIF70(-). The results of these fusion experiments are presented in Figure 16 and Figure 17, and summarized in Table 5.

Three distinct fusion phenotypes were observed. The first was a wild-type fusion phenotype indistinguishable from HPIV3-infected cells, or vTF7-3 infected cells transfected with pIBIHN + pIBIF70(-) (or pIBIF70 + pIBIHN) (Figure 17, panel A). The second phenotype was a negative fusion phenotype in which monolayers did not exhibit fusion. The third phenotype was a novel phenotype which was designated as intermediate fusion. Intermediate fusion was characterized by syncytia that contained 6-30 nuclei, which were smaller than those observed in the

wild-type fusion phenotype, and, typically, 30% or less of the monolayers were involved in syncytium formation. Multinucleated cells occurred in clusters throughout the infected monolayer.

The heptad repeat region of the HPIV3 F protein has the potential to form an α-helix beginning with ALA^{137} and terminating at PRO^{185} (Chambers et al., 1990). To disrupt the predicted α-helical nature of the heptad repeat, a helix-breaking proline residue was substituted for either alanine at position 137 (137/PRO) or SER^{160} (160/PRO). HeLa $T4^+$ cells transfected with plasmid 137/PRO did not fuse (Figure 16, panel B), whereas cells transfected with plasmid 160/PRO (Figure 16, panel E) exhibited an intermediate fusion phenotype. Substitution of serine for PRO^{185} was also intended to modify local conformation by removing a kink or bend in the protein sequence near the end of the heptad repeat region. HeLa $T4^+$ cells expressing mutant protein 185/SER also had an intermediate fusion phenotype (Figure 16, panel A).

The leucine zipper motif spans the region of the HPIV3 F protein from amino acids 460 to 481. Substitutions at ILE^{474} were predicted to lie within the hydrophobic face of the amphipathic α-helix and have the potential to perturb the structure of the leucine zipper and thus affect fusogenic ability. Replacement of ILE^{474} with glycine (Figure 17, panel B) or aspartic acid (Figure 17, panel C) abolished fusion and substitution of ILE^{474} with serine (Figure 17, panel F) or alanine (Figure 17, panel H) produced F glycoproteins with intermediate fusion phenotypes. Substitution of ILE^{474} with glutamic acid (Figure 17, panel G) or valine

Figure 16. Syncytium formation of HeLa T4$^+$ monolayers infected with vTF7-3 and transfected with mutant F plasmids. HeLa T4$^+$ cells were infected with vTF7-3 at a multiplicity of 10 for 1 hour and then transfected with plasmid DNA. Cultures were fixed with methanol 8 hours post-infection and 1:20 dilution of Giemsa stain for 1 hour. Cells were photographed using a Cambridge PhotoZoom inverted microscope. Panel A, plamid 160/PRO. Panel B, plasmid 137/PRO. Panel C, panel 517/THR. Panel D, plasmid 523/LYS. Panel E, plasmid 185/SER. Panel F, plasmid 506/LYS. Panel G, plasmid 520/ILE. Panel H, plasmid 524/ILE.

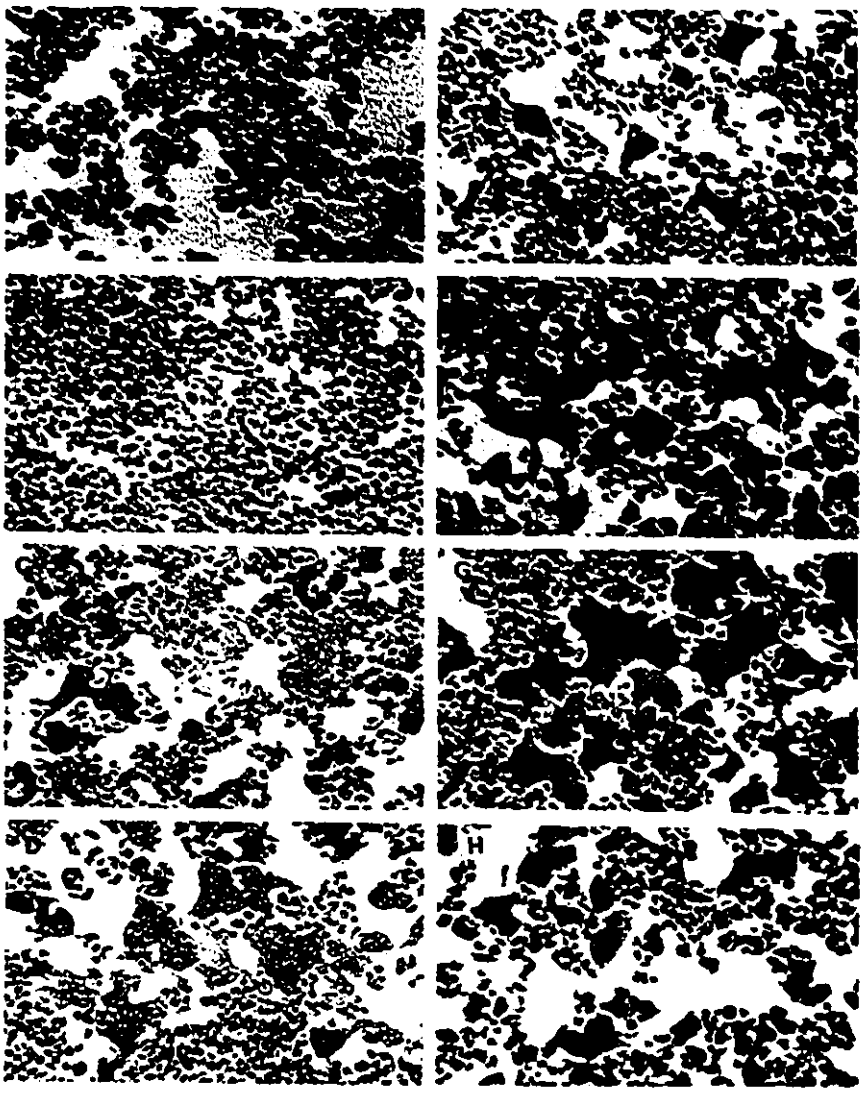


Figure 17. Syncytium formation of HeLa T4$^+$ cell monolayers infected with vTF7-3 and transfected with F plasmids with mutations in the leucine zipper. See legend for figure 16. Panel A, plasmid pIBIF70(-) + pIBIHN. Panel B, plasmid 474/GLY. Panel C, plasmid 474/ASP. Panel D, plasmid 474/VAL. Panel E, vTF7-3. Panel F, plasmid 474/SER. Panel G, plasmid 474/GLU. Panel H, plasmid 474/ALA.

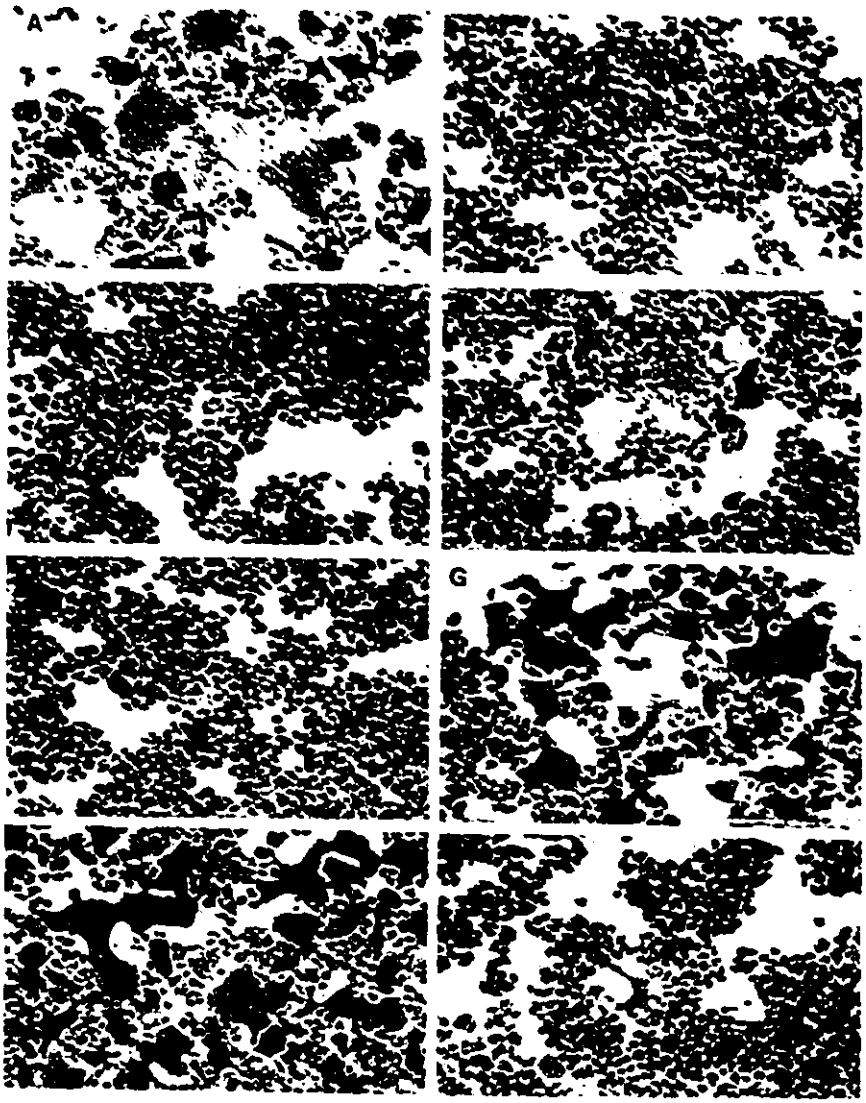

A
G

TABLE 5

MUTATION SITES AND FUSOGENIC ACTIVITY

AA #	AMINO ACID	MUTATION	FUSION ACTIVITY
137	ALA	PRO	NONE
160	SER	PRO	INTERMEDIATE
185	PRO	SER	INTERMEDIATE
474	ILE	GLY	NONE
		SER	INTERMEDIATE
		ASP	NONE
		GLU	WILD-TYPE
		VAL	WILD-TYPE
		ALA	INTERMEDIATE
506	ILE	LYS	WILD-TYPE
517	LYS	THR	WILD-TYPE
520	ARG	ILE	WILD-TYPE
523	LYS	THR	WILD-TYPE
524	ARG	ILE	WILD-TYPE

(Figure 17, panel D) did not appear to have any effect on F protein function and resulted in extensive (wild-type) fusion.

The hydrophobic membrane spanning region of the envelope glycoprotein of HIV-1 (and other lentiviruses), is interrupted by charged amino acids and followed by several positively charged residues in the cytoplasmic tail. Mutation of these residues has been shown to diminish the ability of HIV-1 gp41 to mediate membrane fusion (Helseth et al., 1990). Unlike the transmembrane domain of gp41, the membrane spanning domain of the HPIV3 F glycoprotein is void of charged residues. Substitution of lysine for ILE^{506}, a basic amino acid residue, was introduced into the transmembrane domain of the HPIV3 F protein, but had no detectable effect on fusogenic activity (Figure 16, panel F). Substitution of uncharged isoleucine or threonine residues for lysine (LYS^{517} or LYS^{523}) or arginine (ARG^{520} or ARG^{524}), residues in the cytoplasmic tail also did not appear to interfere with the fusogenic ability of the F glycoprotein (Figure 16, panels C, D, G and H).

III. Cell surface expression of F glycoproteins

Decreased syncytium formation in cells expressing mutant HPIV3 F glycoproteins may have been due to either a reduction in the number of functional F molecules reaching the cell surface or a decrease in the fusogenic properties of

the altered F proteins. To determine if modified F proteins were transported to the cell surface, a mixture of 7 monoclonal antibodies specific for the HPIV3 F protein (a197(A), c145 (AB), c215(B), a640(D), b4(E) and b69(F)) was used in indirect cell surface immunofluorescence assays (Figure 18). Each antibody recognized an epitope on the HPIV3 F protein, in one of 7 previously identified antigenic sites A, AB, B,C, D, E and F (Coelingh and Tierney, 1989). The antigenic sites recognized by each antibody are shown in parentheses. All of the mutant F proteins were detected on the surface of transfected cells (Figure 18, panels B to I and M to Q) with the exception of mutant protein 474/GLY (Figure 18, panel A).

To investigate whether mutant protein 474/GLY was located intracellularly, the panel of monoclonal antibodies was tested on transfected cells permeablized using acetone (Figure 19). Cells transfected with plasmid 474/GLY did not express protein on the cell surface (Figure 19, panel A), however, when fixed using acetone, protein was observed intracellularly (Figure 19, panel C). Decreased immunofluorescence was observed in cells transfected with plasmid 474/GLY, and could reflect either a decrease in the amount of protein present in transfected cells, or a decrease in the ability of the protein to bind one or more of the antibodies in the mixture. Therefore, the protein expressed by mutant 474/GLY is synthesized but not transported to the cell surface. Each of the 7 monoclonal antibodies was tested by immunofluorescence for the ability to recognize each of the mutant F proteins in acetone-permeabilized cells. Representative data are shown in Figure

Figure 18(A). Cell surface expression of mutant F glycoproteins. HeLa T4$^+$ cells were infected with recombinant vaccinia virus vTF7-3 for 1 hour then transfected with plasmid DNAs as indicated. 18 hours post-transfection, cells were incubated with a mixture of monoclonal antibodies specific for the HPIV3 F protein, then with an FITC-tagged secondary antibody and photographed. Panel A, 474/GLY. Panel B, 474/SER. Panel C, 474/ALA. Panel D, 474/GLU. Panel E, 474/VAL. Panel F, 474/ASP.

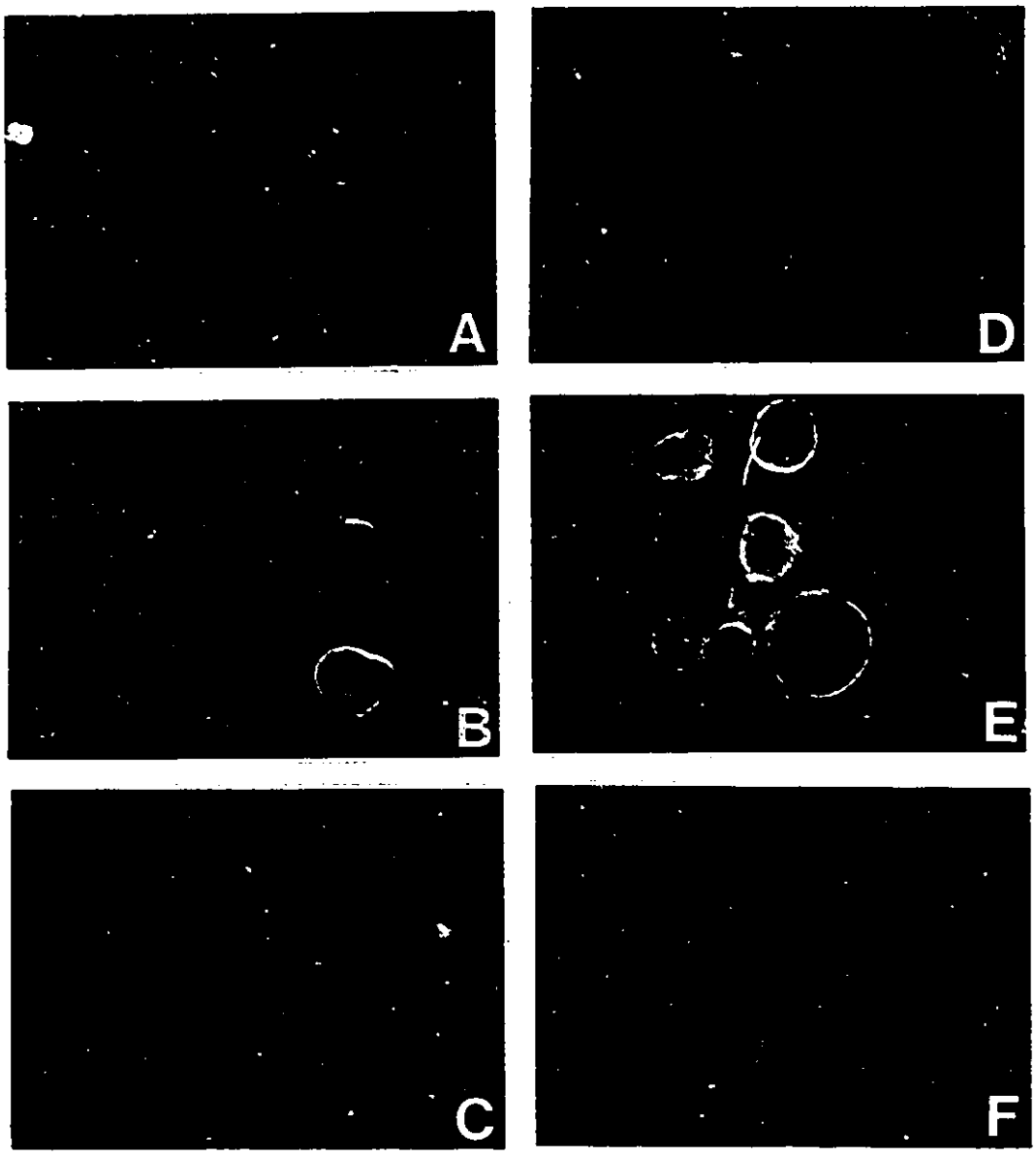

A
D
B
E
C
F

Figure 18(B). Cell surface expression of mutant F glycoproteins. As described in the legend for Figure 18(A). Panel G, 137/PRO. Panel H, 160/PRO. Panel I, 185/PRO. Panel J, mock-infected. Panel K, HPIV3-infected. Panel L, pIBIF70(-).

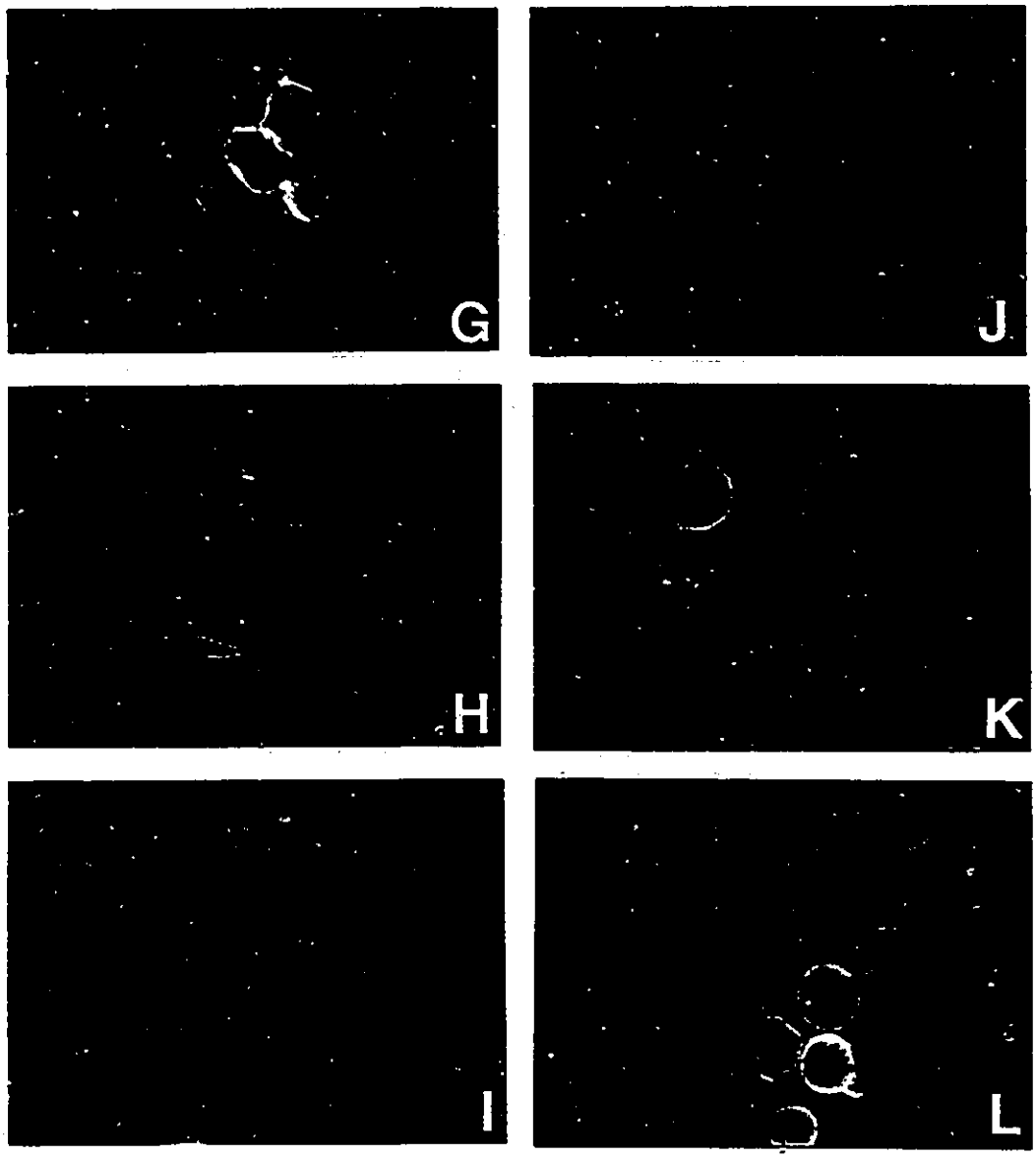

G
J
H
K
I
L

Figure 18 (C). Cell surface expression of mutant F glycoproteins. As described in the legend for Figure 18(A). Panel M, 517/THR. Panel N, 520/ILE. Panel O, 523/THR. Panel P, 524/ILE. Panel Q, 506/LYS. Panel R, pIBI31.

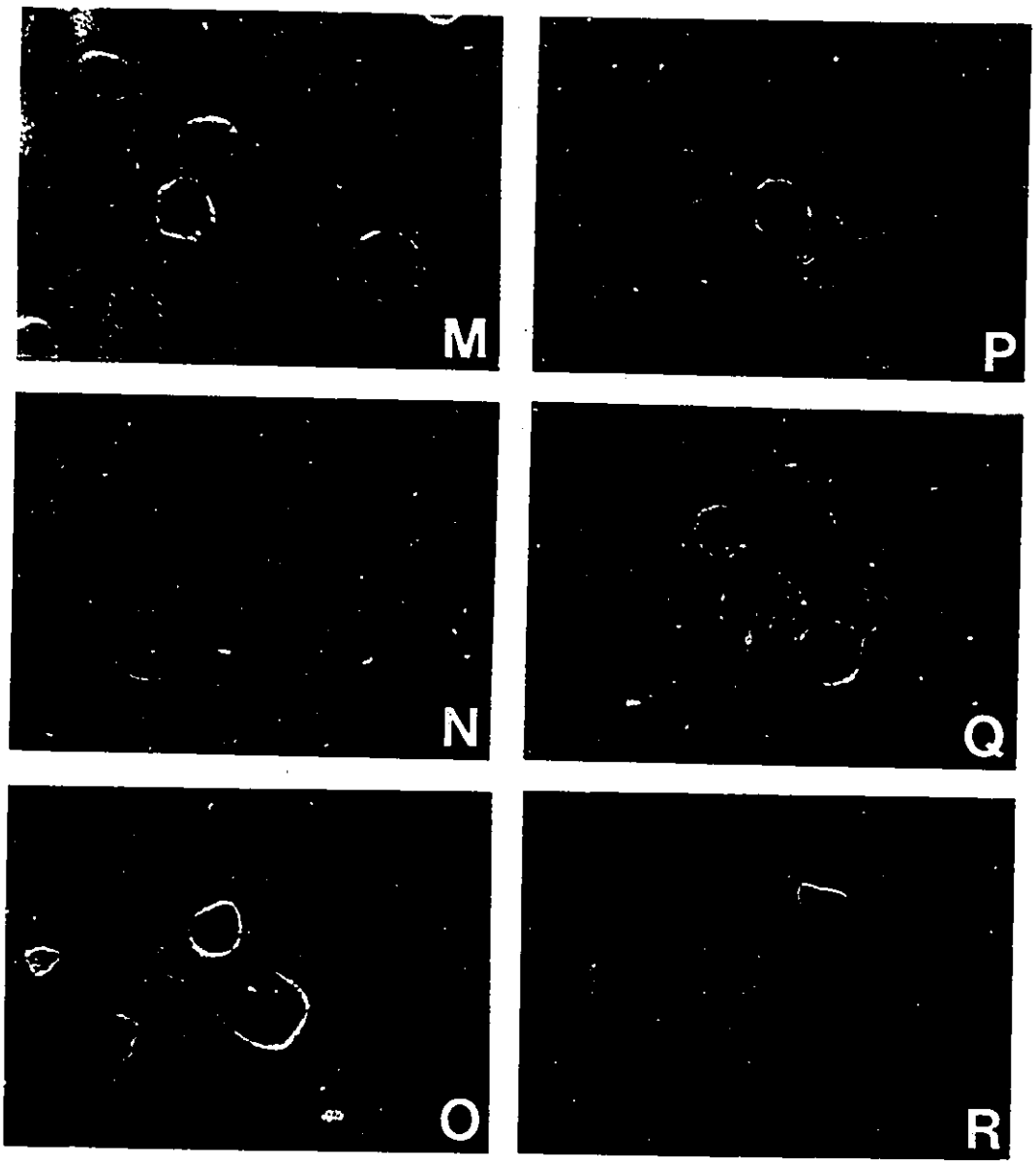

M
P
N
Q
O
R

Figure 19. Intracellular expression of mutant F glycoprotein 474/GLY. HeLa T4$^+$ cells were grown on 8 well slides and infected with HPIV3 or infected with recombinant vaccinia virus vTF7-3 for 1 hour and then transfected with plasmid 474/GLY. Cells were incubated with the panel of 7 monoclonal antibodies specific for the HPIV3 F protein 18 hours post-transfection, then with an FITC-tagged secondary antibody and photographed (Panel A). For permeabilization, slides were immersed in acetone prior to incubation with antibody (Panels B to D). Panel A, 474/GLY. Panel B, HPIV3. Panel C, 474/GLY. Panel D, mock.

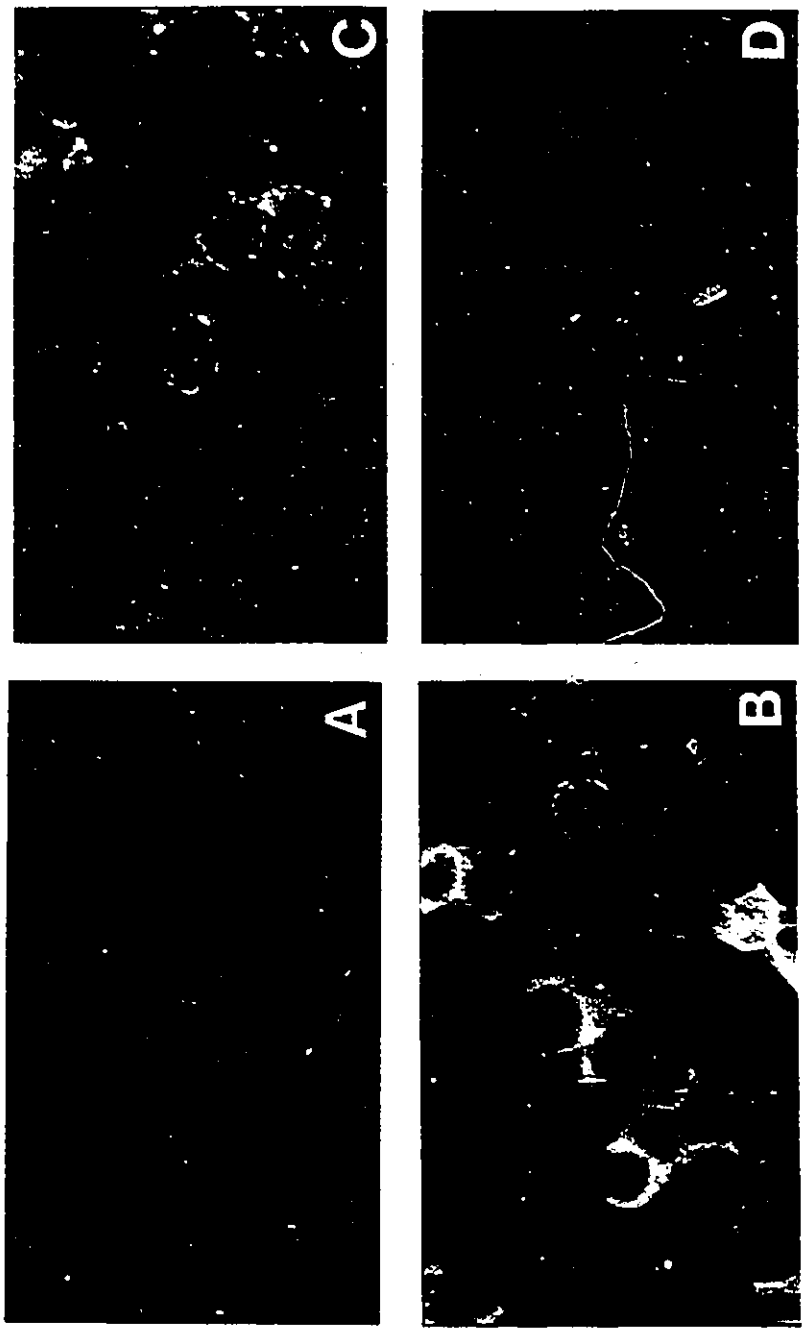
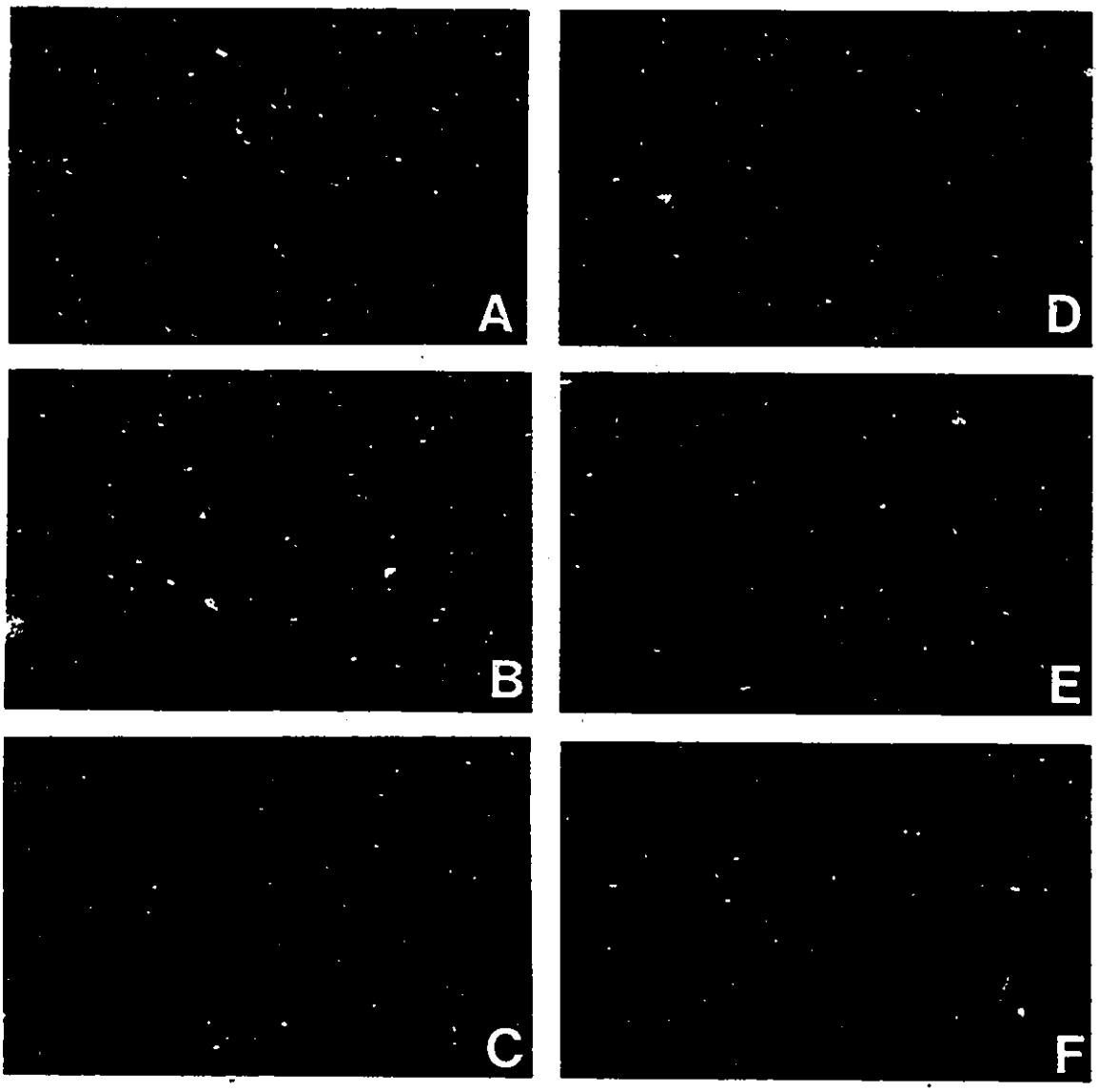

C
D
A
B

Figure 20. Intracellular immunofluorescence of 517/LYS with individual monoclonal antibodies. HeLa T4$^+$ cells were infected with recombinant vaccinia virus vTF7-3 and transfected with the mutant plasmid 517/LYS. Cells were permeabilized with acetone 18 hours post-transfection before incubation with primary monoclonal antibodies and secondary FITC-tagged antibodies. Panel A, a197 (A). Panel B, c215 (B). Panel C, a640 (C). Panel D, a591 (D). Panel E, b4 (E). Panel F, b69 (F).


A
D
B
E
C
F

20, for HeLa T4$^+$ cells transfected with mutant plasmid 517/LYS. Reduced immunofluorescence was observed for acetone-permeabilized cells that expressed certain F mutants using antibodies a197(A), c145(AB) and a640(C) (data not shown). Cells with decreased immunofluorescence were reexamined by flow cytometry, which is a more sensitive method to detect antibody binding.

Antibodies c215(B), a591(D), b4(E) and b69(F), which reacted equally well with all of the mutant F proteins in immunofluorescence assays were not tested further. Instead, three other antibodies, b48(A), c128(B) and b19(E) were included in cytometric analyses. For cytometry, transfected cell monolayers were trypsinized and split into aliquots prior to incubation with primary antibodies to ensure that the populations incubated with each antibody had identical transfection efficiencies. Approximately 10,000 cells were counted for each sample tested. The relative mean fluorescence intensity (MFI) was used as an indicator of cell surface expression of F glycoproteins. The results of cytometric analyses are presented in Table 6 and confirmed that all of the mutant F proteins were expressed on the surface of transfected cells with the exception of mutant protein 474/GLY. Cytometric analyses also confirmed decreased antibody binding for certain combinations of antibody and mutant F proteins.

Monoclonal antibody c128(B) did not react with mutant F proteins 137/PRO, 160/PRO, 185/SER, 474/SER, 474/ASP and 474/ALA. Therefore, mutant F proteins that have aberrant fusion phenotypes (ie. intermediate or negative), also

TABLE 6

FLOW CYTOMETRIC ANALYSIS OF HPIV3 F PROTEINS

	SITE A		SITE AB	SITE B	SITE C	SITE E
	A197	B48	C145	C128	A640	B19
pIBIF70(-)	+(52.3)[1]	+ (34.2)	+ (68.7)	+ (39.7)	+ (69.0)	+ (34.4)
137/PRO	+ (52.1)	+ (71.3)	-[2]	-	-	+ (75.2)
160/PRO	+ (39.2)	+ (20.4)	+ (29.6)	-	+ (85.9)	ND[3]
185/SER	+ (25.0)	+ (24.7)	+ (26.7)	-	+ (25.5)	+ (22.2)
474/GLY	-	-	ND	-	ND	-
474/SER	+ (74.5)	+(100.5)	ND	-	ND	+(100.8)
474/ASP	+ (72.6)	+(107.2)	ND	-	ND	+(107.2)
474/GLU	+ (53.2)	ND	ND	+ (48.2)	ND	+ (36.2)
474/VAL	+ (55.9)	+ (61.8)	ND	+ (37.7)	ND	+ (82.8)
474/ALA	+ (51.8)	+ (70.8)	ND	-	ND	+ (78.4)
506/LYS	+ (74.6)	+ (37.6)	ND	+ (51.7)	ND	+ (39.4)
517/THR	+(126.0)	+ (30.9)	ND	+ (59.1)	ND	+ (33.6)
520/ILE	+ (71.5)	+ (37.1)	ND	+ (58.9)	ND	+ (28.0)
523/THR	+ (61.8)	+ (40.2)	ND	+ (54.2)	ND	+ (38.4)
524/ILE	+ (71.6)	+(103.9)	ND	+ (25.3)	ND	+(102.0)

1 the mean fluorescence intensity (MFI) observed in the negative population of cells was subtracted from the MFI observed in the positive population, the MFI of the negative population of cells was 1.6, 1.8, 2.3, 1.5, 2.0 and 1.6 for antibodies a197, b48, c145, c128, a640 and b19 respectively.

2 negative, less than 1% of cell population bound antibody

3 not determined by flow cytometry

lost the ability to bind this particular monoclonal antibody. Mutant protein 137/PRO, which had no fusogenic ability, also did not react with antibodies c145(AB) and a640(C). Cytometric analysis confirmed the findings of decreased fluorescence (Table 6).

IV. Cleavage of F glycoproteins

The HPIV3 F glycoprotein is synthesized as a precursor protein F_0, which is cleaved into the active form F_1 + F_2, by cellular endoproteases during transport to the cell surface. Both cleaved and uncleaved forms of the F protein are present on the cell surface (Ortmann et al., 1994). Resistance to cleavage activation could explain the intermediate or negative fusion phenotypes of mutant F proteins. To determine if any of the mutant F proteins were present in cells exclusively as uncleaved, inactive precursor molecules, radiolabelled F glycoproteins were immunoprecipitated from cell lysates with a mixture of monoclonal antibodies c215(B) and a197(A). Immunoprecipitation of both F_0 and F_1 had been observed using monoclonal antibodies a197(A), c215(B) and c128(B) (data not shown). Antibody c215(B) had been used previously in immunofluorescence assays and had been shown to immunoprecipitate HPIV3 F oligomers (Russell et al., 1994). Antibody a197(A) was also used for immunofluorescence, flow cytometry and immunoprecipitation of F proteins. Immunoprecipitated proteins were then

analyzed by SDS-polyacrylamide gel electrophoresis. The relative amounts of immunoprecipitated F_0 and F_1 proteins shown in Figures 21 and 22 were measured by densitometry. The extent of cleavage of F proteins was calculated and is shown in Table 7.

As shown in Figures 21 and 22, both the cleaved and uncleaved forms of all of the mutant proteins were present in transfected cells, with the exception of mutant protein 474/GLY where only the F_0 precursor was observed (Figure 21, lane 7). The extent of cleavage of the different F proteins varied in comparison to cleavage observed with the F protein of pIBIF70(-) or HPIV3. Most noticeable was the inefficient cleavage for proteins with mutations in the heptad repeat region, 137/PRO (10% cleavage), 160/PRO (16% cleavage) and 185/SER (13% cleavage) (Figure 22, lanes 5,3 and 4, respectively). Inefficient cleavage was also observed for three proteins with mutations in the leucine zipper, 474/GLY (no cleavage), 474/ASP(7% cleavage) and 474/ALA (< 2% cleavage) (Figure 21, lanes 7, 9 and 12, respectively). All of the mutant proteins that exhibited poor cleavage also had aberrant fusion phenotypes. In contrast, mutant protein 474/SER (Figure 21, lane 8), was efficiently cleaved (54% cleavage) yet still produced an intermediate fusion phenotype. Evidently, even the presence of a small amount of F_1 subunit is sufficient for fusogenic activity (for example cleavage $\leq$ 2%), while cleavage $\geq$18% of the HPIV3 F protein results in wild-type fusion.

Figure 21. Immunoprecipitation of mutant F proteins . Infected and transfected HeLa T4$^+$ cells were labelled with [^{3}H] leucine for 3 hours and lysed. Cell lysates were incubated with protein G sepharose and monoclonal antibodies a197 and c215. Immunoprecipitated proteins were resolved by electrophoresis on a 10% polyacrylamide gel containing SDS under reducing conditions. Lane 1, mock-infected HeLa T4$^+$ cells. Lane 2, 517/THR. Lane 3, 520/ILE. Lane 4, 523/THR. Lane 5, 524/ILE. Lane 6, 506/LYS. Lane 7, 474/GLY. Lane 8, 474/SER. Lane 9, 474/ASP. Lane 10, 474/GLU. Lane 11, 474/VAL. Lane 12, 474/ALA. Lane 13, HPIV3-infected. Lane 14, pIBIF70(-).

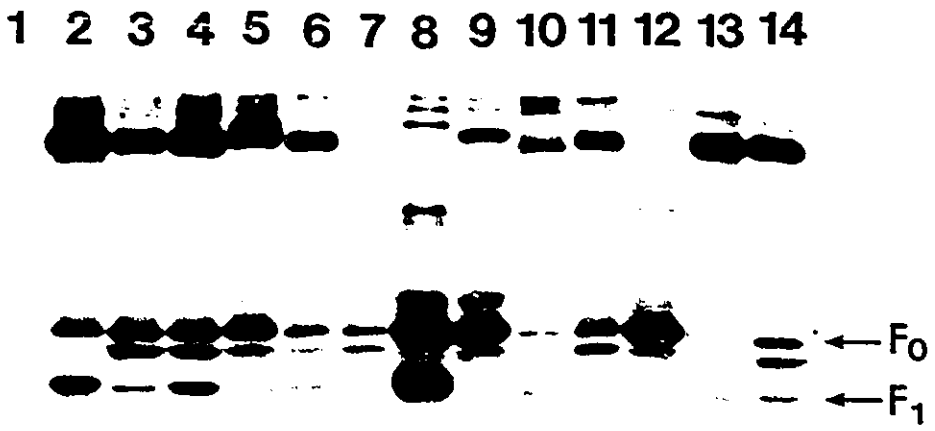

1 2 3 4 5 6 7 8 9 10 11 12 13 14
F_0
F_1

Figure 22. Immunoprecipitation of F proteins with mutation in the heptad repeat. See legend for Figure 21. Lane 1, HPIV3-infected. Lane 2, mock-infected. Lane 3, 137/PRO. Lane 4, 160/PRO. Lane 5, 185/SER. Lane 6, pIBIF70(-).

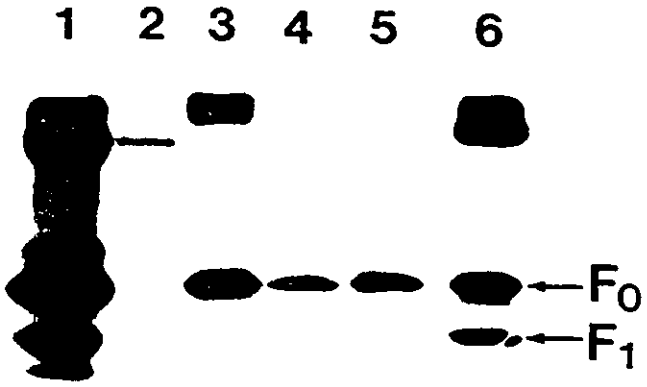

1 2 3 4 5 6
F_0
F_1

TABLE 7

MUTATIONS SITES, FUSOGENIC ACTIVITY AND CLEAVAGE

AA #	AMINO ACID	FUSION ACTIVITY	% CLEAVAGE	
			FIG 14	FIG 15
137	ALA→PRO	NONE		10%
160	SER→PRO	INTERMEDIATE		16%
185	PRO→SER	INTERMEDIATE		13%
474	ILE→GLY	NONE	-[1]	
	→SER	INTERMEDIATE	54 %	
	→ASP	NONE	7 %	
	→GLU	WILD-TYPE	47 %	
	→VAL	WILD-TYPE	36 %	
	→ALA	INTERMEDIATE	<2 %	
506	ILE→LYS	WILD-TYPE	44 %	
517	LYS→THR	WILD-TYPE	58 %	
520	ARG→ILE	WILD-TYPE	37 %	
523	LYS→THR	WILD-TYPE	48 %	
524	ARG→ILE	WILD-TYPE	18 %	
		HPIV3	79 %	34%
		pIBIF70(-)	48 %	48%

[1] No cleavage observed

V. Oligomerization of F glycoproteins

The HPIV3 F protein forms oligomeric complexes, most probably trimers, in infected cells (Russell et al., 1994) and failure of a mutant F protein to oligomerize could explain a negative or intermediate fusion phenotype. To test for the formation of oligomeric complexes, radiolabelled F glycoproteins were chemically cross-linked using DTSSP prior to immunoprecipitation. Immunoprecipitation was carried out using a mixture of monoclonal antibodies a197(A) and c215(B), and oligomers were analyzed by electrophoresis under nondenaturing conditions in boric-acetate polyacrylamide gels. Cross-linked species immunoprecipitated from lysates of HPIV3-infected cells (Figure 23, lanes 1 and 2) and vTF7-3 infected cells transfected with pIBIF70(-) (Figure 23, lanes 5 and 6) were indistinguishable. Three major protein species were observed that migrated with apparent molecular masses of approximately 195, 155 and 72 kDa, similar to cross-linked oligomeric species previously identified for the HPIV3 F protein (Russell et al., 1994). These three species are believed to be homotrimers, homodimers and monomers of the F glycoprotein, respectively.

When transfected cells were lysed in the presence of the detergent NP40 prior to cross-linking, an increase in the amount of F protein present as both trimers and a species larger than trimers was noted. Concomitant with the increase in these two species, there was a decrease in the amount of protein present as monomer, again, similar to observations by Russell and coworkers (1994). Since F proteins

resulting from mutations in the transmembrane region and the cytoplasmic tail did not appear to alter fusogenic activity, these mutant proteins were not tested for oligomerization. All of the F glycoproteins with amino acid substitutions in either the heptad repeat region or leucine zipper that were expressed at the surface of transfected cells produced electrophoretic patterns similar to those seen for the unmodified HPIV3 F protein.

Figure 23 shows the electrophoretic patterns of mutant proteins with amino acid substitutions in the leucine zipper domain. Mutant protein 474/GLY (Figure 23, lanes 1 and 2), which was not transported to the transfected cell surface, was only detected as monomer. Mutant protein 474/GLU, which had a wild-type fusion phenotype produced an oligomerization pattern (Figure 23, lanes 7 and 8), similar to those of leucine zipper mutants with aberrant fusion phenotypes (Figure 23, lanes 1 to 6 and lanes 9 and 10) or to that of unmodified F protein (Figure 23, lanes 11 and 12). Mutant F proteins that had amino acid substitutions in the heptad repeat region also exhibited patterns similar to the other F proteins (Figure 24).

Immunoprecipitation of lysates from cells cotransfected with pIBIF70(-) + pIBIHN with monoclonal antibodies specific for the HPIV3 F protein, produced an oligomerization pattern that did not differ from the pattern produced by immunoprecipitation of lysates from cells infected with HPIV3 or transfected with pIBIF70(-) alone. It did not appear that the coexpression of the F and HN proteins altered the oligomerization pattern of the F proteins. Similarly, immunoprecipitation of lysates from cells cotransfected with plasmids pIBIF70(-) + pIBIHN using a

monoclonal antibody specific for the HPIV3 HN protein produced an oligomerization pattern different to that produced when these cell lysates were immunoprecipitated with monoclonal antibodies specific for the F protein (data not shown). Therefore, it did not appear that the HPIV3 F and HN proteins formed heterooligomers that could be detected using the chemical cross-linking agent DTSSP.

Figure 23. Oligomerization of F proteins with mutation in the leucine zipper. HeLa T4 cells were infected with recombinant vaccinia virus vTF7-3 and transfected with F plasmid DNAs encoding altered HPIV3 F glycoproteins as indicated. Cells were radiolabelled with [^{3}H]-leucine and treated with the chemical cross-linking agent DTSSP, prior to (-) or subsequent to (+) NP40 lysis. Cell lysates were incubated with protein G Sepharose and monoclonal antibodies a197 and c215, and immunoprecipitated proteins were resolved by electrophoresis on a 3.5% boric acetate polyacrylamide gel under non-reducing conditions. Lanes 1 & 2, 474/GLY, -/+ NP40, 8 day and 3 day exposures. Lanes 3 & 4, 474/SER, -/+ NP40. Lanes 5 & 6, 474/ASP, -/+ NP40. Lanes 7 & 8, 474/GLU, -/+ NP40. Lanes 9 & 10, 474/ALA, -/+ NP40. Lanes 11 & 12, pIBIF70(-), -/+ NP40.

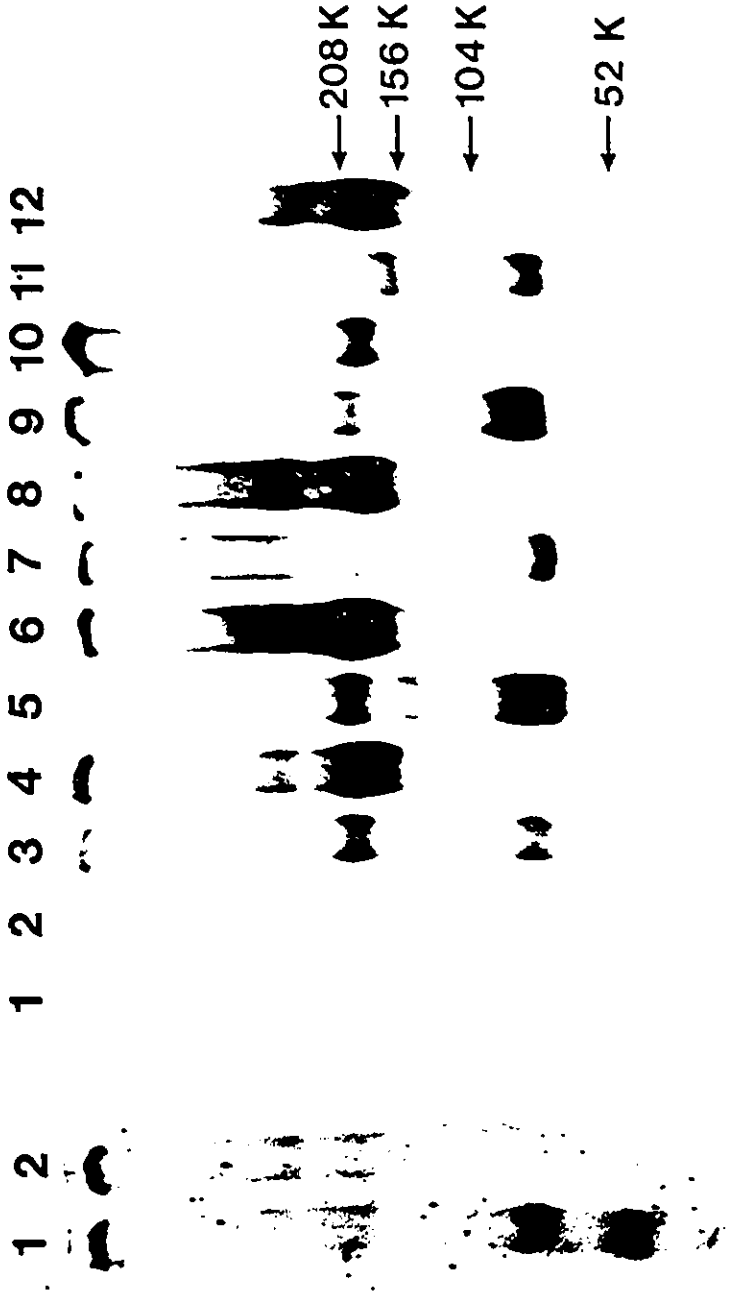
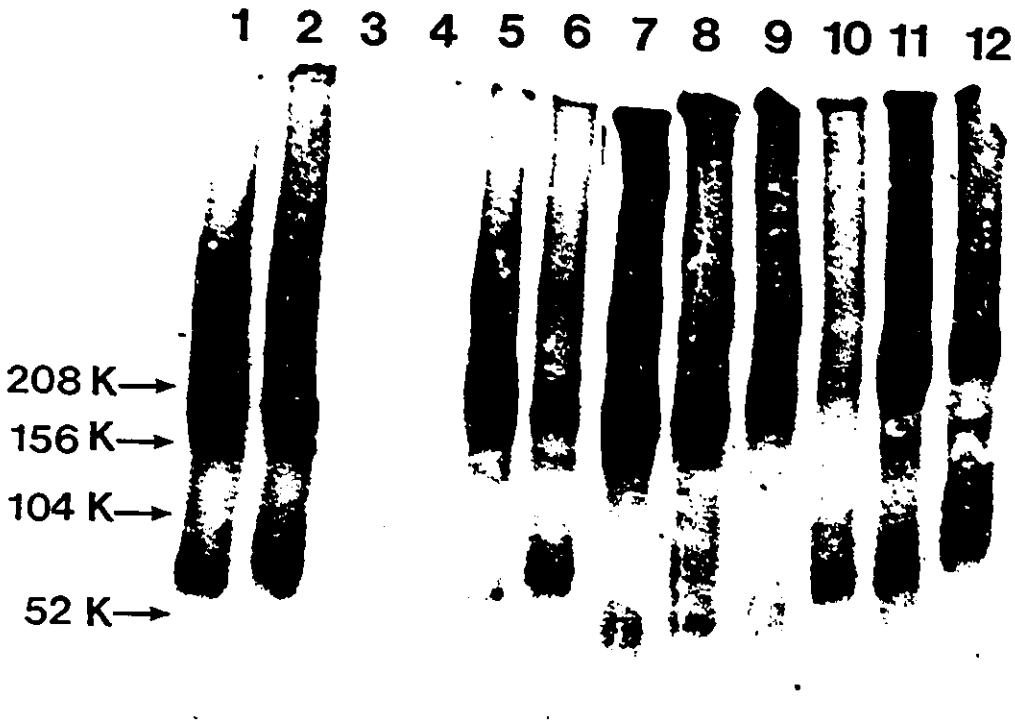

208 K
156 K
104 K
52 K
1 2 3 4 5 6 7 8 9 10 11 12
1 2

Figure 24. Oligomerization of F proteins with mutation in the heptad repeat. See legend for Figure 23. Lanes 1 & 2, HPIV3-infected, +/- NP40. Lanes 3 & 4, mock-infected, +/- NP40. Lanes 5 & 6, pIBIF70(-), +/- NP40. Lanes 7 & 8, 137/PRO, +/- NP40. Lanes 9 & 10, 160/PRO, +/- NP40. Lanes 11 & 12, 185/SER, +/- NP40.


1 2 3 4 5 6 7 8 9 10 11 12
208 K
156 K
104 K
52 K

DISCUSSION

Mutations were introduced by site-specific mutagenesis into three domains of the HPIV3 F protein, with the purpose of identifying regions of the F protein that are important for fusogenic activity. Paramyxovirus F glycoproteins possess two regions of conserved sequence motifs that are predicted to form α-helices and may be essential for fusogenic activity, the heptad repeat region contiguous with the fusion peptide and the leucine zipper adjacent to the hydrophobic transmembrane anchor. Amino acid substitutions were designed to perturb the predicted helical or amphipathic nature of these two regions. Positively charged residues in the transmembrane anchor and cytoplasmic tail of the gp41 protein of HIV-1 had previously been shown to be important for syncytium formation. Therefore, the last region of mutagenesis was the transmembrane domain/cytoplasmic tail of the HPIV3 F protein.

Recombinant vaccinia virus vTF7-3 was used to transiently express the HPIV3 HN glycoprotein and the various forms of the HPIV3 F protein. Syncytia produced following transfection of pIBIF70(-) + pIBIHN plasmids, that expressed HPIV3 F and HN proteins, respectively, were indistinguishable from those seen in HPIV3-infected cells.

All but one of the fifteen mutations resulted in F proteins that were oligomerized and transported to the cell surface similar to authentic HPIV3 F proteins. As expected, some mutant proteins were not fusogenic, and an intermediate fusion

phenotype was also observed. The intermediate fusion phenotype was characterized by syncytia that not only were smaller in size than wild-type syncytia (pIBIF70(-) + pIBIHN) but also involved a smaller percentage of the transfected monolayer. The high sensitivity of HeLa $T4^+$ cells to fusion and their reduced susceptibility to vaccinia virus cytopathic effects may have made it possible to observe this intermediate fusion phenotype.

The decrease in the fusogenic ability of some of the mutant F proteins, 160/PRO, 185/SER, and 474/ALA, for example, was most probably due to poor cleavage of the precursor F_0 protein. Cleavage of F is believed to be a prerequisite for F protein mediated fusion (Homma and Ohuchi, 1973). Although the amount of F_1 subunit present in cells expressing these mutant proteins was low, clearly, fusion was still evident. In an effort to increase cleavage of the F_0 protein, 10 µg/mL of trypsin was added to infected and transfected cell monolayers, however, this treatment had no effect on the extent of syncytium formation mediated by any of the F proteins (data not shown).

Poor cleavage-activation was not, however, responsible for the decreased fusogenic activity of mutant F proteins such as 137/PRO and 474/ASP since substantial amounts of the F_1 subunits of both these proteins were detected in cell lysates. Mutant protein 474/SER was produced in abundance and cleaved as efficiently as the F protein in HPIV3-infected cells and pIBIF70(-) transfected cells but was poorly fusogenic.

All mutant HPIV3 F proteins with reduced fusogenic activity lost the ability to bind monoclonal antibody c128, which recognizes an epitope in antigenic site B. Therefore, disruption of the epitope in antigenic site B resulted from amino acid substitutions in the heptad repeat or the leucine zipper. It was previously proposed that the fusion peptide and the adjacent heptad repeat region are closely associated with the leucine zipper in the tertiary structure of the functional measles virus F protein (Wild et al., 1994b). Coelingh and Tierney (1989) have also shown that amino acid substitutions in the HPIV3 F protein at position 73 (within the F_2 subunit) and/or 452 (upstream of the leucine zipper motif), prevented binding of antibody c128.

These observations are consistent with a model in which interactions between distal sequences of the HPIV3 F protein are necessary for the formation of the epitope that is recognized by antibody c128. It is not known whether the epitope directly involves sequences in the heptad repeat region or the leucine zipper motif, or if the epitope is distal to both of these domains. The results presented here also suggest that an association between these two domains is critical for the HPIV3 F protein to possess optimal fusogenic activity.

I. Mutations in the leucine zipper motif

When these studies were initiated, two sequences predicted to form amphipathic α-helices, a leucine zipper motif (Buckland and Wild, 1989) and a second heptad repeat (Chambers et al., 1990), had been identified in the F proteins of members of the family *Paramyxoviridae*.

Amino acid substitutions in the leucine zipper of the gp41 protein of HIV-1 had shown that this structure was critical for membrane fusion and for virus entry (Dubay et al., 1992). Substitutions in the isoleucine of the third heptad repeat of the gp41 protein had no effect on synthesis, oligomerization, transport to the cell surface or processing of the HIV glycoprotein complex. Replacement of the isoleucine with either valine or leucine had no effect on fusogenic activity, however, substitution with aspartic acid, glutamic acid, glycine or serine resulted in mutant gp41 proteins that were properly processed and transported to the cell surface yet were nonfunctional. Substitution with alanine resulted in a mutant protein that produced an intermediate fusion phenotype, characterized by a reduction in both syncytium formation and infectivity. It appeared that the degree of hydrophobicity of the residue at this position correlated with fusogenic activity of the mutant gp41 proteins. As the hydrophobicity of the substituted residue decreased, so too did its ability to mediate cell fusion.

Similar amino acid substitutions were created at the isoleucine at position 474 in the third heptad repeat of the leucine zipper of the HPIV3 F protein. The

substitution of glycine for isoleucine resulted in the only mutant F protein that was not transported to the cell surface. This mutant protein was not oligomerized nor cleaved and was most probably improperly folded and retained in an intracellular compartment. Two substitutions at position 474, glutamic acid and valine, did not appear to affect the fusogenic ability of the F protein. However, substitution of either serine or alanine resulted in F proteins with intermediate fusion phenotypes and substitution of aspartic acid rendered the F protein nonfunctional. Unlike what was observed for the HIV-1 gp41 protein, there was no correlation between the degree of hydrophobicity of the amino acids substituted for isoleucine and their effects on fusion.

Substitution of amino acids that mimic the bulkiness of isoleucine, such as glutamic acid or valine did not affect the function of the F protein. Decreasing the bulkiness of the R group of the residue reduced fusion activity, despite the fact that the proteins were cleaved and formed oligomeric structures. The substitution of glycine, which had the smallest R group, for isoleucine produced a nonfunctional protein that was neither oligomerized nor cleaved. Cohen and Parry (1986) have stated that a decrease in bulkiness of apolar residues in positions *a* or *d* of a heptad repeat could result in a break in the α-helical structure, and this could explain the concomitant reduction in fusogenic activity.

While single amino acid substitutions in the leucine zipper domain of the HPIV3 F protein and the HIV-1 gp41 protein were detrimental to fusogenic activity, substitution of alanine for leucine in each repeat of the leucine zipper of the NDV

F protein did not significantly alter the fusogenic ability. Fusogenic activity was, however, abolished by double substitutions within the leucine zipper motif of the NDV F protein (Reitter et al., 1995).

With the exception of the 474/GLY mutant, mutation of the leucine zipper motif of the HPIV3 F protein did not appear to affect the ability of mutant proteins to assemble into oligomers. It had been suggested that the leucine zipper contributes to the oligomerization of viral proteins (Buckland and Wild, 1989) although the leucine zipper of the measles virus F protein was not involved in the tetramerization of the protein (Buckland et al., 1992). Similarly, deletion of the leucine zipper motif, the transmembrane region and the cytoplasmic tail of the NDV F protein resulted in the secretion of oligomeric F proteins, and demonstrated that the protein ectodomain amino terminal to these regions are necessary for oligomerization (Reitter et al., 1995).

The decreased fusogenic activity of HPIV3 F proteins with mutations in the leucine zipper domain was not due to the failure of the mutant proteins to form oligomers. It is possible that the structure of the leucine zipper was not sufficiently altered to disrupt oligomerization, however, it is unlikely that this domain is involved in oligomerization of the HPIV3 F protein but may contribute to the stability of the oligomers.

II. Mutations in the heptad repeat region

The heptad repeat region of the HPIV3 F protein also has the potential to form an amphipathic α-helix. Substitution of proline, a strong helix-breaking amino acid for ALA^{137} or SER^{160}, was expected to disrupt this α-helix (Cohen and Parry, 1986). Mutant protein 137/PRO had no fusion activity while mutant protein 160/PRO had an intermediate fusion phenotype. It is possible that the additional conservative change of alanine for VAL^{132} in the fusion peptide of protein 137/PRO may have exacerbated the effects of the mutation at position 137. However, it is unlikely that this second mutation abrogated fusogenic activity of the mutant protein in view of the observation that substitutions in the fusion peptide of the SV5 F protein were tolerated without appreciable loss of biological function (Horvath and Lamb, 1992).

Replacing PRO^{185}, which lies at the carboxyl end of the heptad repeat region with a serine residue also reduced fusogenic activity and may have altered local structure by removing a kink or a bend from the polypeptide sequence (Cohen and Parry, 1986). The heptad repeat region is located between the fusion peptide and CYS^{192}, which presumably forms the disulfide bridge with the F_2 subunit, similar to CYS^{199} of the Sendai virus F protein (Iwata et al., 1994). Mutations in the heptad repeat region could have altered the conformation of this region of the protein sufficiently to result in reduced cleavage of 185/PRO and diminished fusogenic activity.

Cleavage-resistant F proteins of NDV have also been associated with mutations in the heptad repeat region (Wang et al., 1992). Temperature-sensitive mutants of Newcastle disease virus were nonfusogenic due to the lack of cleavage-activation and transport at the nonpermissive temperature, and sequence analysis of the mutant F genes revealed that the mutations were located in the heptad repeat region. Nonconservative mutations along one face of the predicted α-helix of the heptad repeat produced NDV F proteins that were properly folded and transported to the cell surface but lacked fusogenic activity. In one mutant, the substitution of lysine for leucine at position *d* in the 4th heptad repeat resulted in an F_0 protein that was poorly cleaved (Sergel-Germano et al., 1994).

The mutations that were created in the heptad repeat region, like mutations to the leucine zipper motif, also did not alter the pattern of oligomerization and therefore, this region does not appear to be involved in the formation of multimers.

III. Association of the leucine zipper and the heptad repeat regions

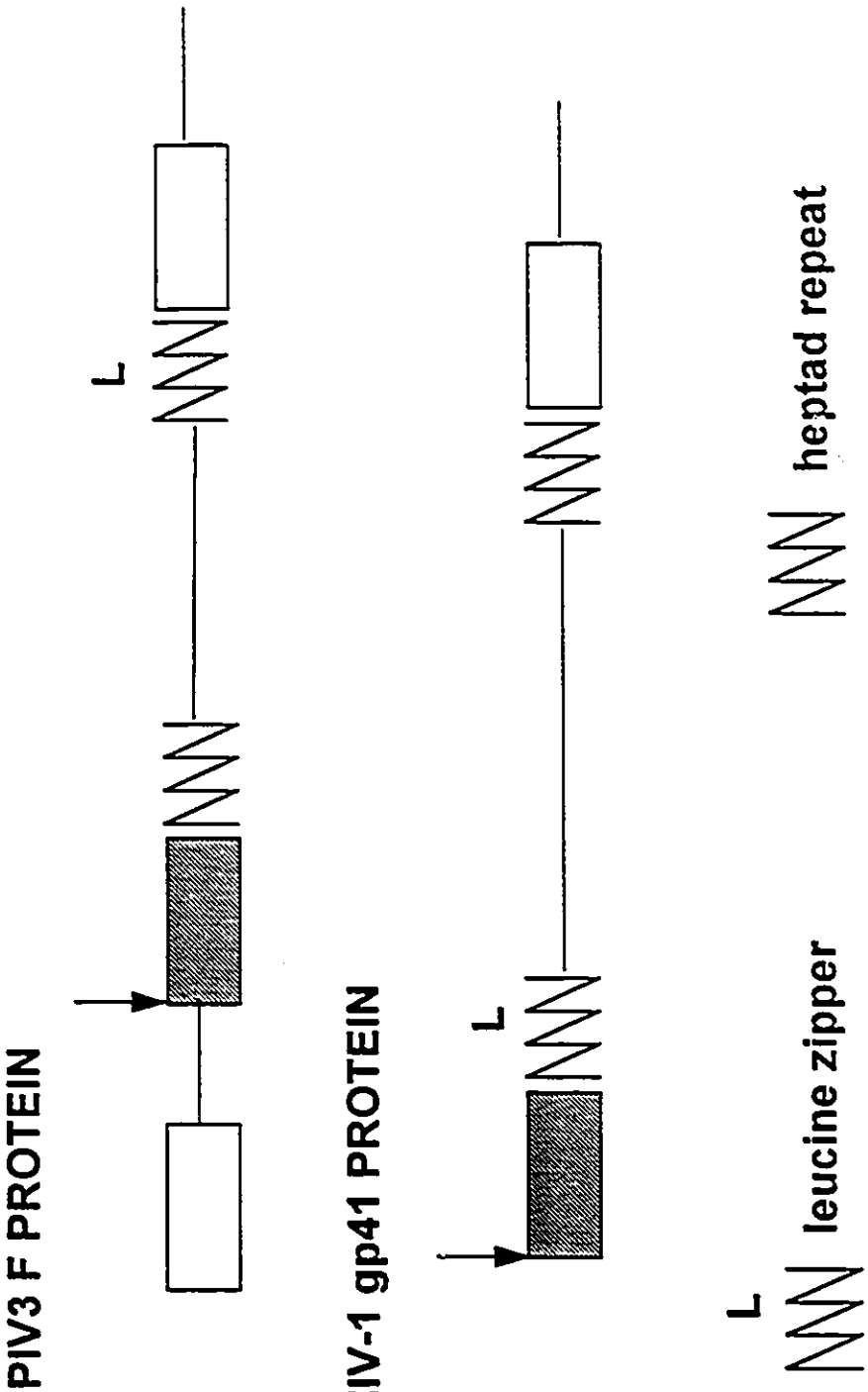
Mutation of either the leucine zipper motif or the heptad repeat domain of the HPIV3 F protein resulted in mutant F proteins that were not only poorly fusogenic or nonfusogenic but also were unable to bind antibody c128. The leucine zipper domain and the heptad repeat appear to be critical for the formation of the epitope recognized by antibody c128.

Antibody c128 normally binds to an epitope within antigenic site B of the HPIV3 F protein and Coelingh and Tierney (1989) have shown that escape mutants to antibody c128 have amino acid changes at position 73 within the F_2 subunit and at position 453, which is near the leucine zipper motif. It is tempting to suggest that the leucine zipper domain and the heptad repeat bring distal regions of the HPIV3 F protein into close proximity. As a result of the interaction of these two domains, the F protein adopts a conformation that is fusion 'competent', and perhaps, the epitope recognized by antibody c128 is a marker for a correct F protein conformation that results in optimal fusogenic activity.

A second heptad repeat has also been identified within the HIV-1 gp41 protein and a schematic of the structures of the gp41 protein and the HPIV3 F protein are shown in Figure 25. Synthetic peptides corresponding to either the gp41 protein heptad repeat (DP-178, Wild et al., 1994) or the leucine zipper motif (DP-107,Wild et al., 1992) inhibited virus-cell fusion but were more potent inhibitors of cell-to-cell fusion. These peptides did not interfere with $CD4^+$ binding nor did they interfere with the interaction of gp41 protein with the gp120 protein. Substitution of a helix-breaking proline residue within peptide DP-107 abolished fusion-inhibiting activity (Wild et al., 1992).

Peptide DP178 was shown to inhibit cell fusion by interacting with sequences located near or within the leucine zipper domain of the same molecule. Similarly, substitution of a proline residue in the leucine zipper of the gp41 protein also inhibited interaction with peptide DP178 (Wild et al., 1994). Circular dichroism

Figure 25. Structural domains of the HPIV3 F protein and the HIV-1 gp41 protein. This is a schematic of the structural domains of the HPIV3 F protein and the HIV-1 gp41 protein. The arrows represent the sites of cleavage. The empty box represents the signal sequence. The hatched boxes represent fusion peptides. The dotted boxes represent the transmembrane anchor. (Adapted from Chen et al., 1995; and Kuby, 1991).


HPIV3 F PROTEIN
L
HIV-1 gp41 PROTEIN
L
L
leucine zipper
heptad repeat

studies have shown that peptide DP178 altered the helical conformation of DP107 and suggests that the mode of action of DP178 involves direct interaction with the leucine zipper domain (Wild et al., 1994)

The two helical domains of the HIV-1 gp41 protein have been shown to be stably associated with each other, this interaction acts as a molecular clasp that maintains a conformation in the gp41 protein that is recognized by a human recombinant Fab fragment (Chen et al., 1995). Mutation or deletion of either of the α-helical domains resulted in loss of a conformational and/or discontinuous epitope and severely reduced Fab reactivity (Chen et al., 1995). The epitope recognized by the Fab could be reconstituted by mixing peptide DP178, which represents the heptad repeat with the protein fragment containing the leucine zipper, and is consistent with the interaction of these two domains. The similarities between mutations in the α-helices of the HPIV3 F protein and the HIV-1 gp41 protein are striking. Loss of an epitope was also observed upon mutation of either the heptad repeat domain or the leucine zipper motif of the HPIV3 F protein and correlated with decreased fusogenic activity. These results suggest that the two α-helical domains of the HPIV3 F protein are also interacting with each other.

IV. Mutations in the cytoplasmic tail and transmembrane region

Substitutions of uncharged or hydrophobic amino acids for positively charged residues in the cytoplasmic tail proximal to the transmembrane hydrophobic region of the HPIV3 F protein had no apparent effect on cleavage, transport or fusogenic activity. In contrast, substitution of hydrophobic amino acids for two positively charged amino acids in the cytoplasmic tail of the HIV-1 gp41 protein inhibited syncytium formation and led to a decrease in the complementation of an HIV-1 provirus with a large deletion in the *env* gene (Helseth et al., 1990).

The transmembrane anchor of the gp41 protein of HIV-1 also contains two positively charged residues that are critical for syncytium formation (Owens et al., 1994). The transmembrane region of the F protein of HPIV3 has no charged residues. The insertion of a lysine residue into the HPIV3 F transmembrane domain had no apparent effect on protein processing or fusogenic activity.

The cytoplasmic tail and approximately 30% of the transmembrane domain of HIV-1 gp41 protein are not essential to membrane anchoring or cell surface transport, yet are necessary for fusogenic activity (Owens et al., 1994). Single amino acid substitutions within the cytoplasmic tail that did not involve charged amino acids did not affect function. Yao and Compans (1995) have shown that removal of the entire HPIV3 F protein cytoplasmic tail abolishes both cell surface expression and fusogenic activity. Truncation of the leucine zipper, the transmembrane domain and the cytoplasmic tail of NDV F protein resulted in the

secretion of an oligomeric protein (Reitter et al., 1995). Similar changes to the cytoplasmic tail of the F protein of HPIV2 had no effect on either the transport of the truncated F protein or fusogenic activity (Yao and Compans, 1995). Clearly, the role of the transmembrane region and the cytoplasmic tail in cell fusion is different for these viruses. For the HPIV3 , these regions are essential to transport, for HIV-1 gp41 protein, they are not critical to protein transport but are essential for cell fusion and for the NDV and HPIV2 F proteins, these regions are not required for either transport or function.

CONCLUSIONS

The initial objective of these studies was to elucidate which HPIV3 proteins were required to mediate syncytium formation. The results presented here showed that both the F and HN proteins of HPIV3 are required for cell fusion irrespective of the expression system that was chosen. The F proteins that were expressed from recombinant vaccinia viruses, recombinant adenoviruses or recombinant plasmids were not fusogenic in the absence of the HN protein.

Additional experiments suggested that the F and HN proteins must be contained within the same membrane in order to be fusogenic. Also, the fusion promotion function of the HPIV3 HN protein could not be replaced by the lectin, wheat germ agglutinin, indicating that the role of the HN protein in fusion is more complex than as a simple receptor binding protein.

The final objective of these studies was the creation of HPIV3 F glycoprotein mutants that were properly processed and transported to the cell surface but lacked fusogenic activity. Site-specific mutagenesis was used to create amino acid substitutions in three regions of the F protein that are conserved among paramyxoviruses and that were predicted to be important for fusogenic activity.

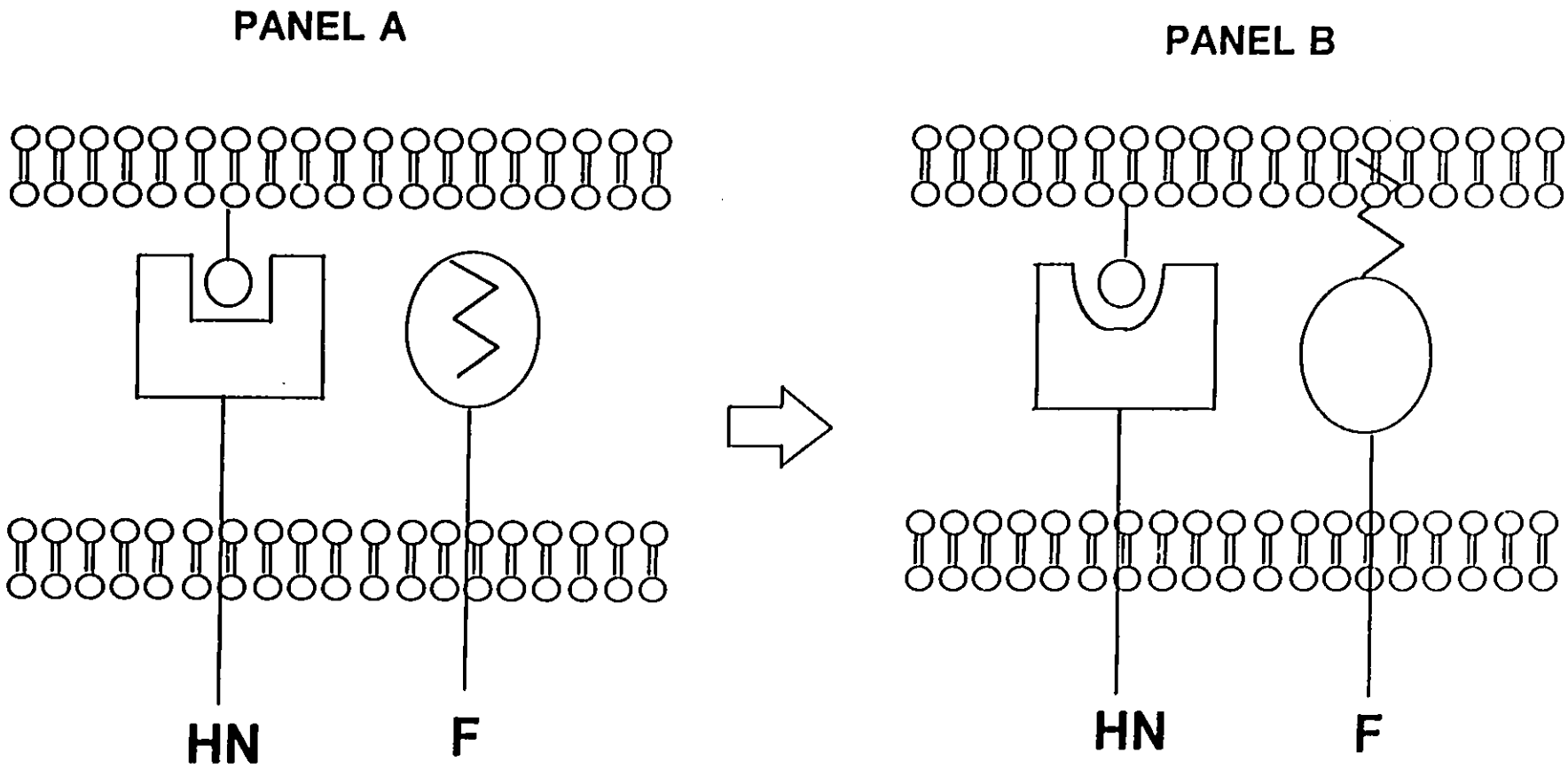
Amino acid substitutions within the heptad repeat region and the leucine zipper domain produced several mutant F proteins that exhibited intermediate or negative fusion phenotypes. Significantly, the mutant F proteins with aberrant fusogenic

activity also lost the ability to bind one, c128(B), of a panel of F-specific monoclonal antibodies.

These data suggest that the leucine zipper motif and the heptad repeat region, which are predicted to form α-helices, interact with each other leading to the formation of the epitope recognized by antibody c128. The absence of this epitope correlated with impaired fusogenic activity. It is possible that c128 binds directly to one or both of these domains when they are interacting. Alternatively, the epitope may be distal to the domains and that the antibody binds elsewhere on the structure formed by their association.

A general model depicting the role of the envelope glycoproteins of paramyxovirus in membrane fusion has been proposed by Lamb (1993) and is presented in Figure 26. In this model for cell-to-cell fusion, the receptor binding protein interacts with its cellular receptor and undergoes a conformational change (Panel A). This change, in turn, triggers a conformational change in the fusion protein. This conformational change in the F protein allows the fusion peptide, which was previously inaccessible, to interact with the cellular membranes and mediates cell fusion (Panel B). The use of circular dichroism to study the conformation of Sendai virus glycoproteins showed that the conformations of the F and HN proteins contained within the same membrane are different from conformations of F or HN proteins alone, or if they are mixed together but contained in different membranes (Citovsky et al., 1986). This suggests that the F and HN proteins exist in two states. Therefore, the F protein can exist in the native state or

Figure 26. Model for conformational change of HPIV3 F and HN proteins. The HN protein interacts with the host cell receptor and undergoes a conformational change. This change, in turn, induces a conformational change in the F protein which results in the interaction of the fusion peptide with the cell membrane. Adapted from Lamb, 1993.


PANEL A
PANEL B
HN
F
HN
F

a state that is fusion-competent but not fusogenic, and the fusogenic state, which mediates membrane fusion.

HPIV3 glycoproteins are cotranslationally translocated into the endoplasmic reticulum where they undergo glycosylation and oligomerization. The F protein can also undergo cleavage before arrival at the cell surface. Because F and HN proteins are produced within the same membranous compartments, it is possible that they become associated with each other before reaching the cell surface. However, since the HN protein must interact with the host cell receptor before being 'activated', any association of these two proteins before they reach the cell surface would not induce conformational change in the F protein.

The question arises whether the heptad repeat and the leucine zipper interact in the native F_0 or $F_1 + F_2$ protein or if their interaction is subsequent to activation and is characteristic of the fusogenic state. The epitope recognized by antibody c128 is present on both cleaved and uncleaved F proteins in the absence of the HN protein. Therefore, the epitope must be present in the native state of the F protein and the α-helices must interact before any conformational change can be triggered by the HN protein.

Is the interaction of the heptad repeat and leucine zipper and the formation of the c128 epitope necessary for cleavage? Clearly, for some F mutants, amino acid substitutions may have affected the efficiency of cleavage and decreased syncytium formation could be explained by reduction in the number of $F_1 + F_2$ subunits that

are fusion competent. However, since mutant protein 474/SER shows efficient cleavage and the mutant F protein is produced at levels equal or greater to those observed with pIBIF70(-), it can be concluded that the formation of the epitope is not a prerequisite for cleavage. Also, since antibody c128 immunoprecipitates the F_0 protein precursor from lysates of HPIV3-infected cells or pIBIF70(-) transfected cells, cleavage is therefore not a prerequisite for the formation of the epitope.

It appears, therefore, that the interaction of the heptad repeat region and the leucine zipper motif occurs prior to cleavage and may be considered to be part of the native conformation. The epitope recognized by the antibody c128 may, however, be a marker for fusion competence. Amino acid substitutions that alter the ability of these α-helices to interact also abolish the c128 epitope and result in decreased fusogenic activity.

Since most of the mutant F proteins exhibited poor cleavage activation, this provides the simplest explanation for decreased fusogenic activity. The addition of exogenous trypsin did not alter fusion phenotypes, however, I did not investigate whether additional cleavage of the proteins had occurred. Amino acid substitutions altered the folding pattern of the F protein, evidenced by the loss of the c128 binding site as well as cleavability, which in turn, decreased the fusogenic activity. Under these circumstances, even after additional cleavage of altered F proteins, they might still be folded aberrantly since the epitope is present in uncleaved F_0 precursor proteins and its formation is not dependent on cleavage.

It is possible that the altered F proteins are fusion competent but fail to interact properly with the activated HN protein and therefore cannot adopt a fusogenic conformation. For the measles virus F protein the interaction with the H protein involved sequences located within the first five cysteine residues of the cysteine rich region (Wild et al., 1994b). The cysteine rich region is situated between the heptad repeat and the leucine zipper and is presumably involved in the formation of secondary structure due to cysteine pairing and the interaction of the α-helices. Conversely, the region on the HPIV3 HN protein which has been identified as the fusion promotion domain is the transmembrane anchor and the first 82 residues of the ectodomain (Deng et al., 1995).

A second explanation for altered fusogenic activity of mutant F proteins is that they are able to interact properly with the HN protein, but are not fusion competent and are unable to undergo a conformational change necessary for liberation of the fusion peptide and membrane fusion.

Chemical cross-linking of HPIV3 F and HN proteins was not observed using DTSSP, although it has been observed for measles virus F and H proteins (Malvoisin and Wild, 1993). It is possible that the use of different chemical cross-linking agents would be able to detect the interaction of HPIV3 F and HN proteins that results in F protein activation. Mutant F proteins that cannot be cross-linked with the HN protein might therefore not be able to interact properly and as a result exhibit decreased fusogenic activity.

Finally, an in-vitro packaging system would provide additional information about the effect of an altered F protein on such mechanisms as host cell binding or viral replication. It would be most interesting to know whether decreased fusogenic activity of the mutant F proteins would affect the pathogenesis of HPIV3.

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APPENDIX A

OLIGONUCLEOTIDE SEQUENCES

517/THR	5' ATTATAATTGCAGTTACGTATTAC 3'
520 /ILE	5' TATTACATAATTCAAAAGAGAAATCGA 3'
523/THR	5' CAAACGAGAAATCGAGTGGATCAA 3'
524/ILE	5' ATAAATCGAGTGGATCAAAATGATAAA 3'
506/LYS	5' ATAATTATATTGTTTAAAATTAATGTAACGATA 3'
474/GLY	5' GAGTCAAAAGAATGGGGAAGAAGGTCAAATCAA 3'
474/SER	5' GAGTCAAAAGAATGGTCAAGAAGGTCAAATCAA 3'
474/ASP	5' GAGTCAAAAGAATGGGACAGAAGGTCAAATCAA 3'
474/GLU	5' GAGTCAAAAGAATGGGAGAGAAGGTCAAATCAA 3'
474/VAL	5' GAGTCAAAAGAATGGGTAAGAAGGTCAAATCAA 3'
474/ALA	5' GAGTCAAAAGAATGGGCAAGAAGGTCAAATCAA 3'
137/PRO	5' GCGGCTCTGGTTGAACCCAAGCAGGCAAGATCA 3'
160/PRO	5' AACAAAGCAGTGCAGCCAGTCCAGAGCTCCATA 3'
185/SER	5' AACAAAGAAATCGTGTCATCAATTGCGAGATTA 3'